Addis Ababa University
Addis Ababa Institute of Technology
School of Chemical and Bio Engineering

Extraction of camel's milk bioactive component (Lactoferrin) and application for extending storability of whole milk

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University, Institute of Technology, in Partial Fulfillment of the Requirements for the Degree of Masters of Science in School of Chemical and Bio-Engineering

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DECLARATION

I declare that this thesis work titled “Extraction of camel’s milk bioactive component (Lactoferrin) and application for extending storability of whole milk” submitted for master degree at Addis Ababa university is my original work and has not previously been submitted for degree at this or any other university, and that all resources of materials used for this thesis has been dully acknowledged /referred.

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Abstract

Milk must have a desirable chemical composition and must be of satisfactory hygienic quality. Lactoferrin is a multifunctional glycoprotein, occurring as whey protein in milk secretions of mammals. It shows a variety of antimicrobial, antioxidant and antiviral properties. The presence of iron in the environment is essential for bacterial growth. Lactoferrin binds to iron to make it out-of-reach for the bacteria this mechanism can destroy the bacteria. The antibacterial property of lactoferrin makes it suitable for potential exploitation in a variety of food applications. Since the importance of natural antimicrobial is increasing in demand, the present study was designed to extract lactoferrin from whole camel milk so as to use it as a natural preservative of whole milk before processing. Microfiltration, ammonium sulphate precipitation, and protein dialysis technology was used for the extraction of lactoferrin. Whole camel milk produced 0.93 mg/ml of lactoferrin, this result showed that the purification by dialysis technology was moderately good for the recovery of lactoferrin. The lactoferrin was further characterized through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The quantification of camel lactoferrin was done using Bradford Assay. The antibacterial property of camel milk lactoferrin in whole milk was evaluated by the application of Total bacterial count. The evaluated whole milk with the addition of lactoferrin as a natural antimicrobial stayed for 8h at a storage temperature of 30°C with a total bacterial load of 5.36log cfu/ml. the inhibition effect of camel lactoferrin against pathogenic milk bacteria E. coli and Salmonella typhi. was evaluated by the application of disc diffusion method. The different concentration of lactoferrin inhibited both pathogenic microbes, with increased lactoferrin concentration. The results showed that higher concentration of camel lactoferrin had an increased inhibition zone of 28 mm and 19 mm for both E. coli and Salmonella typhi. respectively. The lactoferrin extracted from camel milk exhibited promising antibacterial activity against E. coli and Salmonella typhi.

Keywords: Lactoferrin, SDS-PAGE, Dialysis, Antibacterial effect, camel milk

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List of Abbreviations

ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BLF	Bovine Lactoferrin
BSA	Bovine serum albumin
CFU	Colony forming units
CLF	Camel Lactoferrin
FAO	Food and agricultural organization
FDA	Food and drug administration
HPLC	High performance liquid chromatography
HTST	High temperature short time
LTLT	Low temperature long time
MSNF	Milk solid non fat
MW	Molecular weight
Rf	Relative migration
SCC	Somatic cell count
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel-electrophoresis
SNF	Solids not fat
SPC	Standard plate count
TBC	Total bacterial count
UHT	Ultra heat treatment
WPC	Whey protein concentrates
WPI	Whey protein isolates

Chapter One

1. Introduction

1.1 Background

Milk is a mixture of nutrients required for human nutrition which contains water, milk fat, lactose, milk proteins including casein and small amounts of vitamins and minerals. Milk proteins are composed of casein and whey proteins (Ibrahim et al., 2017). Milk also contains natural bioactive substances, including oligosaccharides, hormones, growth factors, mucins, endogenous peptides, conjugated linoleic acids, triglycerides, transfatty acids, polar lipids, and nucleotides (Trybek et al., 2016). Milk can be obtained from different mammals like cow, goat, sheep, horse, donkey, camel, rabbit, rat, whale etc.

Camels produce milk which can stay for a longer period of time than other species. Camel milk is unique containing various protective proteins like lysozyme, lactoferrin, lactoperoxidase, Immunoglobulins which exert antioxidatives, antibacterial, antiviral, antifungal, hypoglycaemic, antiparasitic, growth promotion, aging prevention, autoimmune diseases and anti-tumor activity than the other milks (Reiter, 1985).

