T.C.

ÇANAKKALE ONSEKİZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

DETERMINING THERAPEUTIC PROTEINS AND PREBIOTIC *N*-GLYCAN CONCENTRATIONS IN BOVINE COLOSTRUM

MASTER OF SCIENCE THESIS

Ayşenur ARSLAN

Thesis Supervisor:

Assoc. Prof. Sercan KARAV

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The study titled "**Determining Therapeutic Proteins and Prebiotic *N*-Glycan Concentrations in Bovine Colostrum**" written by Ayşenur ARSLAN under the direction of Assoc. Prof. Sercan KARAV was defended on 20/06/2022. This thesis has been approved as **Master of Science Thesis** in **Department of Molecular Biology and Genetics** at Çanakkale Onsekiz Mart University, School of Graduate Studies by the Examining Jury Members.

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23/06/2022

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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.

I declare that this thesis is prepared according to the current research thesis manual at Çanakkale Onsekiz Mart University, School of Graduate Studies. I declare that the data, information, and document are obtained within the academic and ethical rules, all information, documents, evaluations, and results are presented in accordance with scientific ethics and moral codes, the appropriate references are cited, any changes are not found in the used data, the information and findings specified in this study are original. I declare above mentioned issues and accept all rights losses that may arise against me.

(İmza)
Ayşenur ARSLAN
23/06/2022

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A journey of thousand miles begins with a single step.

Confucius

I am appreciated to everyone with me in all my steps…

Ayşenur ARSLAN

Çanakkale, Haziran 2022

ÖZET

SIĞIR KOLOSTRUMUNDA BULUNAN TERAPÖTİK PROTEİN ve PREBİYOTİK *N*-GLİKAN KONSANTRASYONLARININ BELİRLENMESİ

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Sığır kolostrumu, doğumdan hemen sonra meme bezlerinden salgılanan süttür ve immünoglobulin G (IgG), laktoferrin, oligosakkaritler, büyüme faktörleri ve temel besinler gibi zengin immünolojik olarak aktif bileşenler içermektedir. IgG ve laktoferrin, sığır kolostrumunda *N*-glikan kaynağı olan terapötik proteinlerdir. Biyoaktif *N*-glikanlar, faydalı mikroorganizmaların büyümesini teşvik etmek için prebiyotik maddeler olarak etki sağlamak gibi önemli biyolojik aktivitelere sahiptir. Bu tezin amacı, sığır kolostrumunda bulunan toplam protein, IgG ve laktoferrin gibi terapötik proteinler ve *N*-glikan konsantrasyonunun kolostrumdan olgun süte geçişte nasıl değiştiğini incelemektir. Bu amaçla, ULUOVA Süt Ticaret A.Ş.'den 28 Holstein inekten doğumdan sonraki 6 gün içinde sığır kolostrum örnekleri toplandı ve örnekler her örneğin günlerine göre havuzlandı. Daha sonra bu örneklerden toplam proteinler izole edildi. Numunelerin toplam protein konsantrasyonu, BCA yöntemi ile belirlendi ve proteinlerin görüntülenmesi SDS-PAGE ile gerçekleştirildi. IgG ve laktoferrin konsantrasyonu, ELISA testi kullanılarak tespit edildi. *N*-glikanlar, yeni *N*-glikosidazlar kullanılarak glikoproteinlerden salındı ve fenol-sülfürik asit yöntemi ile kantifiye edildi. Bu çalışmanın sonucunda erken kolostrumun en yüksek protein, laktoferrin, IgG ve *N*-glikan konsantrasyonuna sahip olduğu ve sonraki 3 gün boyunca konsantrasyonlarında azalma olduğu saptanmıştır.

Anahtar Kelimeler: Sığır kolostrum, terapötik protein, IgG, laktoferrin, *N*-glikan

ABSTRACT

DETERMINING THERAPEUTIC PROTEINS and PREBIOTIC *N*-GLYCAN CONCENTRATIONS in BOVINE COLOSTRUM

Ayşenur ARSLAN

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School of Graduate Studies

Master of Science Thesis in Molecular Biology and Genetics

Advisor: Assoc. Prof. Sercan KARAV

01/06/2022, 69

Bovine colostrum is produced by milking gland directly after calving and rich source of immunologically active components, such as immunoglobulin G (IgG), lactoferrin, oligosaccharides, growth factors and essential nutrients. IgG and lactoferrin are therapeutic proteins which are source of *N*-glycans in bovine colostrum. These bioactive *N*-glycans have significant biological activities such as acting as prebiotic substances to stimulate growth of beneficial microorganisms. The overall aim of this thesis is that assessment of the changes in the concentration of total protein, therapeutic proteins, including IgG and lactoferrin, and *N*-glycan of bovine colostrum through transition from colostrum to mature milk. For this purpose, bovine colostrum samples were obtained during the six days after parturition from twenty-eight Holstein Friesian cows from ULUOVA Milk Trading Co. and the samples were pooled according to days of each sample. Then, the total proteins were isolated from these samples. The concentration of total protein in samples was detected using BCA assay and the proteins were visualized via Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The concentrations of IgG and lactoferrin were determined with ELISA assay. After that, the *N*-glycans were released from bovine colostrum glycoproteins using novel *N*-glycosidases and quantified using phenol-sulfuric acid assay. As a results of this study, early colostrum involves the highest concentration of protein, lactoferrin, IgG and *N*-glycan and with subsequent decreases concentration over the next 3 days.

Keywords: Bovine colostrum, therapeutic proteins, IgG, lactoferrin, *N*-glycans

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LIST of ABBREVIATIONS and SYMBOLS

IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
LF	Lactoferrin
IGF	Insulin-like growth factor
EGF	Epidermal growth factor
N-glycans	*N*-linked glycosylation
O-glycans	*O*-linked glycosylation
HexNAc	*N*-acetylglucosamine
GlcNAc	*N*-acetylglucosamine
Asn	Asparagine
Ser	Serine
Thr	Threonine
ER	Endoplasmic reticulum
PNGases	Peptide-*N*-Glycosidases
Endo	Endoglycosidase
EndoBI-1	Endo-β-*N*-acetylglucosaminidase
Neu5Ac	*N*-acetyl-neuraminic acid
Neu5Gc	*N*-glycolyl neuraminic acid
Gal	Galactose
Man	Mannose
Fuc	Fucose
GalNAc	*N*-acetyl galactosamine
BCA	Bicinchoninic Acid Assay
ELISA	Enzyme-Linked Immunosorbent Assay
OD	Optical density
MS/MS	Tandem mass spectrometry
BSA	Bovine Serum Albumin
RT	Room temperature
TEMED	Tetramethyl ethylenediamine
APS	Ammonium persulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

mg	Milligram
mL	Milliliter
µL	Microliter
mM	Milimolar
v	Volume
%	Per cent
min	minutes
sec	seconds
nm	nanometer
˚C	Degrees Celsius
dH_2O	Distilled water
WR	Working reagent
pH	Power of hydrogen
HSTS	High-temperature short-time
LTLT	Low-temperature low-time
UHT	Ultra-high temperature
URTIs	Upper respiratory tract infections
NSAID	Nonsteroidal anti-inflammatory drug

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CHAPTER 1
INTRODUCTION

Bovine colostrum is produced by milking gland immediately after post-partum and rich source of immunologically active components, such as immunoglobulin G (IgG), lactoferrin, oligosaccharides etc., growth factors and nutrients. Bovine colostrum has several functions for human health owing to this unique nutritional and biologically activities. According to studies in the literature, bovine colostrum supplementation is useful to gain health benefit for individuals that have gastrointestinal disorders, immunodeficiency, and also athletes. It is the most valuable during the first 24–48 h after parturition and the composition varies evenly during the transition to mature milk.

The IgG, which is not only provide passive immunities but also modulate both the adaptive and innate immunity, is the primary immunoglobulin present in bovine colostrum, and significant bioactive protein lactoferrin, which has iron-binding capacity, are glycoproteins, and these proteins are the mainly source of *N*-glycans in bovine colostrum. These glycans present in bovine colostrum can assist protection against pathogens due to various biological functions such as acting, especially as prebiotic agents, promoting the beneficial microorganisms growth found in the healthy gut microbiota. Additionally, these bioactive compounds can act as inhibitory factors against toxin producing microorganims by binding sites on gut epithelial cells through simulating carbohydrate structures found on the epithelial cells. These bioactive compounds can be processed into various food products as ingredients to produce fortified infant formulas, and design innovative food supplements and high-protein alternatives in support of athletes and convalescents as well as in neutraceuticals and cosmetics due to their various biological activities. Therefore, bovine colostrum is super food that has the potency for the further development of the ingredients, and evaluating the concentration of protein, IgG, lactoferrin and glycans is important for the study and use of colostrum proteins.

1.1. Bovine Colostrum

1.1.1. Content of Bovine Colostrum

Colostrum as a secretion from the mammary gland is a superfood produced within the first few days after calving. Colostrum is a natural source of essential macro- and micro-nutrients, antimicrobial peptides in addition to growth factors (Rathe, Müller, Sangild, & Husby, 2014). Bovine colostrum is a complex milky fluid secreted instantly after parturition and immediately changes to mature milk (Arslan, Kaplan, et al., 2021).

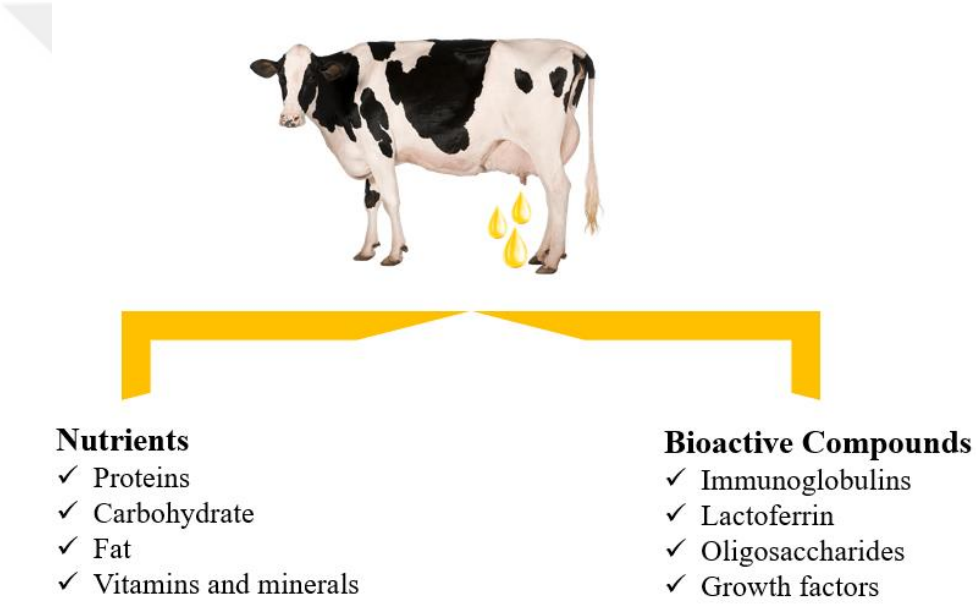

Figure 1. Bovine colostrum composition.

The composition and quality of colostrum change gradually through transition to mature milk, especially varies subsequently birth most significantly within the 24 h after parturition (Zhang et al., 2015). Bovine colostrum involves less lactose and higher amount of protein fat, peptides, vitamins and minerals as well as growth factors by comparison to mature milk (Playford & Weiser, 2021). It provides many important nutrients and bioactive components which support the growth and development in addition to immune function (Kehoe, Jayarao, & Heinrichs, 2007). The content and quality of bovine colostrum are based on many factors as individuality, parity, health status, length of dry period of cows,

induction of parturition, pre-partum nutrition post-partum time, and colostral handling factors (Tittle, 2002).

Figure 2. Stages of transition to mature milk.