Camel milk contains all essential nutrients. Camel milk is known to be a rich source of vitamin C; the vitamin content was reported to be three times higher than that in bovine milk (Omar et al., 2010). The milk has potential therapeutic properties and useful for preparation of milk products such as yoghurt, cheese, butter, ice-cream etc. (Patel et al., 2016).

The study of these bioactive components in milk has been difficult because of their biochemical complexities, the small concentrations in milk, the need to develop special methods to quantify certain factors due to their particular forms in milk, and the dynamic effects of length of lactation and other maternal factors upon the concentrations or functions of the components of the systems (Young, 2009).

Among the bioactive components of camel milk, Lactoferrin is an 80 kDa iron binding glycoprotein of the transferring family. It is one of minor protein of milk which can be obtained in whey and has a vast application in the inhibition of spoilage and infective bacteria. It has unique properties of iron binding which results in deficiency of iron for gram negative bacteria. Due to its

bacteriostatic properties it can be used for extending the shelf life of milk and meat products (Taylor et al. 2004).

The concentration of citrate in camel milk (128 mg/100 mL) (Moslah, 1994) is lower than in cow milk (160 mg/100 mL). The low level of citrate in camel milk increases the activity of lactoferrin. Several studies showed that lactoferrin concentration in camel milk varies widely in normal milk from 0.02 to 7.28 mg/mL (El - Agamy 1994b; El - Agamy et al. 1996; Abd El - Gawad et al. 1996; Kappeler et al. 1999a; El - Hatmi et al. 2007).

Quality of whole milk is a main factor for the yield of lactoferrin. Milk and dairy products may be contaminated by mycotoxins either directly by contamination of milk with fungi followed by their growth or indirectly which is by contamination of animal feed with subsequent passage of the mycotoxin to milk (Van Egmond, 1989).

1.2 Statement of the problem

Ethiopia has a large number of cattle, but milk and dairy processing industries are challenged by many problems including the shortage of quality whole milk to be used for processing. Milk is a good source of nutrients which is prone to perish easily by growth of microorganisms and spoil during storage at high ambient temperature.

In Ethiopia, high spoilage is reported in milk coming from lowland areas due to high ambient temperature prevalent in the region combined with difficulties in applying refrigeration system, because of the high initial investment, running costs, and technical problems, including the lack or unreliability of an electricity supply (Lumadede et al., 2010). These problems cause difficulties in expanding their milk production system. In a situation like this, it would be beneficial to have access to a method, other than refrigeration, for retarding bacterial growth in raw milk during collection and transportation to the dairy processing plant.

In the past alternative approaches like using synthetic preservatives and antimicrobials has been used to overcome this problem. Nonetheless, this method has not achieved any general acceptance since it has several shortcomings, which can cause severe health effects. Currently attentions have been focused on the native protective proteins antibacterial systems in milk to determine if these could be applied practically to preserve raw milk. But in Ethiopia, there is only limited number of research done with regarding to these important natural preservatives.

The camel milk can stay longer time without getting sour, that is, it takes 8hrs to reach a pH of 5.8 at 30ºC. Although bovine milk takes 3hrs to turn sour (pH of 5.7) at 30ºC. This may be because camel milk contains a greater content of antimicrobial bioactive components such as lysozyme, lactoferrin and immunoglobulins than do bovine or buffalo milk (Benkerroum, 2008).

Lactoferrin is naturally occurring compounds which have been proven to be both bacteriostatic and bacteriocidic to a variety of microorganisms while being harmless to proteins and enzymes of the host organism (González-chávez et al., 2009). Lactoferrin contents of camel milk (0.22 mg/mL) which is significantly higher than goat, sheep, buffalo and cow milk (El -Agamy et al., 1997).The antimicrobial activity of camel milk lactoferrin on cow whole milk should be checked by different microbial assay. However, in Ethiopia, there is a limited research done on using camel milk lactoferrin for whole milk conservation purpose.

Considering the importance of camel milk and the unparallel contribution of lactoferrin in the application antimicrobial property, the present research was persuaded in the following general and specific objectives.

1.3 Objectives of the study

1.3.1 General objective

The general objective of this research was extraction of Camel's milk bioactive component (lactoferrin) and application for extending storability of whole milk

1.3.2 Specific objectives

Specific objectives of this study were:

- ✓ To analyze microbial quality, aflatoxin present and proximate values of whole camel milk
- ✓ To analyze physicochemical properties of whole camel milk
- ✓ To extract bioactive component (lactoferrin) from camel's milk and determine its molecular weight by using SDS-page
- ✓ Applying lactoferrin extract to whole milk as preservative and analyze its antimicrobial effect

1.4 Significance of the study

Camels are one of the few animals not only to survive, but also benefit human beings in hot areas. As the nutritional value is high, the camel therefore provides a nutritious and diluted food, supplying calories, minerals and water, which are greatly needed. A herd of camels producing such amounts of milk would be sufficient to keep numerous people alive in times of drought.

Extending the storability of dairy products till it reaches the consumer is the best option for reducing losses. The use of chemicals in foods is a well-known method of food preservation. Wide varieties of chemicals or additives are used in food preservations to control pH, as antimicrobials and antioxidants, and to provide food functionality as well as preservation action. Most additives are entirely synthetic, such as phenolic antioxidant tertiary butylhydroquinone. But instead of using artificial preservatives, natural based preservatives are appreciated and beneficial for health of human.

Camel milk naturally contains several bioactive components which act as bacterial inhibitors. From this application of naturally occurring bioactive milk whey protein (lactoferrin) for food preservation (antimicrobials) is gaining more and more attention because of consumer's trend of taking natural compounds in their foods.

Recently, lactoferrin has attracted more attention because of the increased problems of antibiotics such as antibiotic resistance, direct toxicity, hypersensitivity, antibiotic induced immune suppression and super infections. Even though lactoferrin can also be found in bovine milk, the yield in camel milk is higher, because the proportion of citrate to bicarbonate in camel milk is low (Moslah, 1994).

Ground-breaking technologies are required to resolve issues with regarding to food preservation in developing as well as developed countries. Therefore, this research work focus on the development of natural preservatives (lactoferrin) for preservation of whole milk. And this paper work can be also used as a secondary document for further study of camel milk bioactive components and product development.

Chapter Two

2. Literature review

2.1 Camel milk properties and composition

Camel milk, generally opaque and white, has an acceptable taste. The milk normally has a sweet and sharp taste, but sometimes can also have a salty taste due to the type of plants eaten in the desert by the camels (Khaskheli, et al, 2005). The changes in taste are mainly caused by the type of fodder and availability of drinking water (Farah, 1996). The average density of camel milk is 1.029 g/cm^3, which is less viscous than bovine milk. The viscosity of camel milk at 20ºC is 1.72 mPa s, whereas the viscosity of bovine milk at the same dry matter content and under the same conditions is 2.04 mPa s. The pH of fresh camel milk ranges from 6.5 to 6.7, a slightly lower pH of 6.4 and 6.0 have also been recorded. The buffering capacity of skim camel milk was reported to be lower than that of bovine milk (Al-Saleh & Hammad, 1992).

Camel milk is more resistant to heat compared to bovine milk. The proximate composition of camel milk and milk of other species is given in Table 2-1. The unprocessed camel milk has shelf life of 5 days at 7°C. But shelf life of pasteurized milk is 22 days, when heated at 65°C for 20 minutes and kept at 7°C. The whole milk can also be stored for one year in frozen condition.