Bovine colostrum consists of various protein fractions, including immunoglobulins (Igs), α-lactalbumin, β-lactoglobulin, lactoferrin, and several growth factors such as, majorly, insulin-like growth factor (IGFI and IGF-II), transforming growth factors (TGF) and also epidermal growth factor (EGF) etc. (Arslan, Kaplan, et al., 2021). Bovine colostrum contains ~40 mg/mL of total protein with a ranging from 169.55 mg/mL to 11.26 mg/mL (Zhang et al., 2015). The protein content comprises whey proteins (124.00 mg/mL) and caseins (26.00 mg/mL). Fractions of the whey proteins in bovine colostrum mainly characterized with three primary types of immunoglobulins including IgG, IgM, and also IgA presenting the ratio of 85-90%, 7%, and 5%, respectively (Playford & Weiser, 2021). The predominant immunoglobulin in bovine colostrum is IgG, and has function on modulating both adaptive and innate immune systems as well as stimulates passive immunities by absorption in the calf's intestines and transportation to the bloodstream (Arslan, Kaplan, et al., 2021). The other significant therapeutic protein is lactoferrin which is an glycoprotein with iron-binding capacity present in bovine colostrum, and responsible for enhancenment of iron absorption, binding lipopolysaccharide, promoting the proliferation of intestinal epithelial cells, and modulating immune systems (Zhao et al., 2019). The carbohydrate content of bovine colostrum consists of lactose, oligosaccharides, glycolipids, glycoproteins, and nucleotide sugars. The major carbohydrate structure in bovine colostrum is identified as lactose which is primarily energy source of the newborn. The mean percentage of lactose in bovine colostrum is reported as 27.00 mg/mL with increment until the fifth day of parturition

(Kehoe et al., 2007). Oligosaccharides are found with a concentration of 1 g/L in bovine colostrum (Arslan, Kaplan, et al., 2021) and act as prebiotics due to being non-digestible in the intestine. These carbohydrate structures can pass as a intact form into the colon, and metabolizing as a substrate by colonic bacteria. They can bind gut epithelial cells and mimic carbohydrate structures on surface of epithelial cell; therefore, they can act as inhibitory factors against toxigenic microorganisms (Morrin et al., 2019). Bovine colostrum also contains approximately 64 mg/mL fat including short-chain fatty acids, polyunsaturated fatty acids, conjugated linoleic acid as well as gangliosides, and phospholipids. In addition, it involves fat- and water-soluble vitamins, especially higher levels of D-carotene and also vitamins A, B, D and E. High amount of most vitamins such as vitamins B2, B12, D, and E are present in bovine colostrum as comparison to mature milk. Bovine colostrum is known as a rich source of essential minerals, involving iron, calcium, magnesium, phosphorus etc. The concentration of copper, iron and zinc in bovine colostrum are significantly higher than mature milk with decrement over the first 336 h after parturition (Arslan, Kaplan, et al., 2021). Additionally, bovine colostrum contains important bioactive elements such as cytokines, which are the peptides impact on immune response, cellular signaling, and pathogen recognition, including maternal leukocytes, for instance B and T lymphocytes that are responsible for the protection of the body against enteric pathogens (Dinarello, 1994). These compounds have also significant roles in stimulating the immune response owing to the production of cytokines, antibodies, antimicrobial and growth factors that take roles in promoting growth, differentiation, and development of the neonate (Playford & Weiser, 2021).

Table 1

Bovine colostrum and bovine mature milk contents

		Bovine Colostrum				
Components	n*	Mean	Minimum	Maximum	SE	Mature Milk
Fat mg/mL	54	67.00	20.00	265.00	41.60	39.00
Protein mg/mL	55	149.20	71.00	226.00	33.20	36.00
Casein mg/mL	-	26.00				29.5
Whey mg/mL		124.00				6.30
Lactose mg/mL	55	24.90	12.00	52.00	6.50	49.00
Dry matter mg/mL	55	276.40	183.00	433.00	58.40	125.00
Ash mg/mL	55	0.50	0.20	0.70	0.10	7.00
IgG1 mg/mL	55	34.96	11.80	74.20	11.80	0.30-0.40
IgG2 mg/mL	55	6.00	2.70	20.60	2.70	0.03-0.08
IgA mg/mL	55	1.66	0.50	4.40	0.50	0.04-0.06
IgM mg/mL	55	4.32	1.10	21.00	1.10	0.03-0.06
Oligosaccharides mg/mL			0.70	1.20		
Lactoferrin mg/mL	55	0.82	0.10	2.20	0.10	0.10-0.30
Lactoperoxidase mg/mL			11.00	45.00		13-30
Ca mg/kg	55	4,716.10	1,775.10	8,593.50	1,898.00	1,220.00
P mg/kg	55	4,452.10	1,792.40	8,593.5	1,706.29	1,520.00
Mg mg/kg	55	733.24	230.30	1,399.60	286.07	120.00
Na mg/kg	55	1,058.93	329.70	2,967.80	526.02	580.00
K mg/kg	55	2,845.89	983.20	5,511.40	1,159.89	1,520.00
Zn mg/kg	55	38.10	11.20	83.60	15.90	5.30
Fe mg/kg	55	5.33	1.70	17.50	3.09	0.80
Vitamin $IU.g^{-1}$		1.20				0.36
Vitamin A mg/kg	55	4.90	1.40	19.30	1.82	460.00
Vitamin E mg/kg	55	77.17	24.20	177.90	33.52	2.10
Vitamin B12 µg/mL	5	0.60	0.20	1.10	0.35	4.50

*Different colostrum samples from bovine in Pennsylvania.

1.1.2. Potential of Bovine Colostrum for Human Health

Bovine colostrum is not only the primary source of essential nutrients but also is an important source to produce ingredients for infant formula and protein supplements human health due to bioactive constituents. It plays important roles in maintaining gastrointestinal integrity, preventing, and resolving microbial infections, reducing the infections such as upper respiratory tract infections (URTIs) as well as diarrhea, binding and neutralizing human respiratory syncytial virus and modulating immunity (Playford & Weiser, 2021). Particular clinical studies have been reported that bovine colostrum supplementation has impact on reducing raised glucose in Type-II diabetic individuals, increasing bone density, and provide a therapeutic effect for nonsteroidal anti-inflammatory drug (NSAID) gut injury an and inflammatory bowel disease as well as short bowel syndrome and chemotherapy-induced mucositis (Arslan, Kaplan, et al., 2021).

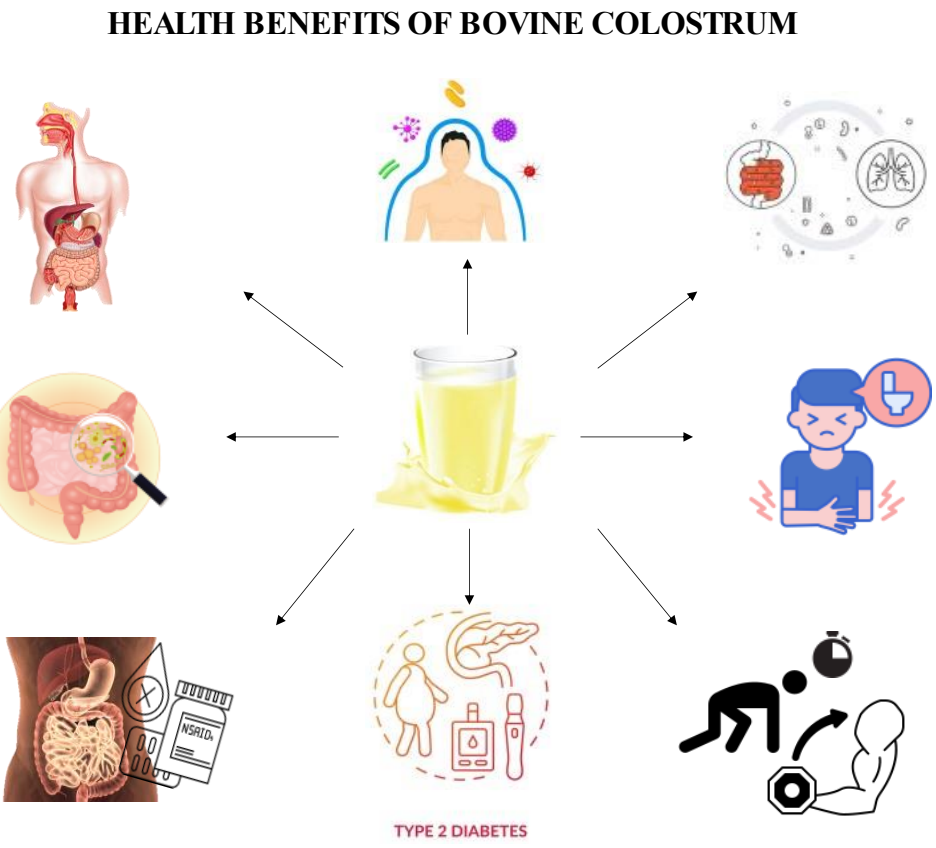

Figure 3. Health benefits of bovine colostrum.

Due to its various biological activities, bovine colostrum is utilized in industrial applications as ingredients or supplements for human consumption. A variety of processes, including thermal treatment and drying methods are involved to produce numerous end-products. Preference of proper processes is crucial to manufacture high-quality, safety in addition to extended shel-life bovine colostrum supplement and ingredients without any contamination (Kaplan et al., 2022).

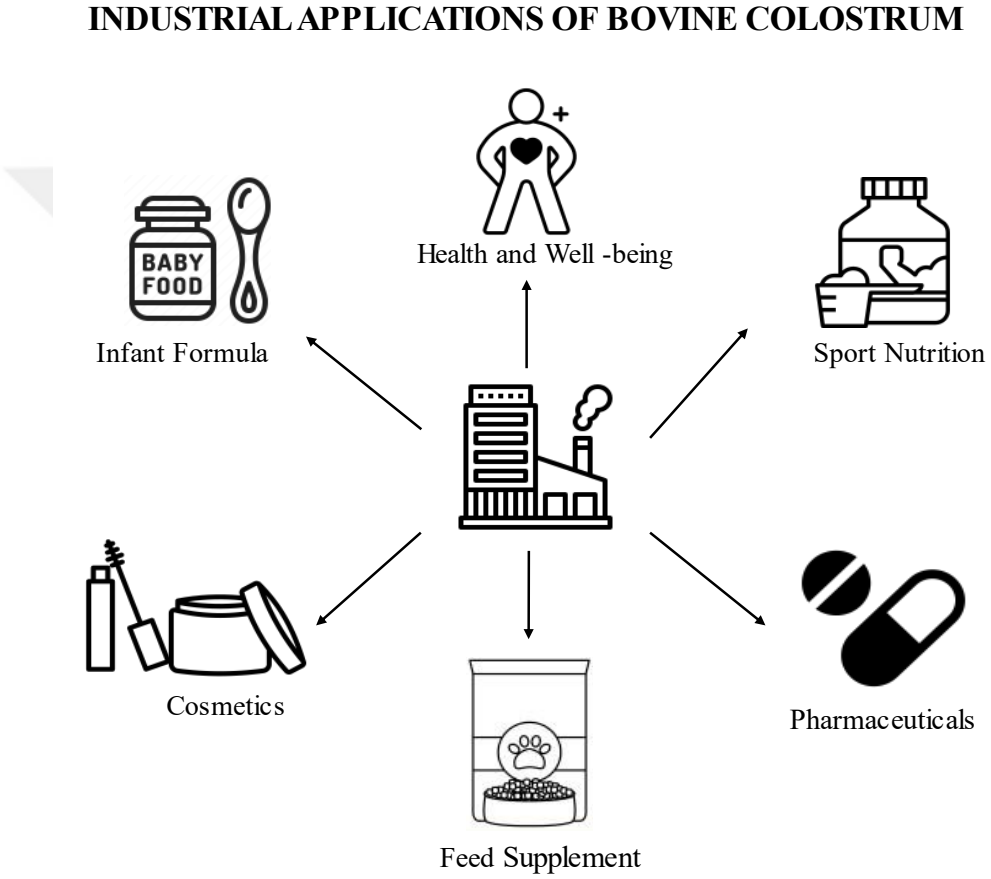

Figure 4. Industrial applications of bovine colostrum for various fields.

1.1.3. Processing of Bovine Colostrum for Human Consumption

Bovine colostrum is processed using assorted approaches, including themal treatment methods such as pasteurization and sterilization, and drying techniques which are majorly freeze drying and spray drying (Kaplan et al., 2022).