Table 2-1 Proximate chemical composition of camel milk and other species milk

Proximate	Water %	Protein %	Fat %	Ash %	Lactose %
Camel	86-88	3.0-3.9	2.9-5.4	0.6-1.0	3.3-5.8
Cow	85-87	3.2-3.8	3.7-4.4	0.7-0.8	4.8-4.9
Buffalo	82-84	3.3-3.6	7.0-11.5	0.8-0.9	4.5-5.0
Sheep	79-82	5.6-6.7	6.9-8.6	0.9-1.0	4.3-4.8
Goat	87-88	2.9-3.7	4.0-4.5	0.8-0.9	3.6-4.2
Human	88-89	1.1-1.3	3.3-4.7	0.2-0.3	6.8-7.0

Source: (Fox, 2003)

- **Proteins**

Total protein content of camel milk ranges from 2.15 to 4.90 percent (Konuspayeva et al., 2009) Camel milk protein i classified into two main groups as described in the following sections.

i. Caseins

Casein is a major part of protein in camel milk. It has 1.63 to 2.76 percent casein protein that constitutes 52 to 87 percent of total milk protein. In whole casein portion, β-CN is 65 percent and αs1-CN is 21 percent, Camel milk has more digestibility and less allergic reactions in infants as αs-CN slowly hydrolyze than β-CN. 3.47 percent k-casein is present in camel milk casein, while 13 percent is found in milk of bovine (Omar et al., 2010). The amino acid compositions of camel milk caseins are similar to cow milk casein fractions.

ii. Whey proteins

Whey protein in camel milk constitutes 20 to 25 percent that make it the second biggest fraction of protein found in the milk of a camel. Camel milk has a whey protein in range of 0.63 and 0.80 percent. Camel milk β-lactoglobulin is found in traces, while α-lactalbumin comprises the major portion of camel milk. In the milk of bovines, α-lactalbumin constitute only 25 percent, while β-lactoglobulin made 50 percent of the total whey protein that make it the major whey protein of bovine milk. Whey protein of camel milk consists of some other main components such as peptidoglycan recognition protein, immunoglobulins, lactoferrin, lactoperoxidase and serum albumin. Camel milk whey has an acidic protein (12.5 kDa) possessing a potential protease inhibitor. Depending on these findings it is suggested that the higher level of natural preserving agents may bring about longer storage or shelf life (El-Agamy, 2009).

Fats

Milk fat serves nutritionally as an energy source, acts as a solvent for the fat-soluble vitamins and supplies essential fatty acids. The fat content of camel milk is between 1.2 and 6.4 percent and its contents can be reduced from 4.3 to 1.1 percent in the milk of thirsty camels (Konuspayeva et al., 2009). The lipid fraction in camel milk is characterized by a high proportion of long chain fatty acids, which accounts for 96.4 percent compared to 85.3 percent in bovine milk. It is reported that the cholesterol level of fat of camel milk ($34.5 mg.100 g^{-1}$) is higher as compared to cholesterol level ($25.63 mg.100\ g^{-1}$) of bovine milk fat (Konuspayeva et al., 2008). Milk fat of dromedary camels carries a lower level of carotene and lesser concentrations of short chain fatty acids as compared to milk of bovine (Stahl et al., 2006). This lower carotene content could explain the whiter color of camel milk fat (Abu-Lehia, 1989).

Melting point (41.9ºC) and solidification (30.5ºC) temperature is higher in camel milk fat, compared with bovine milk fat (32.6 and 22.8 ºC respectively), probably because camel milk fat contains a lower amount of short chain fatty acids (C4-C12) and a higher amount of long chain fatty acids (C14-C22) compared with bovine milk fat in addition to the differences in isomeric properties of oleic acid (Omar et al., 2010).

Butter was reported to be only produced from camel cream at a high churning temperature of (20-25) ºC. These temperatures are higher than those values reported for bovine milk butter manufacture of 8ºC-12ºC. Also, camel milk fat has been reported to be more viscous (Attia et al., 2000).

- **Lactose**

Lactose is the major carbohydrate fraction in milk and is a source of energy for the young calf. It is made up of two sugars, glucose and galactose, which are fermented to lactic acid when milk goes sour. Camel milk lactose contents ranged between 2.40 to 5.80 percent (Konuspayeva et al., 2009). The nature of vegetation eaten by the camels in desert areas could be a significant factor for extensive variation in lactose contents. Camels generally like to take *halophilic* plants like *Salosa*, *Acacia* and *Artiplex* to fulfill their physiological necessities of salts (Yagil, 1982).