Thermal treatment approaches are used in processing of bovine colostrum to eliminate the pathogens that are mainly consisting of *Mycoplasma spp., Salmonella spp.,*

Paratuberculosis as well as *E.coli* (Houser, Donaldson, Kehoe, Heinrichs, & Jayarao, 2008). Pasteurization is one of the heat treatment approaches to minize microbial load by destroying pathogens, involving bacteria, yeasts in addition to molds in food industry (Jay, 1992). It is characterized as high-temperature short-time (HTST) and low-temperature long-time (LTLT) pasteurization. In HTST method, 72 ˚C thorough 15 sec treatment is adequate to eliminate extensive pathogens found in bovine colostrum. However, this approaches can cause the reduction in IgG concentration as approxiamtely 25% (Stabel, Hurd, Calvente, & Rosenbusch, 2004). In LTLT pasteurization, 63 ˚C thorough minimum 30 min treatment is performed. In a study, 34% reduction in IgG concentration of bovine colostrum has been observed after 63˚C thorough 30 min heat treatment while no changes were reported as 60˚C thorough 120 min (McMartin et al., 2006). Additionally, elimination of pathogens was carried out in 60 ˚C thorough 15 min for *E.coli*, 30 min for *M. bovis, Listeria spp., Salmonella spp.,* and 60 min for *Mycobacterium spp.* without any changes in activitiy and concentration of IgG in bovine colostrum (Godden et al., 2006). Donahue et al., (2012) has been demonstrated that 60 ˚C thorough 60 min treatment is efficient to reduce microbial load with no significant shifting in IgG concentration in bovine colostrum. Besides, maintaining of IgG concentration is carried out after 60 ˚C during 30, 60 and 90 min heat treatment (Hesami, Shahraki, Zakian, & Ghalamkari, 2021). Sterilization is known as thermal treatment approach used in destructing all viable microorganisms in dairy industry. Ultra-high temperature (UHT) is a common sterilization technique, which is characterized at 135 ˚C thorough between 2 to 5 sec. UHT provides elimination of all microorganisms, however, several biologically active compounds are destroyed within this treatment (Li-Chan, Kummer, Losso, Kitts, & Nakai, 1995).

Drying strategies are utilized to provide high-quality and durable products subsequently pasteurization. This strategies are crucial to avoid loss in bioactive contents of bovine colostrum. Spray drying and freeze drying are common used drying strategies in dairy industry to produce powder products. Spray drying is related with the transition of liquid form to dried particles, and products, which are produced within this technique are temperature-sensitive. This technique is mostly utilized in processing of milk, merely, in the case of processing of colostrum, significant reduction in the concentration of bioactive compounds is reported with some disadvantages such as loss of aroma, alteration of morphology and particle size etc. (Stewart et al., 2005). Freeze drying, which is also known as lyophilization, is a well-known advanced drying technology, and based on the

evaporation of water in the food under low temperature and pressure. Especially, nutritional value is preserved as well as aroma, texture and appearance within this method. Freeze drying also enable the production of products with a long shelf life without the need for any additives or preservatives (Ciurzyńska & Lenart, 2011). As considering drying strategies, freeze drying is more efficient to maintain bioactivity of immunological contents of bovine colostrum, particularly IgG and lactoferrin. In addition to thermal strategies, the biological, physicochemical and pharmacological activities of therapeutic proteins such as lactoferrin and IgG are affected from biological processes such as glycosylation (Karav, German, Rouquié, Le Parc, & Barile, 2017).

1.2. Glycosylation

Protein glycosylation is a most abundant and also structurally diverse co- or post-translational modifications on extracellular and excreted membrane-assaociated protein structures. It includes free or attached of glycans to proteins. Protein glycosylation is characterized as *N*-linked and *O*-linked glycosylation (*N*- and *O*- glycan) based on the glycosidic linkages between the sugar residues and amino acid. Free glycans contain common lactose core and extended with sialic acid, *N*-acetylglucosamine (HexNAc), and also fucoses. In *N*-glycosylation, *N*-acetylglucosamine (GlcNAc) is attached to the carboxamide group of the asparagine (Asn) residue in conserved chain Asn-Xaa-Ser/Thr (X can be any amino acid except of proline), whereas in *O*-glycosylation, attachment is formed between glycan and the OH group of serine (Ser) or threonine (Thr) residues of a protein (Varki et al., 2017).

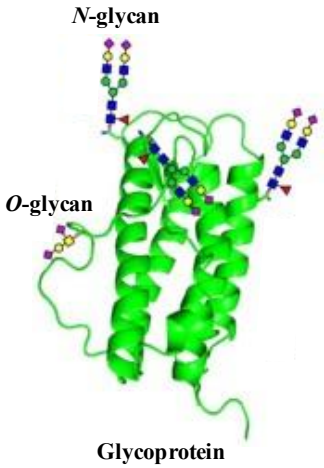

Figure 5. Structure of glycoprotein (Varki et al., 2017).

1.2.1. *N*-linked glycosylation

N-linked glycosylation is the common glycosylation process among eukaryotes. Biosynthesis of *N*-glycosylation is occurred consecutively in both endoplasmic reticulum (ER) and also Golgi apparatus. *N*-glycosylation is initially processed in the ER, where formation of glycosidic linkage between the carboxamide group in Asn side chain residue and GlcNAc at the reducing end is occurred. The specific structurally modifications, such as branching and fucosylation) are introduced with combinatorial expression of subsets of glycosidases and glycosyltransferases in the secretory pathway of the Golgi apparatus. *N*-glycans are characterized in three distinct groups as high-mannose, complex and hybrid according to further processing in the Golgi apparatus with a common core pentasaccharide, $Man_3GlcNAc_2$. High-mannose glycans include five to nine mannose extend the core, complex glycans are characterized as six branches terminated with sialic acid residues, and hybrid glycans contain both unsubstituted and substituted mannose residues with an *N*-acetylglucosamine linkage (Varki et al., 2017).

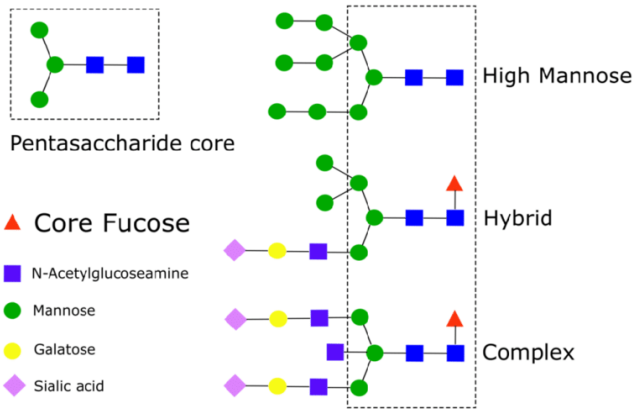

Figure 6. *N*-glycan structures (Varki et al., 2017).

1.2.2. *O*-linked glycosylation

O-linked glycosylation is characterized into two common types as O-GalNAc-type and *O*-GlcNAc-type glycosylation. Biosynthesis of *O*-glycosylation is processed in the lumenal part of the ER membrane, where a mannose residue is transferred to the Ser or Thr residue of a protein, and Golgi apparatus where addition of the *O*-glycans to the core structure is occurred. In *O*-GalNAc type glycosylation, GalNAc is linked to Ser or Thr side chain of the protein with α-glycosidic linkages, whereas GlcNAc is attached to Ser or Thr with β-glycosidic linkage (Peter-Katalinić, 2005). *O*-glycan structures consist of eight distinct cores and further modifications can be introduced such as addition of fucose to obtain various *O*-glycan structures.

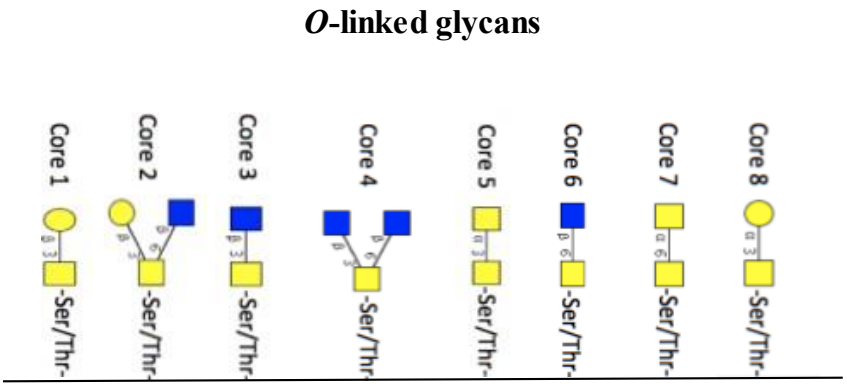

Figure 7. *O*-glycan cores (Varki et al., 2017).

1.2.3. Roles of glycosylation

Protein glycosylation impacts the various biological functions, including protein folding, protein stability, solubility of protein, receptor binding and activation, and cell adhesion and trafficking, and immune responses etc. (Varki & Lowe, 2009). Free glycans inhibit the pathogen attachment to the intestinal epithelial cells, therefore, growth of beneficial microorganisms found in the gut microbiota of newborns is promoted (Ruhaak & Lebrilla, 2012). Free glycans have also role in biological processes as a signaling molecules (Etzler & Esko, 2009). Additionally, sialic acid present in the free glycans responsible for the prevention of rotavirus infection and decreasing the number of leukocytes, as well as brain development of the infants (Bode, 2009). *O*-glycans contribute to protection and maintanance of mucosal epithelial integrity as well as protection against pathogen colonization. Besides, *O*-glycans are associated with various health problems, including cystic fibrosis and inflammatory disease (Brockhausen, 2006). For instance, alterations in sialylation and sulfation of the *O*-glycans cause detrimental effect on the protective barrier function of the mucus, thus, cystic fibrosis and inflammatory bowel diseases can be occurred. *N*-glycans are crucial for vairous biological processess. *N*-glycans released from glycoconjugates can stimulate the growth of crucial gut symbionts, for instance, *Bifidobacterium longum subsp. infantis* (*B, infantis*) owing to their specificity and complexity in terms of composition and structure. Specifically, *N*-glycans have important roles in several biological functions that are related to health and disease. Various studies demonstrated that the biological functions of glycans in cell-cell or cell-host recognition, protein folding and degradation, calnexin-calreticulin cycle, immune signaling, protection against different infections, as well as modulation and development of infant gut microbiome (Karav et al., 2017).

1.2.4. Deglycosylation Techniques

Releasing of glycans from glycoproteins is an essential process in glycobiology studies. Glycans are released from glycoproteins by treating with chemical or enzymatic deglycosylation methods. Chemical methods such as β-elimination and hydrazination which are common chemical deglycosylation approaches are used due to their some advantages such as highly substrate specificity, convenient, and also cost-effective (Sojar

and Bahl, 1987). β-Elimination is a deglycosylation method based on the releasing of glycans under mild alkaline circumstances. Degradation of released glycan chains can be observed because of the high pH (peeling actions). However, the degradation can be avoided with the used of sodium borohydride as a reducing agent to provide stabilization. The limitation of β-elimination is identified due to a single labeling group, which is converted to alditols with the acting of the reducing agent, and can bind to a fluorophore or a chromophore. This situation induces challenges to visualize the released glycans. β-Elimination can also cause sample losses in the step of elimination of high salt components. Hydrazination is associated with the hydrolysis reactions which provide releasing of glycans with a free reducing end. According to reports, hydrazine treatment is more efficient deglycosylation process than β-elimination for releasing *O*-glycans in terms of acitivity and selevtivity (Karav et al., 2015). Additionally, anhydrous forms of hydrogen fluoride is assessed within chemical deglycosylation methods as well as trifluoroacetic acid treatment (Sojar and Bahl, 1987).

Enzymatic methods to release *N*-glycans from glycoproteins is mainly carried out with Peptide-N-Glycosidases (PNGases) and endoglycosidase (Endo). Generally, a PNGase cleaves asparagine-linked glycans to hydrolyze the carboxamide side chain. Releasing of *N*-glycans with commercially available PNGases is carried out of their charge and size (Morelle, Faid, Chirat, & Michalski, 2009), the cleavage ability of these enzymes is limited on fucose linked *N*-acetylglucosamine glycans. The another limitation of PNGases activity is dependent on the glycoprotein structures. PNGase activity involves harsh conditions, such as high heat and detergent to denature of glycoproteins for increasing the accessibility of enzymes. Though, the released glycans and remaining polypeptide structures can be disrupted due to harsh conditions, and also biological acitivities associated with the these compounds can be affected. On the other hand, various endoglycosidases including Endo F1, F2, and F3 are more effective on the native state of the glycoproteins than PNGaseF. However, the activity of these enzymes is not sufficient on multiple-antennary glycan structures. Unlike other deglycosylation approaches, activity of EndoBI-1 can not be affected by fucosylation of glycan core, and harsh reaction conditions such as heat treatment (Garrido et al., 2012).