- **Minerals**

Milk mineral salts are mainly chlorides, phosphates and citrates of sodium, calcium and magnesium. The total content of minerals is usually expressed as total ash; this amount varies from 0.60 to 0.90 percent in Dromedary camel milk (Konuspayeva et al., 2009). The mean values for zinc, manganese, magnesium, iron, sodium, potassium and calcium in mineral contents of camel milk ($100g^{-1}$) are 0.53, 0.05, 10.5, 0.29, 59, 156 and 114 mg respectively (Omar et al., 2010).

The minerals Na, K, Fe, Cu and Mn in camel milk are substantially higher than that reported for bovine milk. Fe was reported to play an essential role in a number of biological systems, including oxygen transport and storage as well as DNA synthesis (Miller, 1996).

- **Vitamins**

Numerous vitamins such as D, E, A, C and vitamins of B group are found in dromedary camel milk (Stahl et al., 2006). Rich amount of vitamin C is present in camel milk. Camel milk contains three to five times more vitamin C as compared to bovine milk. The mean value of vitamin C

concentration present in camel milk is 34.16 mg.L^{-1}. Camel milk contains higher concentration of niacin (B3) as compared to bovine milk (Farah and Atkins, 1992).

2.2 Camel milk production

Camels are known to occupy the arid and desert countries. The world's total population of camels was reported to be 22,000,000 in 2010 that could produce about 300 million liters milk representing 0.2% of world's total produced milk in 2010 (IDF, 2010). Also, according to FAO data, the production of camel milk is 5.3 million per liter in the world. At the present time, depending on the camel cultivation camel milk production is also becoming increasingly common.

Under these harsh conditions, camels have the capability to produce more milk than any other species and for longer periods of time, while their feed requirements are modest (Omar et al., 2010). Each camel produce between 1000-2000 L of milk per lactation period of 8-18 months (FAO, 2010). Their daily milk production average is estimated to be between 3 and 10 kg during a lactation period of 12-18 months (Omar et al., 2010). The yield could increase to 20 L per day under improved feed, husbandry practice, water availability and veterinary care (FAO, 2010).

East Africa is the first region in the world in camel milk production (66%) followed by West Africa (20%), Asia (9%) and North Africa (5%). Ethiopia possesses about 925,000 camels, the annual camel milk production in Ethiopia is estimated to be 170,000 tone and rank fourth by camel milk production in Africa next to Somalia 1,100,000 tone, Kenya 937,000 tones and Mali 242, 911 tones (FAO, 2010).

The dwellers of these areas are mainly pastoral and the camels travel according to the range conditions. The major ethnic groups owning camels in Ethiopia are the Beja, Rashaida, Afar, Somali and Borana (Sisay & Awoke, 2015). There are some agents that are involved in the camel milk production and marketing. These sectors are found in Ethiopian Somali region and other pastoral areas. Most of the camel milk is sold to restaurants, cafes, householders, and drivers for making tea or for direct consumption by their customers. In addition, the producers also use the milk for private home consumption.

Camel milk is one of the basic sources of income, food and other socio-economic and cultural needs both for rural and urban dwellers in the region. Nevertheless, despite its significant contribution to the livelihood of the pastoralist society who does have petite alternative mode of

production system, up until recently the camel is one of the neglected domestic livestock by scientific community in Ethiopia.

2.2.1 Camel milk dairy products

Camel milk is suitable for drinking and various other products can be produced from camel milk including soft cheese, fermented milk, yoghurt, ice cream and butter. Yoghurt produced from camel milk (with no additives) has a thin, flowable and very soft texture. The addition of both 0.75% sodium alginate and 0.075% calcium chloride to camel milk produce acceptable firmness and body similar to that for yoghurt produced from bovine milk (Hashim et al., 2008).

Ice cream can be produced successfully from camel milk using a mixture of 12% fat, 11% milk solids not fat (MSNF) and 37% total solids. The overrun of camel milk ice cream was found to significantly depend on the fat and MSNF levels in the mixture (Abu-Lehia et al., 1989). For example, the increase in fat and MSNF content in the mixture leads to an increase in viscosity. These products are still not well developed enough to reach a commercial scale, and there is also a need to examine consumer acceptability of these products.