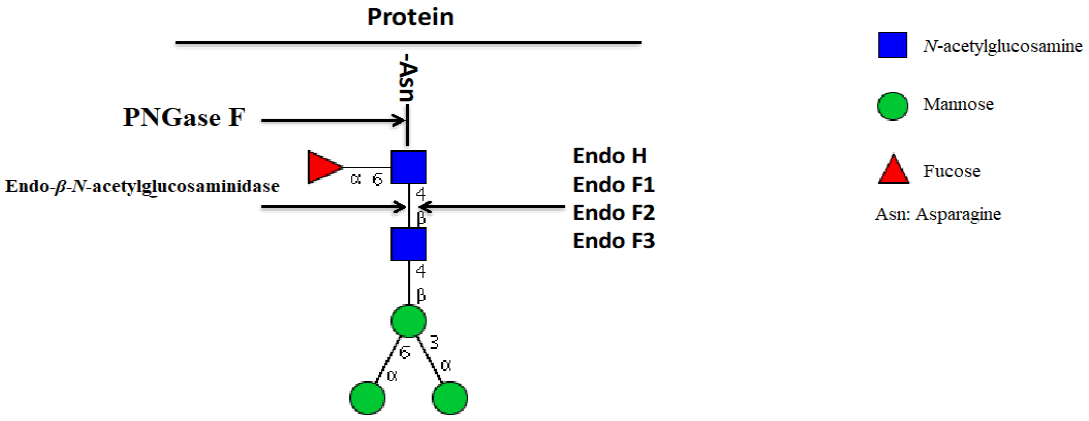

Figure 8. The various glycosidases activities on *N*-glycan core structures (Karav et al., 2017).

1.3. Bovine Colostrum Glycans

Bovine colostrum contains free and conjugated glycans. The free glycans are introduced as neutral (uncharged) and acidic glycans, which consist of *N*-glycolyl neuraminic acid (Neu5Gc) in addition to sialic acid *(N*-acetyl-neuraminic acid (Neu5Ac) residues. To date, more than forty glycan structures which consist of prominently acidic glycans such as include 3′-sialyllactose, 6′-sialyllactose and sialyllactosamine (Arslan, Kaplan, et al., 2021). On the other hand, conjugated glycans are divided majorly as glycolipids and glycoproteins. Bovine colostrum involves *N*- and *O*- linked glycans. Bovine colostrum glycoproteins are characterized as lactoferrin, Igs, κ-casein, caseinomacropeptide, the mucins, milk fat globule membrane, butyrophilin, lactadherin, cluster of differentiation 36 and proteose peptone component 3. Lactoferrin and IgG are the main *N*-linked glycosylated glycoproteins in bovine colostrum (Karav et al., 2017).

1.3.1. Lactoferrin

Lactoferrin is a multifunctional glycoprotein, which has iron-binding capacity. Lactoferrin concentration of bovine colostrum changes markedly thorough the lactation stages, and also depended on the breed of cows (Karav et al., 2017). Lactoferrin is *N*-linked glycosylated glycoprotein, and the glycosylation sites vary from one to five depended on the lactoferrin species. For instance, bovine lactoferrin involves five potential glycosylation sites while human lactoferrin involve three glycosylation sites. Bovine

lactoferrin consists of glycan content as 6.7-11.2% of total molecular weight (Van Veen, Geerts, Van Berkel, & Nuijens, 2002). Highly heterogenous glycan structures are present in the bovine lactoferrin, including Gal, Man, Fuc, GlcNAc, GalNAc, Neu5Ac and Neu5Gc. According to different studies, 42 *N*-glycan structures are identified by Van Veen et al., (2002), and 59 *N*-glycan structures are detected, additionally. Bovine colostrum includes high mannose, complex and hybrid type glycans. Bovine lactoferrin glycosylation is characterized as 65% high-mannose type while 35% hybrid and complex type, and these glycans are introduced 76% neutral, 15% di-sialylated and 9% mono-sialylated (Van Veen et al., 2002).

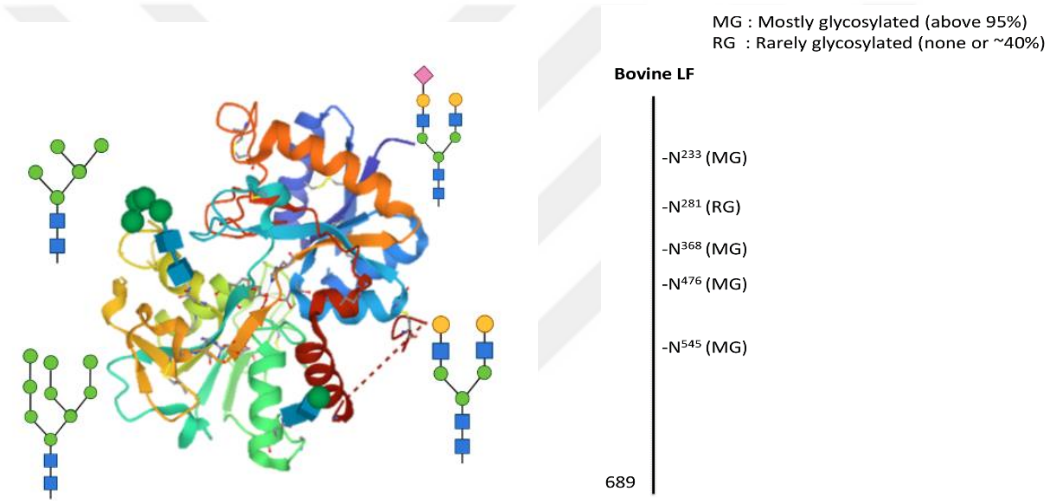

Figure 9. Bovine lactoferrin glycosylation (Protein Data Bank), (Karav et al., 2017)

Glycan moieties provide protection of lactoferrin from digestion by proteolytic enzymes. Bovine lactoferrin is associated with various biological functions, including antimicrobial by inhibition of growth of pathogens due to its iron binding capacity and ability to destruct the membrane of the microorganisms, immune responses, anti-inflammatory activities, antioxidant, modulation of cell growth etc. (Karav et al., 2017).

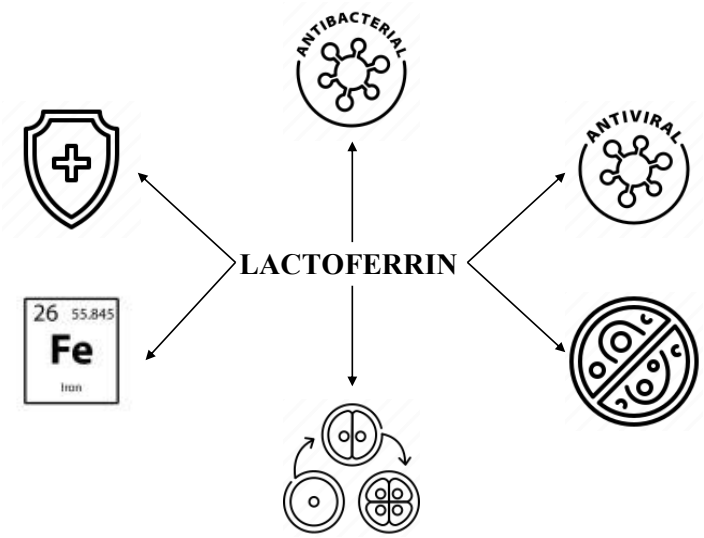

Figure 10. Biological activities of lactoferrin (Karav et al., 2017).

1.3.2. Immunoglobulin G

IgG is the another significant glycoprotein abundant in bovine colostrum which is characterized four polypeptide chains, including two identical heavy and light chains (Sørhaug, Langsrud, Stepaniak, & Vegarud, 1993). IgG includes a *N*-glycosylation site at Asn297 of the Fc region in the each heavy chain (Takimori et al., 2011). Although glycosylation is usually taken place in the Fc region, 20% antibodies is also include glycosylation sites within the Fab (van de Bovenkamp, Hafkenscheid, Rispens, & Rombouts, 2016). IgG contains high mannose glycans which are composed of two sequential GlcNAc moieties as a core glycan attached at Asn297, and two additional mannoses with GlcNAc. Further modifications can be occurred in IgG glycans by fucosylation to the primary GlcNAc, galactosylation to the antennary GlcNAc, and sialylation (Varki et al., 2017). Glycosylation provides protection of IgG from proteolysis, thus, IgG antibodies reach the colon as intact or partially digested form. Bovine IgG exhibits antimicrobial functions against pathogens, supporting intestinal barrier function, anti-inflammatory responses against gastrointestinal inflammation, immune responses, assist shape of the microbiota etc. (Arslan, Kaplan, et al., 2021).

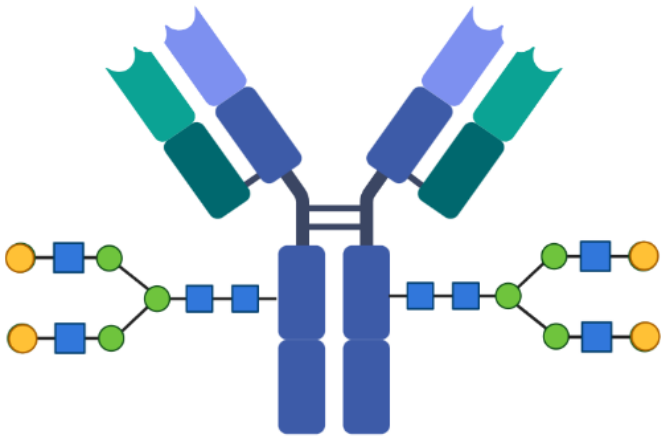

Figure 11. Schematic illustration of IgG *N*-glycosylation adapted from (van de Bovenkamp et al., 2016).

1.4. The Overall Aim of This Thesis

The overall aim of this thesis is that determine the changes of total protein, IgG, lactoferrin and *N*-glycan concentration in bovine colostrum in the time of transition to mature milk.

The overall aims of this thesis;

- ✓ Isolation of protein from bovine colostrum samples
- ✓ Determination of the total protein concentration and visualization of the proteins
- ✓ Determination of the lactoferrin concentration
- ✓ Determination of the IgG concentration
- ✓ Releasing of *N*-glycans from bovine colostrum glycoproteins using novel *N*-glycosidases
- ✓ Quantification of obtained *N*-glycans with phenol-sulfuric acid assay

The objectives of this thesis;

- ✓ Obtain proteins from bovine colostrum samples to evaluate the protein concentration and visualize the protein profile and indicate the changes through the lactation stages.

- ✓ Determine the amount of lactoferrin and IgG glycoproteins which are rich source of *N*-glycans significant for human health.
- ✓ Isolate and purify of *N*-glycans from glycoproteins found in bovine colostrum by using novel *N*-glycosidases and quantify the amount of *N*-glycan concentration of each sample.
- ✓ Evaluate the changes of the *N*-glycan concentration during the 6 days after parturition.

CHAPTER 2
PREVIOUS STUDIES

2.1. Bovine Colostrum and Importance for Health and Nutrition

Bovine colostrum is the first milk produced thorough first days after post-partum. Bovine colostrum is unique due to composition of essential macro- and micro- nutrients that are necessary for the benefits of growth, development and immune response of the newborns (Arslan, Kaplan, et al., 2021). The composition of colostrum is distinct as compared to mature milk, and severely changes throughout the lactation period (Kehoe et al., 2007).

Bovine colostrum is consumed by humans as a health food and medical purposes, for instance, in India, Scandinavia and US (Rocha, 2016). The well-characterization of bioactive molecules of colostrum provides enhancement of demand in consuming bovine colostrum and the end product forms as a dietary supplement, neutraceuticals and dairy products (Arslan, Kaplan, et al., 2021). Thus, revealing of the changes in the concentration of total and therapeutic proteins through transition from colostrum to mature milk is crucial. As a result of the literature searches, there are studies concerning with the changes in the protein, IgG and lactoferrin concentration. In a study, the bovine colostrum proteome in the first 9 days is analyzed with BCA assay. The samples are taken from first-parity, 4 healthy Holstein-Friesian cows. According to results, the protein concentration is approxiamtely 10 fold decrease thorough the 9 days, including maximum value is 169.55 and the minimum value is 13.39, and the significant changes is observed especially in the first three days (Zhang et al., 2015). In another study, decreasing in the total protein and whey protein concentration is observed as 75.99%, and 94.12% in bovine colostrum as well as decreasing in the concentration of the IgG and lactoferrin by 97.90% and 77.44% after five days of parturition, respectively. The changes in the concentration of these therapeutic proteins have been determined by several various studies (Arslan, Duman, et al., 2021).