2.3 Health benefit of camel milk

Camel milk contains disease fighting immunoglobulins which are small in size, allowing penetration of antigens and boosting the effectiveness of the immune system. It is a rich source of insulin which makes it a great treatment option for Type-1 and Type-2 diabetics as well as gestational diabetes (Agrawal et al., 2003). Camel milk can be used for the treatment of different types of tuberculosis. Camel milk is supposed a precautionary in ulcers.

The milk protein lactoferrin, which is present in large quantities in camel milk (ten times higher than in cow milk), does have some antiviral and antibacterial properties. There is considerable evidence that oxidative stress causes many neurological diseases including Parkinson, Alzheimer, amyotrophic, strokes, seizures as well as rheumatoid arthritis, fatigue and cancer. The presence of lactoferrin as a mediator in the control of oxidative stress and its role in immune homeostasis reduce neurological diseases and increase longevity (Jahani, Shakiba, & Jahani, 2015). Camel milk helps in reducing coronary heart diseases. Camel milk also benefits in infection, gastroenteritis and cancer (Arjita & Desh, 2016).

Autism disease is general terms for a group of complex disorders of brain development. The etiology of many autistic cases is based primary on autoimmune disease, affecting an intestinal enzyme responsible for the formation of amino acids from the milk protein casein. The most prominent cerebral symptoms are caused by a malfunction in the formation of amino acids from two caseins in cow milk, beta-casein and beta-lactoglobulin. Instead, a powerful opioid, casomorphine, is formed (Yagil and Reuven, 2013). This opioid elicits the cerebral symptoms of the autism syndrome. But Camel milk does not contain the two caseins that form casomorphine from cow milk, so symptoms do not develop (Al-Juboori et al., 2013). In addition, camel milk contains protective proteins, including Igs necessary for initiating the immune system and nutritional advantages for brain development (Al-Juboori et al., 2013). Furthermore, camel milk has emerged to have potential therapeutic effects in autism (Sharma and singh, 2014).

Camel milk was recently suggested as a healthy food alternative to children with allergenicity to bovine milk. Hypoallergenicity of mothers' milk was reported to be due to the high percentage of β-CN, low percentage of αs-CN, deficiency of β-lactoglobulin, and similarity of the immunoglobulins (El-Agamy et al., 2009). Camel milk has been used for the treatment of food allergies and autism (Patel et al., 2016). Camel milk contains higher amount of zinc. The rapidly dividing cells of the immune system are sensitive to zinc deficiency. The role of Zn in the development and maintenance of a normally functioning immune system has been well established.

Camel milk has positive effects in controlling high blood pressure and helps in the management of Arteriosclerosis and Osteoporosis. Camel milk lysozyme showed a higher lysis value towards Salmonella typhimurium compared to egg white and bovine milk lysozymes. Camel milk is used for treating dropsy, jaundice, spleen ailments, tuberculosis, asthma, anemia and piles (Patel et al., 2016). The patients suffering from chronic hepatitis had improved liver functions after drinking of camel milk (Sharmanov et al., 1978).

Camel milk has powerful bactericidal properties and can rehabilitate the immune system. It was observed that drinking non-pasteurized camel milk is beneficial to people with all the variety of symptoms associated with an infection of the alimentary canal (Shabo et al., 2005).

2.4 Quality of raw milk

Milk is an important raw material for the production of a variety of dairy products. It is therefore important that the milk used for processing has acceptable quality characteristics. Microbial growth primarily bacteria, also some molds and yeasts are the primary cause for loss of acceptability of milk and milk products. Quality characteristics for raw milk include compositional quality, microbial contamination levels, somatic cell count, freedom from inhibitory substances, and reception temperature (Shafiur M., 2011).