Lactoferrin and IgG are highly glycosylated proteins found in bovine colostrum. In 1939, isolation of lactoferrin was firstly carried out from bovine milk (Sorensen and Sorensen, 1940). Lactoferrin has bacteriostatic activity. For instance, lactoferrin has antimicrobial effects against Gram-positive such as *Staphlycoccus epidermis* and *Bacillus cereus* as well as Gram-negative bacteria, including *Campylobacter jejuni* and Salmonella. Additionally, lactoferrin can act as a selective anti-microbial agent which provides inhibiting the pathogen growth and stimulating the beneficial bacterial growth, including Lactobacillus and Bifidobacterium species (Petschow, Talbott, & Batema, 1999).

Lactoferrin and IgG are used as a supplement or nutraceuticals due to their various biological activities, and bovine milk is a common source to obtain these therapeutic proteins. Therefore, the examination of the concentration of the bovine colostrum is crucial. Glycosylation process affects the biological, physicochemical and pharmacological activities of these therapeutic proteins (Karav et al., 2017).

2.2. Glycosylation and Importance

Glycosylation is a common and complex forms of co- or post-translational process and associated with the significant roles in several biological pathways, involving protein functions, enzymatic protection and biological recognition as well as pathogen binding (Moremen, Tiemeyer, & Nairn, 2012). Glycosylation is characterized as two major classifications, including *N*-linked glycosylation and *O*-linked glycosylation among eukaryotes. *N*-glycans are attached into Asn side chain of a protein and classified into three groups as high-mannose, complex and also hybrid while *O*-linked glycans are linked with Ser or Thr residue of a protein, and consists of eight core structures. Glycans can be found as free or conjugated forms, such as glycoproteins or glycolipids (Varki et al., 2017).

In a study, lactoferrin of bovine colostrum (1 day) has been obtained with proteolysis and glycan analysis has been performed by MS/MS. According to result of this study, bovine colostrum lactoferrin contains biantennary complex glycans with terminal sialylation and fucosylation as well as non-sialylated glycans, and also high mannose glycans were detected (Gnanesh Kumar and Mattad, 2021). Cao et al., (2019) have been

evaluated how the *N*-glycome changes during lactation. In this study, bovine colostrum samples, which are 0-5 days after parturition, and mature milk samples have been analyzed. Bovine colostrum contains *N*-glycosylated proteins involving nine *N*-glycosylation sites while the five glycosylation sites are determined in matura milk *N*-glycosylated proteins. According to results, the whey *N*-glycoproteomes dramatically changes during lactation stages. Valk-Weeber, Eshuis-De Ruiter, Dijkhuizen, & Van Leeuwen, (2020) have been revealed that lactoferrin, which is isolated from bovine colostrum, glycoprofiles were predominantly with oligomannose glycans such as Man-5 to Man-9 as well as hybrid structures that is formed by expansion with a GlcNAc, and galactose or GalNAc. Besides, complex type glycans including diantennary structures of varying compositions were found, and these hybrid and complex types of glycans were sialylated. Fucosylated and sialylated glycan moieties exist as highest level in early colostrum samples, and reduced based on the lactation times. Variation in the glycosylation profiles of bovine colostrum lactoferrin are also demonstrated (O'Riordan et al., 2014).

Karav et al., (2016) have been demonstrated that 40 mL of bovine colostrum whey contains 80 mg of *N*-glycans, and 18 glycan species were identified as 6 neutral and 12 sialylated moieties. Besides, bovine colostrum whey *N*-glycans, released with EndoBI-1 was utilized by Bifidobacterium as a entirely carbon source. In other studies, bovine milk derived *N*-glycans possess anti-pathogenic function as well as bifidogenic activity (W.-L. Wang et al., 2017). Current studies have been demonstrated that bovine milk *N*-linked glycans obtained with activity of EndoBI-1 enzyme selectively stimulate the growth of *B. infantis*. Specifically, *N*-glycan structures, namely such as 3Hex-5HexNAc, 3Hex-5HexNAc-1Fuc, 5Hex-3HexNAc-1NeuGc, 3Hex-5HexNAc-1NeuAc, 5Hex-3HexNAc-1Fuc-1NeuAc, 5Hex-3HexNAc-1Fuc-1NeuGc and 5Hex-4HexNAc-1NeuAc-1NeuGc, are preferably consumed by *B. infantis*, whereas *B. animalis* is not capable of utilizing these structures. Wang, Du, et al., (2017) demonstrated that whole skimmed bovine milk *N*-glycans have function as bifidogenic activity. The different concentration of *N*-glycans were combined with the growth medium of the Bifidobacteria. As a conclusion, bovine milk *N*-glycans have growth promoting effects for Bifidobacterium species with significant levels of activity starting at 40 nmol/mL and reaching to 80 nmol/mL. Recent *in-vivo* study indicated that lactoferrin and immunoglobulins linked *N*-glycans promote the growth of predominantly *B. infantis*. This study also revealed that EndoBI-1 isolated from *B. infantis*

exhibits unique function as well as structural specificity concerning with the releasing of *N*-glycans from milk glycoproteins (Karav et al., 2019). According to these outputs, many microorganisms in the intestinal microbiota improve different glycan metabolism and the glycans shape the microbiota throughout our lifespan.

CHAPTER 3
MATERIALS and METHODS

3.1. MATERIALS

Table 2

List of materials used in this thesis

Materials	Code	Information
BCA Assay	23225	Determination of Protein Concentration
Lactoferrin ELISA Kit	LS-F4884	Determination of Lf Concentration
IgG ELISA Kit	OKIA00005	Determination of IgG Concentration
2X Laemmli Sample Buffer	TGS10	SDS-PAGE
Sure Cast 40% Acrylamide	HC2040	SDS-PAGE
Sure Cast Ammonium Persulfate	HC2005	SDS-PAGE
Sure Cast TEMED	HC2006	SDS-PAGE
10X Running Buffer	TGS10	SDS-PAGE
10% SDS	S100	SDS-PAGE
Tris	TRS001.1	SDS-PAGE
Acetic Acid	27225	SDS-PAGE
Methanol	947.046.25000	SDS-PAGE
Coomassie Brilliant Blue (R-250)	0472	SDS-PAGE
Sodium Phosphate Monobasic Anhydrous	0471	Preparation of Sodium Phosphate Buffer
Sodium Phosphate Dibasic Dihydrate	04272	Preparation of Sodium Phosphate Buffer
Amicon Centrifugal Tubes	UFC9010	Elimination of Carbohydrate Residue
Ethanol	920.027.2501	Protein Precipitation
Phenol	P4557	Quantification of Glycan Concentration
Sulfuric Acid	1.007.132.500	Quantification of Glycan Concentration

3.2. EQUIPMENTS

Table 3

List of laboratory equipments used in thesis

Equipment List	Brand
Incubator	Indem Nuve EN 400
Autoclave	Nuve
Fume Cupboard	Setra
-80 °C Freezer	Operon
-20 °C Freezer	Vestel
Pure Water System	Millipore
Electrophoresis System and Power Supply	Bio-Rad
NanoQuant	Tecan Infinite M200 PRO
Cooling Centrifuge	Hettich
Heated Stirrer	Wisd
Minicentrifuge	CF-5 Wisd
Vortex	Vortex Genic 2
Block Heater	BSH1002 Benchmark
Centrifuge	Beckman Allegra-X-15R
Shaker	Benchmark-Incu10LR
pH Meter	IsoLab
Ice Machine	Izmak

3.3. METHODS

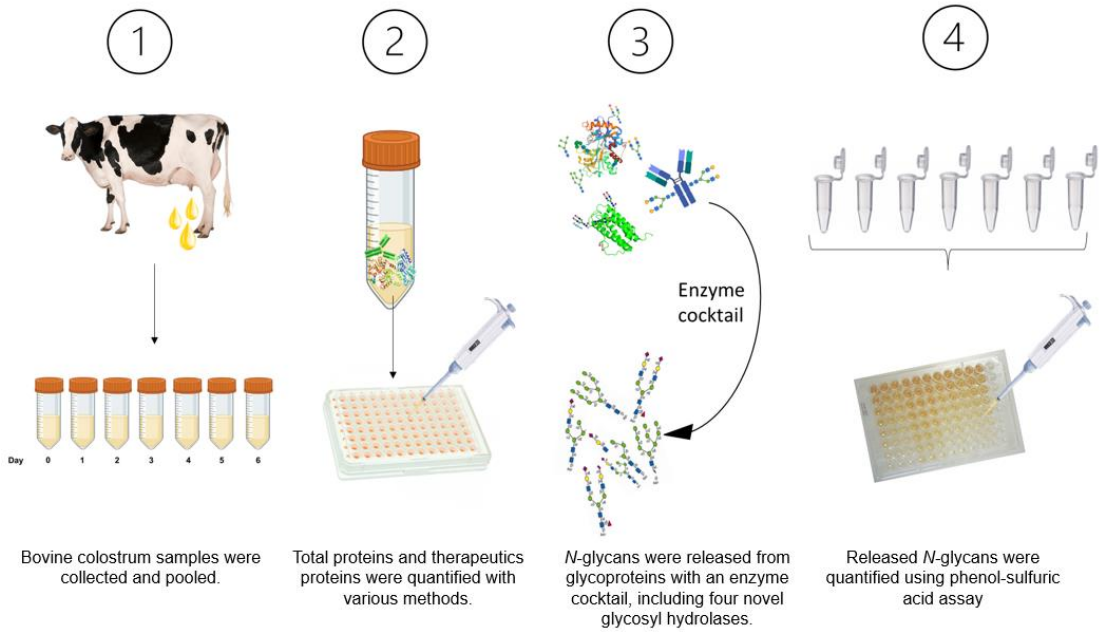

Figure 12. General scheme of methods used in the thesis.

3.3.1. Obtaining of Bovine Colostrum Samples

Bovine colostrum samples were collected during 6 days after post-partum from 28 Holstein Friesian cows at the period July-August, from Uluva Milk Trading Co. in Çanakkale, and all samples were frozen for further studies.

Figure 13. Bovine colostrum samples collected from Uluova Milk Trading Co.

3.3.2. Protein Isolation from Bovine Colostrum Samples

The bovine colostrum samples were pooled based on the day of each sample. Firstly, the colostrum samples were diluted with dH_2O as a 1:3, and all samples were centrifuged at 4 °C, 1000 x g for 30 min. Then, the middle phase from diluted samples was used to determine concentration of total protein by using Bicinchoninic Acid Assay (BCA), lactoferrin with Enzyme-Linked Immunosorbent Assay (ELISA), and visualize proteins by SDS-PAGE. The middle phase of undiluted samples was used to evaluate IgG concentration with ELISA assay.

Figure 14. General scheme of protein isolation from bovine colostrum samples.

3.3.3. Evaluation of Total Protein Concentration

The total protein concentration of pooled bovine colostrum samples was evaluated with BCA assay. Bovine Serum Albumin (BSA) standards were diluted within ranging from 20-2000 µg/mL final BSA concentration using dH_2O as a diluent (Table 4). The middle phase of diluted samples was appropriately diluted as 1:100 (total dilution factor is 1:300), and BCA assay was performed according to microplate procedure. BCA working reagent (WR) was made by mixing both BCA Reagent A with BCA Reagent B (50:1 ratio). 25 µL of each BSA standard or each sample was put into a 96-well plate. Then, 200 µL of the WR were put into the each well. Then, the plate was covered and incubated at 37 °C for 30 min. After that, the plate was cooled to RT to equilibrate the reaction around 10 min, and optical density (OD) value was measured at a wavelength of 562 nm. According to absorbance, standard curve of BSA was created, and the total protein concentration of the samples was evaluated based on the standard curve.

Table 4

Dilution of BSA standards

Vial	Volume of Diluent (µL)	Volume of BSA (µL)	Final Concentration (µg/mL)
V1	0	300 µL of stock	2.000
V2	125,0	375 µL of stock	1.500
V3	325,0	325 µL of stock	1.000
V4	175,0	175 µL of V2	750,0
V5	325,0	325 µL of V3	500,0
V6	325,0	325 µL of V5	250,0
V7	325,0	325 µL of V6	125,0
V8	400,0	100 µL of V7	25,0
V9	400,0	0	0 = Blank

3.3.4. Visualization of The Proteins

The proteins of bovine colostrum were visualized with the SDS-PAGE. 4-12% Bis Tris gel was prepared, including 4% of stacking gel (involving 40% Acylamide/Bisacrylamide, 1M Tris Buffer (pH 6.8), 10% SDS, 10% Ammonium persulfate (APS), Tetramethyl ethylenediamine (TEMED), and dH_2O), 12% of separating gel (involving 40% Acylamide/Bisacrylamide, 1.5 M Tris Buffer (pH 6.8), 10% SDS, 10% APS, TEMED, and dH_2O) according to Laemmli procedure (U K Laemmli, 1970). 5 µL of colostrum samples were treated with 5 µL of Laemmli Sample Buffer and incubated at 95 ˚C for 5 min for degradation of proteins. After incubation, all samples and 5 µL of SeeBlue™ Pre-stained Protein Standard (Invitrogen) ladder were loaded onto the wells. Tris-glycine SDS Running Buffer (Novex) was used in order to run the gel. Then, the gel was treated with Coomassie Brilliant Blue staining solution at RT, 55 rpm for 30 min. Then, destaining solution, including 50% dH_2O, 10% glacial acetic acid and 40% methanol was used during approximately two hours to remove the stain from the gel.

Figure 15. Materials used in visualizing of bovine colostrum proteins.

3.3.5. Evaluation of Lactoferrin Concentration

The lactoferrin concentration of colostrum samples was evaluated with sandwich-type ELISA assay. The middle phase of diluted samples will be diluted appropriately. 0. day bovine colostrum samples were diluted as 1:10.000, 1. Day bovine colostrum samples were diluted as 1:2.000 or 1:6.000, other samples were diluted as 1:1.000 (total DF are 1:30.000, 1:6.000 or 1:12.000, and 1:3.000, respectively). 100 µL of lactoferrin standards or samples were put into the coated 96-well plate and precisely incubated at 37 °C for 1 h. Then, the liquid was aspirated, and 100 µL of detection reagent A working solution was added to wells and incubated at 37 °C for 1 h. Then, wash steps were performed for 3 times with approximately 350 µL of 1 X wash buffer. 100 µL detection reagent B working solution was put into the wells and incubated at 37 °C for 30 min after. After incubation, washing steps were performed as 5 times. After washing steps, 90 µL of TMB substrate solution was put into the wells and incubated at 37 °C for approximately 20 min protecting from light. 50 µL stop solution was mixed with the TMB substrate after incubation. Absorbance was measured at 450 nm. Standard curve of lactoferrin was generated, and lactoferrin concentration in these samples was evaluated based on the standard curve.

Table 5

Dilution of bovine lactoferrin standards

Vial	Volume of Diluent (µL)	Volume and Source of Lf (µL)	Final Concentration (ng/mL)
L1	0	500 µL of stock	100.00
L2	250	250 µL-L1	50.00
L3	250	250 µL-L2	25.00
L4	250	250 µL-L3	12.5
L5	250	250 µL-L4	6.25
L6	250	250 µL-L5	3.125
L7	250	250 µL-L6	1.563
L8	250	0	0 = Blank

3.3.6. Evaluation of IgG Concentration

The IgG concentration in colostrum samples were analyzed with ELISA assay. The middle phase of undiluted samples was diluted with appropriate diluent as 1:400.000. 100 µL of IgG standards or colostrum samples were put into the 96-well plate and incubated at RT for 30 min. After incubation, the liquid part of the each well was aspirated, and wash step was performed as 4 times with 1x wash buffer. Then, 100 µL of diluted Enzyme-Antibody Conjugate was put into the wells and incubated at RT for 10 min. After incubation, wash steps were performed and 100 µL of TMB substrate solution was put into the wells and the plate was incubated at RT for 10 min away from light. Finally, 100 µL of stop solution was put into the TMB substrate after incubation. Absorbance value was measured at 450 nm. Standard curve of IgG was generated, and concentration of IgG in these samples was evaluated based on the standard curve.

Table 6

Dilution of IgG standards.

Vial	Volume of Diluent (µL)	Volume and Source of IgG (µL)	Final Concentration (ng/mL)
I1	900	100 µL of stock	12.300
I2	900	100 µL of I1	1.230
I3	178	122 µL of I2	500

Table 6 (Continue)

I4	150	50 µL of I3	250
I5	150	150 µL of I4	125
I6	100	100 µL of I5	62,5
I7	100	100 µL of I6	31,25
I8	100	100 µL of I7	15,625
D9	100	100 µL of D8	7,813
D10	100	0	0 = Blank

3.3.7. Isolation and purification of *N*-glycans from bovine colostrum glycoproteins by novel *N*-glycosidases

Conjugated *N*-glycans were released by novel *N*-glycosidases, which are recombinantly produced from *B. pullorum* (OU11_RS07620), *B. kashiwanohense* (BBKW_1881), *B. bohemicum* (BBOH_0438) and EndoBI-1 within TUBITAK 1001 Project, 118Z146, under previously optimized conditions (Karav et al., 2015). Briefly, all samples were mixed with the enzyme cocktail, which involves *N*-glycosidases, in a sodium phosphate buffer (20 mM, pH 5). Then, the releasing of *N*-glycans procedure was carried out at 37 °C for overnight. The released glycans were purified by ethanol precipitation, which eliminates the deglycosylated proteins from the mixture. Obtained purified glycans were kept at -20 °C.

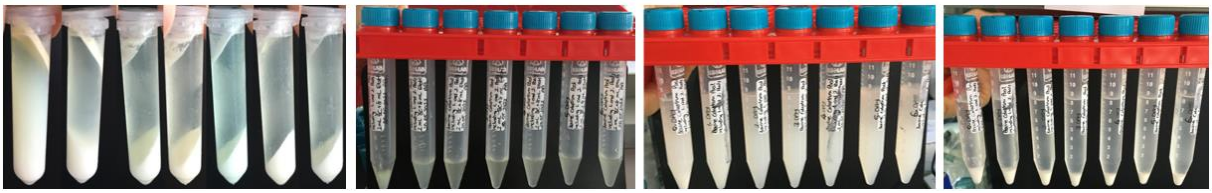

Figure 16. General scheme of protein isolation from pooled colostrum samples.

Table 7

Releasing of *N*-glycans from glycoproteins in bovine colostrum samples

Components	BC				
	BC 0 Control	BC Day 1	BC Day 2	BC Day 3	BC Day 5
dH_2O (µL)	10	10	10	10	10
Sample	150	150	150	150	150
Buffer (µL)	18	16	16	16	16
Enzyme (µL)	2 µL of dH_2O	2 µL of enzyme cocktail	2 µL of enzyme cocktail	2 µL of enzyme cocktail	2 µL of enzyme cocktail
Total Volume (µL)	180	180	180	180	180

3.3.8. Quantification of Purified *N*-glycans

The *N*-glycan concentration was evaluated according to microplate phenol-sulfuric acid assay. Firstly, glucose standards were prepared. Therefore, 1 mg/mL glucose solution (1.55 mg glucose is dissolved with 1.55 mL of distilled water) was prepared and serial dilution was performed to obtain 800, 600, 400, 200 and 100 µg/mL of glucose standards as final volume is 100 µL. 25 µL of standards or colostrum samples were put into a sterile 96-well plate. Then, 25 µL of phenol solution (5%) was put into the wells. 125 µL of sulfuric acid (98%) was put into the wells and mixed immediately at 10 sec. After that, the plate was incubated at least 20 min. After incubation, OD measurement was carried out at 490 nm (Masuko et al., 2005).

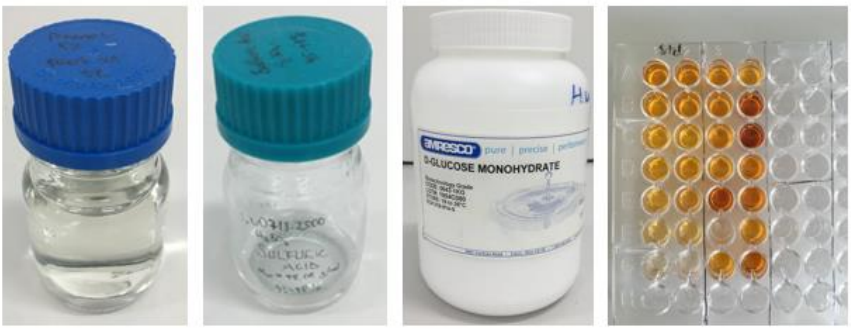

Figure 17. Materials used in phenol-sulfuric acid assay.

CHAPTER 4
RESEARCH FINDINGS

Following the protocol, the concentration of protein, lactoferrin, IgG and *N*-glycans was evaluated in the bovine colostrum samples. The results of analyzes are indicated in Table 8.

Table 8

Protein, lactoferrin, and IgG concentration in bovine colostrum

	Bovine Colostrum		
Days	**Protein (mg/mL)**	**Lactoferrin (mg/mL)**	**IgG (mg/mL)**
0	154.85 ± 43.88	1.72 ± 0.21	78.30 ± 29.01
1	37.75 ± 16.77	0.33 ± 0.04	25.22 ± 16.69
2	23.57 ± 1.42	0.23 ± 0.04	10.24 ± 4.76
3	18.42 ± 5.13	0.15 ± 0.12	5.73 ± 6.07
4	19.28 ± 1.69	0.10 ± 0.11	1.24 ± 0.46
5	16.93 ± 0.93	0.13 ± 0.03	1.60 ± 1.29
6	15.72 ± 0.81	0.12 ± 0.06	0.42 ± 0.09

4.1. Protein Concentration of Bovine Colostrum

The BCA standard curve was indicated in Figure 18. According to BCA standard curve, the equation was y = 0.0012x + 0.0769, and R^2 = 0.9938. The concentration of protein in bovine colostrum was evaluated based on this equation.

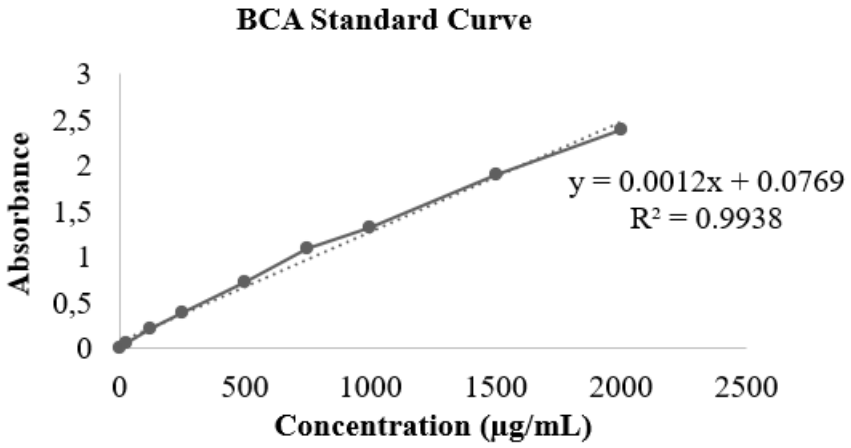

Figure 18. BCA standard curve.

The maximum protein concentration was 154.85, and the minium protein concentration was 15.72 in bovine colostrum during lactation stages. According to results, the avarage concentration of protein was approximately evaluated 41.00 mg/mL. Results of analysis indicated that 10 fold decrement was observed in the concentration of protein thorough 6 days after parturition and the grade of changes was high within 24 hours, especially. These results were similar as shown by (Zhang et al., 2015).

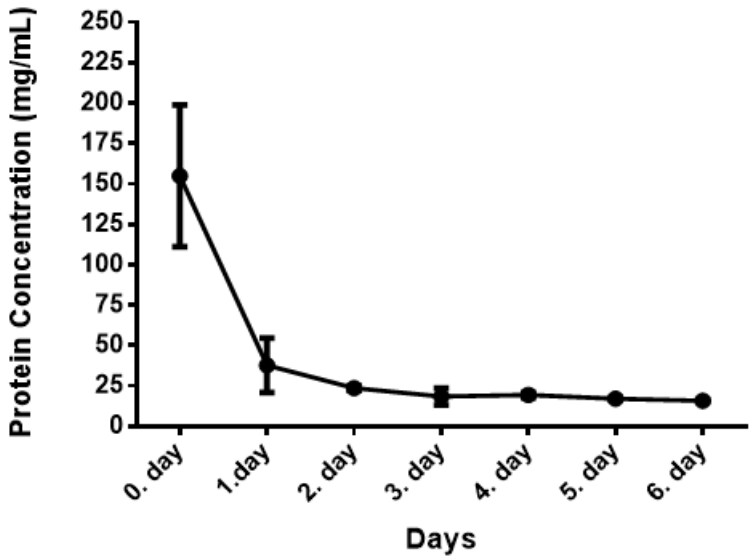

Figure 19. Protein concentration of bovine colostrum

4.2. Visualization of Protein

Molecular weight of bovine colostrum proteins were qualitatively analyzed with SDS-PAGE as well as semi-quantitative analysis of protein fragments. The protein fragments of bovine colostrum were shown in Figure 20.

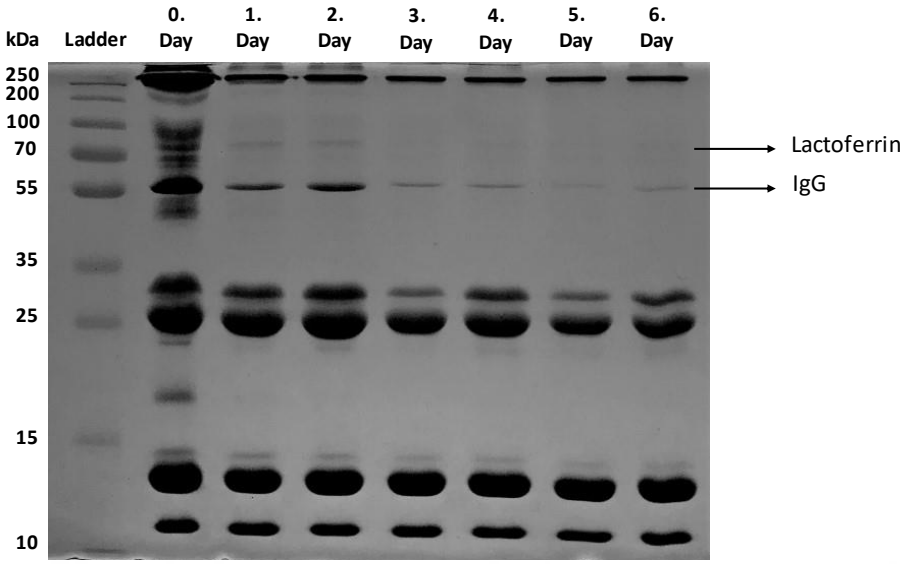

Figure 20. Visualization of bovine colostrum proteins.

4.3. Lactoferrin Concentration of Bovine Colostrum

The LF standard curve was indicated in Figure 21. According to LF standard curve, the equation was y = 71.621x2 – 14.532x + 0.2436, and R^2 = 0.988. The concentration of lactoferrin in bovine colostrum was evaluated based on this equation.

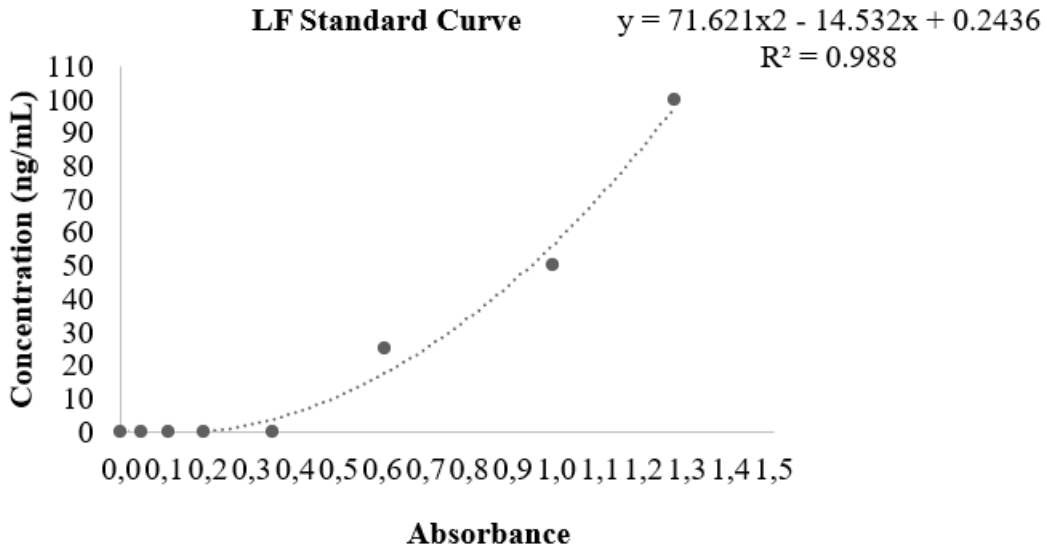

Figure 21. Lactoferrin standard curve.

The lactoferrin concentration of the early bovine colostrum was evaluated as 1.72 mg/mL and reduced to the 0.12 mg/mL during six days after calving as shown in Figure 22. These results were indicated in previous reports (Kehoe et al., 2007).

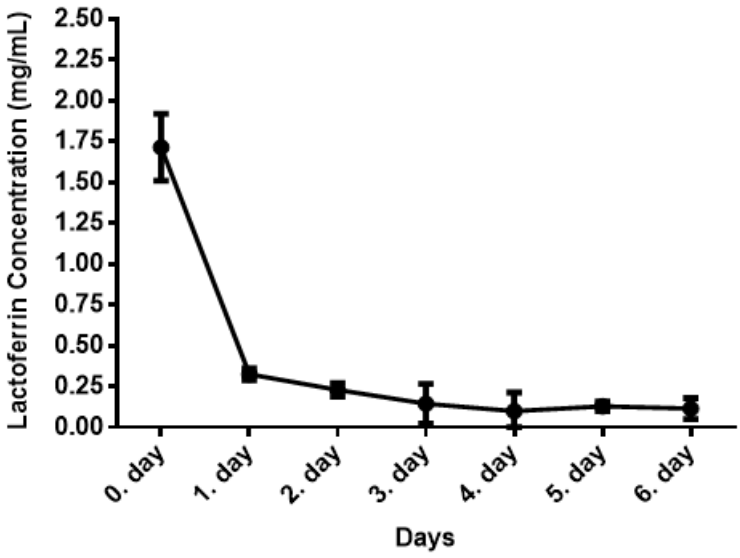

Figure 22. Lactoferrin concentration of bovine colostrum.

4.4. IgG Concentration of Bovine Colostrum

The IgG standard curve was indicated in Figure 23. According to IgG standard curve, the equation was $y = -2E\text{-}05x^2 + 0.0157x + 0.0992$, and $R^2 = 0.993$. The IgG concentration of bovine colostrum samples was evaluated based on this equation.

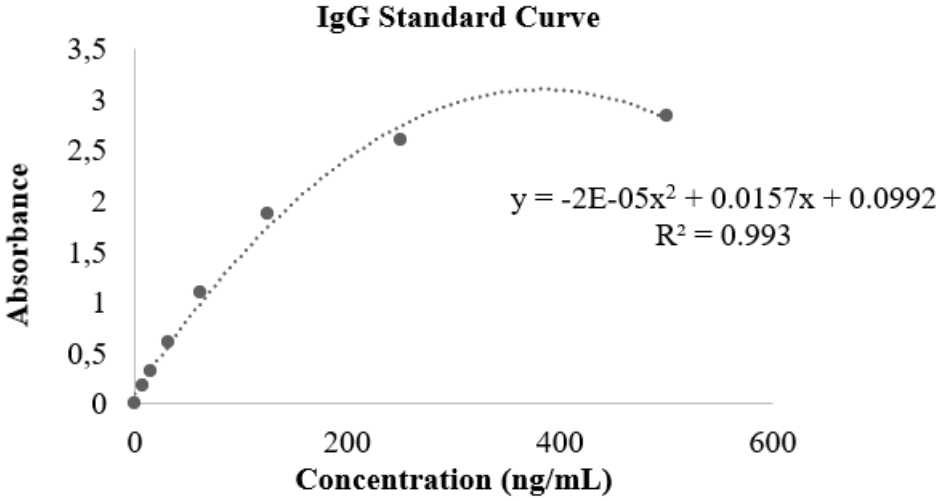

Figure 23. IgG standard curve.

The IgG concentration was found to be as 78.30 mg/mL in the early colostrum collected after post-partum. The highest IgG concentration was found in the first colostrum portions immediately after calving and thereafter were rapidly decreased. Similar results were obtained previously (Newby, Stokes, & Bourne, 1982), and the IgG concentration in bovine colostrum is within the range indicated by various studies (Arslan et al., 2021; Playford and Weiser, 2021).

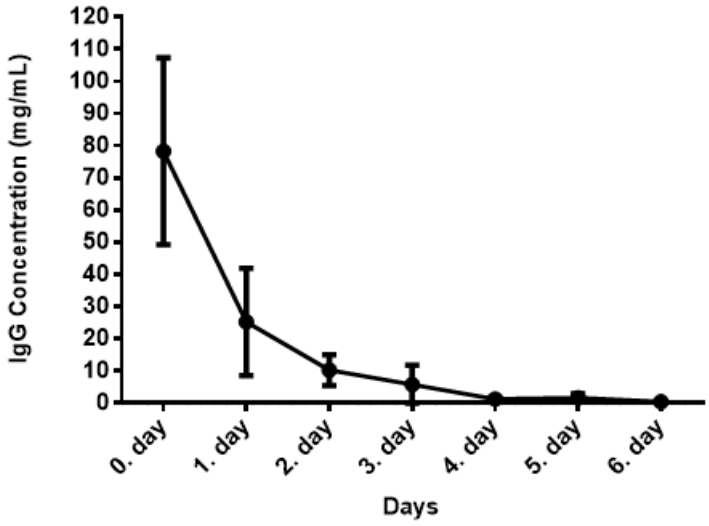

Figure 24. IgG concentration of bovine colostrum.

4.5. *N*-glycan Concentration of Bovine Colostrum

The glucose standard curve was indicated in Figure 25. According to glucose standard curve, the equation was y = 3.9648x, and $R^2 = 0.966$. The *N*-glycan concentration in bovine colostrum was evaluated based on this equation.

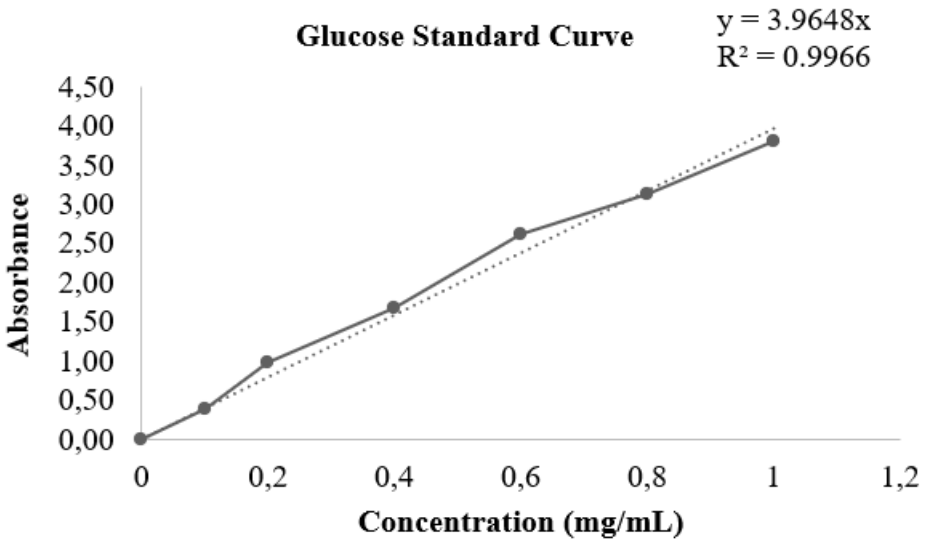

Figure 25. Glucose standard curve.

The *N*-glycan concentration was identified maximum concentration as 1,26 mg/mL and minimum concentration as 0.51 mg/mL. The concentration of *N*-glycans was the highest first days after parturition, and thenceforth decreased during lactation stage.

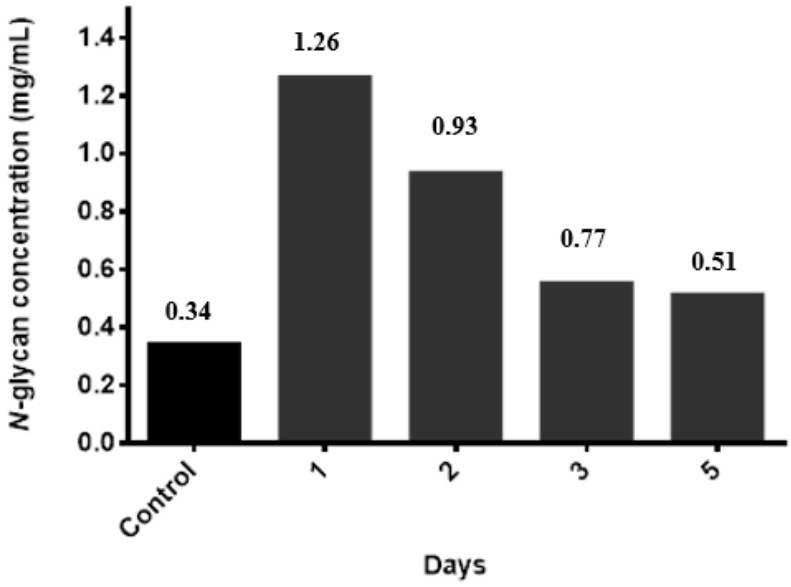

Figure 26. Concentration of *N*-glycan in bovine colostrum samples.

CHAPTER 5
RESULTS and RECOMMENDATIONS

Bovine colostrum is a high nutritional biological fluid used in varioous food and functional applications. Colostrum impacts in wide variety of aspects in human health such as maintenance of gastrointestinal integrity, prevention of microbial infections, reducing the number of symptoms in URTI and diarrheal episodes as well as enhancement of performance and recovery for sportsman due to its bioactive constituents (Arslan et al., 2021; Playford and Weiser, 2021). Consequently, utilization of bovine colostrum and also its therapeutic compounds as a ingredient have attracted in various scientific researches in addition to several industrial fields.

IgG and lactoferrin are the main *N*-linked glycoproteins in bovine colostrum, and have antimicrobial and immunoregulatory functions due to glycan structures. The composition of multifunctional therapeutic proteins found in bovine colostrum changes significantly throughout the lactation stages, consequently *N*-glycan profile is changed. It is reported that glycans contribute to biological functions of these proteins (Karav et al., 2017; W. L. Wang et al., 2017). Bioactive *N*-glycans have significant functions in supporting proliferation and the growth of significant microorganisms which are crucial to neonatal health and shape the gut microbiome composition during the critical period of neonatal development. Several *in-vitro* studies showed that bovine colostrum *N*-glycans stimulate the growth of probiotics such as *B. infantis*, selectively, has several health benefits such as protection against pathogen binding, immune modulation, etc. (Karav et al., 2016). Additionally, various studies have been demonstrated that bovine colostrum derived IgG glycans promote increasing of colonization of various Bifidobacterium species. Takimori et al., (2011) claimed that the glycoprofiles of early colostrum are different as compared to late colostrum, and mature milk due to qualitatively and quantitatively changes in IgG N-glycosylation. For instance, 30% of lactoferrin in bovine colostrum is glycosylated while 15% is glycosylated in mature milk (Wei, Nishimura, & Yoshida, 2000). In early colostrum, higher degree of fucosylation and sialylation are reported for IgG and lactoferrin (Valk-Weeber et al., 2020).

This scientific research provides outputs concerning with the substantial changes of the total protein, therapeutic proteins, including lactoferrin, and IgG, and *N*-glycan concentration in bovine colostrum along the lactation stages. Variaton of the lactoferrin and IgG concentration were detected using sandwich-type ELISA assay, the concentration of total protein was evaluated with BCA assay, the *N*-glycans, which were released with novel glycosidases, were quantified with phenol-sulfuric acid method. As a results in the analyzes, the highest protein, lactoferrin, IgG and *N*-glycan concentration were observed in early colostrum with subsequent decreases in the concentration along three days after parturition. Precise measurements of the bioactive contents are related with the utilization of colostrum for the development of functional products including ice cream, yogurt, butter as well as milk drinks and fermented milks (Ayar, Sıçramaz, & Çetin, 2016). Therapeutic proteins in bovine colostrum such as lactoferrin and Igs in addition to lysozyme, are also used as ingredients in the pharmaceuticals to produce novel neutraceuticals. This study indicated that the composition of bovine colostrum is varied virtually with lactation stages. Therefore, using of efficient, and applicable detection techniques are crucial for assessment of bovine colostrum as well as its bioactive contents used as both supplementary agents and ingredients within the advanced functional food and the pharmaceutical industry.

Detection techniques are essential to determine precisely the amount of the bioactive contents in bovine colostrum. Therapeutic proteins, including lactoferrin and IgG, have been quantified with various methods such as aptasensor, immunoassay, and chromatography radial immunodiffusion as well as electrophoresis. This thesis indicated that application of the well-corroborated and extensively used techniques in scientific researches, including ELISA and BCA assay since assessment of the bioactive proteins of relevant in bovine colostrum. The methods are used due to some advantages, including cost-effective, precise and significantly, are appropriate using for high-throughput applications. On the other hand, in the literature, the releasing of glycans is carried out with various approaxhes using PNGaseF, trypsin digestion or lectin methods. Besides, there are a few studies concerning with the changes in the total protein, IgG and lactoferrin concentration during parturition, however, limited study is found related with changes in the N-glycan concentration thorough transition from colostrum to mature milk for 6 days. Furthermore, in this thesis, a novel enzyme cocktail consisting of three four different *N*-glycosidases was used to release glycans from glycoproteins and using of these

glycosidases provides obtaining structurally and compositionally unique *N*-glycans without any limitations that are observed in another deglycosylation methods.

The demand for colostrum or colostrum derived therapeutic protein products increasing year by year due to its various biological functions such as boosting immune system, improving gut health etc. On the other hand, *N*-glycans have attracted much scientific and industrial interest thoroughout development of state-of-the-art technologies, such as biotechnology. These scientific and technological developments lead to designing of novel functional foods as well as modifying traditional foods. Thus, revealing of changes in the total protein, therapeutic proteins and bioactive *N*-glycan concentration of the bovine colostrum is crucial to provide designing and manufacturing foods which have specific characteristics, and also development of new research.

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APPENDICES

APPENDIX 1

Publication concerning with the thesis issue.

APPENDIX 2

Publication concerning with the thesis issue.

frontiers in Nutrition

REVIEW
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Bovine Colostrum and Its Potential for Human Health and Nutrition

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Colostrum is the first milk produced post-partum by mammals and is compositionally distinct from mature milk. Bovine colostrum has a long history of consumption by humans, and there have been a number of studies investigating its potential for applications in human nutrition and health. Extensive characterization of the constituent fractions has identified a wealth of potentially bioactive molecules, their potential for shaping neonatal development, and the potential for their application beyond the neonatal period. Proteins, fats, glycans, minerals, and vitamins are abundant in colostrum, and advances in dairy processing technologies have enabled the advancement of bovine colostrum from relative limitations of a fresh and unprocessed food to a variety of potential applications. In these forms, clinical studies have examined bovine colostrum as having the substantial potential to improve human health. This review discusses the macro-and micronutrient composition of colostrum as well as describing well-characterized bioactives found in bovine colostrum and their potential for human health. Current gaps in knowledge are also identified and future directions are considered in order to elevate the potential for bovine colostrum as a component of a healthy diet for a variety of relevant human populations.

Keywords: bovine colostrum, human health, bioactive proteins, oligosaccharides, infants

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INTRODUCTION

Colostrum is the earliest milk produced from the mammary glands for the first few days after giving birth and is unique in its composition of essential nutrients, immune factors, and oligosaccharides that benefit the newborn (1, 2). In the case of cows, bovine colostrum is produced immediately after calving and quickly wanes to mature milk (3), which lacks the high level of beneficial nutrients found in bovine colostrum. There are several factors affecting the composition and physical properties of colostrum such as individuality, breed, parity, pre-partum nutrition, length of the dry period of cows, and time post-partum (4). Generally, colostrum has more fat, protein, peptides, non-protein nitrogen, ash, vitamins and minerals, hormones, growth factors, cytokines, nucleotides, and less lactose compared to mature milk content. The concentration of these compounds decreases rapidly in the first 3 days of lactation with the exception of lactose content (5–7).

APPENDIX 3

Publication concerning with the thesis issue.

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REVIEW
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Production of Bovine Colostrum for Human Consumption to Improve Health

Merve Kaplan[1], *Ayşenur Arslan*[1], *Hatice Duman*[1], *Melda Karyelioğlu*[1], *Barış Baydemir*[2], *Bilgetekin Burak Günar*[3], *Merve Alkan*[1,4], *Ayşe Bayraktar*[1,4], *Halil İbrahim Tosun*[4], *Melih Ertürk*[4], *Günay Eskici*[2], *Rebbeca M. Duar*[5], *Bethany M. Henrick*[5,6], *Steven A. Frese*[7] *and Sercan Karav*[1]*

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Colostrum contains all essential nutrients for the neonate during the first days of life, with impacts that continue far beyond these first days. Bovine colostrum has been used for human consumption due to the high concentrations of bioactive proteins, vitamins, minerals, growth factors, as well as free and conjugated oligosaccharides. Processes involved in the preparation of bovine colostrum for human consumption play a pivotal role in preserving and maintaining the activity of the bioactive molecules. As bovine colostrum is a multifunctional food that offers a myriad of benefits for human health, assessing the main processes used in preparing it with both advantages and disadvantages is a crucial point to discuss. We discuss major processes effects for colostrum production on the nutritional value, some advanced technologies to preserve processed bovine colostrum and the end-product forms consumed by humans whether as dairy products or dietary supplements.

Keywords: bovine colostrum, immunoglobulins, thermal processing, drying methods, nanotechnology, liposomal technology, human consumption

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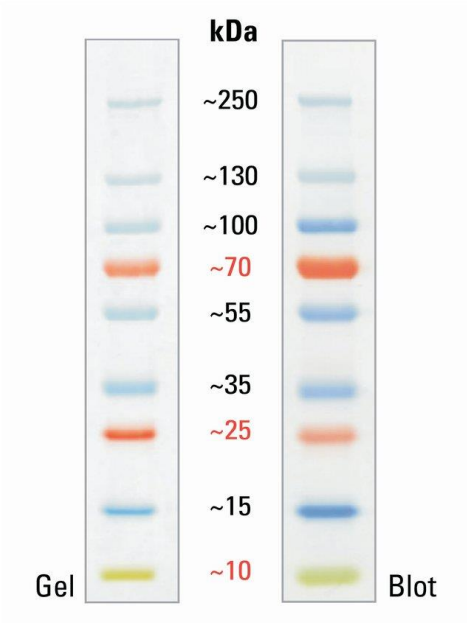
1 INTRODUCTION

Colostrum is the first fluid secreted by mammals for the first few days after parturition (Marnila and Korhonen, 2002; Stelwagen et al., 2009; Godhia and Patel, 2013). This food provides the initial supply of vital nutrients for neonates and plays a crucial part in the nutrition, protection, development, and immunological defense of the newborn (Rasmussen et al., 2016; Juhl et al., 2018). In the absence or limited availability of human milk, bovine colostrum is widely used as an alternative source for infants (Li et al., 2017). As newborn calves do not have an active adaptive immune system of their own, the high concentration of antibodies in colostrum, immunoglobulins, have a major impact on priming the calf's immune system. The small intestine of calves is permeable for the passive transfer of colostrum immunoglobulins through the intestinal wall for only a limited time. The potential for this passive transfer decreases in the first 6–12 h postnatal and becomes impermeable to immunoglobulins by about 24–48 h after birth. Therefore, it is vital to provide colostrum as soon as possible after birth to calves (Sangild, 2003; Baintner, 2007). Even though calves develop a gut closure that stops immunity transfer from mother, colostrum is produced in excess for several days (Marnila and Korhonen, 2002; Stelwagen et al., 2009; Godhia and Patel, 2013). Moreover,

APPENDIX 4

Protein ladder usin as a marker in scientific analysis



BIOGRAPHY