The most common grades of raw milk are Grade A and Manufacturing Grade. Grade A milk must not exceed 100,000 cfu /mL standard plate count (SPC) for an individual milk producer, 300,000 cfu/mL SPC as mixed milk as shown in Table 2-2. The SPC measures all bacteria able to form colonies on standard methods agar within 48 h under aerobic conditions at 32°C.

Table 2-2 Bacteriological Standards for Raw and Pasteurized Milk

Product	**Test**	**Standard**
Grade A raw milk and milk products	Total bacterial count	≤100,000 cfu/mL before mixing ≤300,000 cfu/mL after mixing
Grade A pasteurized and milk products	Total bacterial count Coliform count	≤ 20,000 cfu/mL ≤ 10 cfu/mL

Source: U.S. Public Health Service, 1995.

The milk quality is determined by aspects of composition and hygiene of milk, where breeding, feeding, management system, genetics and many such facts mainly influence the compositional quality. In addition, good-quality milk must not contain pesticides, antibiotics, sanitizers, drug residues, and other abnormalities. Hygienic conditions and improved animal husbandry practices in Ethiopian village households and small farms located at the low lands should be reached, in order to achieve quality milk.

In Ethiopia fast deterioration in milk quality has been perceived by the time it reaches from producer to dairy processing plant. This is due to the fact that under the insufficient storage conditions, nutrients in the milk which are a good medium for the growth and development of individual groups of microorganisms.

In most circumstances, the microorganisms that cause the spoiling of milk reduce the quality of raw milk to some extent, which results in significant economic losses. These reduced quality results from different chemical and biochemical bonds which cause change of appearance, smell, texture, taste, and aroma of the raw milk. The appearance of those bonds is hardened by metabolic degradation of some components of the milk, by microorganisms causing the spoilage or by their enzymes.

Even though pasteurization process can destroy most of bacteria in raw milk, shelf life of pasteurized fluid milk is influenced by the quality of raw milk. For this reason maintaining high quality raw milk at farm level is very important, and hence, the first steps in preserving the quality of milk must be taken at the farm.

2.4.1 Microbiology of raw milk

Milk is an excellent medium for the growth of a variety of microorganisms owing to its high-water content, pH (6.4–6.6), and ample supply of nutrients. Numerous selective and differential tests can be used to determine the presence or absence of specific types of bacteria in raw milk. A significant increase in the SPC after preliminary incubation is considered to be indicative of unsanitary production practices. The coliform count, in which samples are plated on the selective and differential medium Violet Red Bile Agar and incubated for 24 h at 32°C, estimates the number of coliform organism's present (Christen et al., 1992). The presence of these organisms can also indicate unsanitary production and processing practices. The selective and differential Edwards Medium can be used to isolate streptococci, which can be indicative of mastitis in the herd (Atlas, 1993).

Characterization of the bacterial population present in raw milk must always consider the limitation inherent in any analytical technique. No one test can detect all bacteria. Even non-selective tests designed to determine total bacterial numbers cannot detect fastidious organisms that require additional nutrients, slow-growing organisms that require more time to form visible colonies, or poor competitors that require selective media to ensure sufficient nutrient access.

The numbers and types of microorganisms in milk immediately after production (i.e., the initial microflora) directly reflect microbial contamination during production, collection, and handling. In Ethiopia, a standard for very good raw milk bacteriological quality is ≤ 200,000 counts per ml (ES ISO 6610) for milk intended for heat treatment before consumption.

For milk that is to be consumed raw, a more stringent standard generally is required because consumers of raw milk are at a greater risk for contracting a milk-borne illness such as salmonellosis.

The microflora in the milk when it leaves the farm is influenced significantly by the storage temperature and the elapsed time after collection. Where milk is stored at ≤4°C, this low temperature normally will delay bacterial multiplication for at least 24h. The microflora, therefore, is similar to that present initially. However, if unsanitary conditions exist with the milking equipment or storage tank, the low temperature could mask these conditions.

A useful indicator for monitoring the sanitary conditions present during the production, collection, and handling of raw milk is the "total" bacterial count or standard plate count (SPC). The SPC is determined by plating on a standardized plate count agar followed by aerobic incubation for 2 or 3 days at 32°C or 30°C, respectively. Microorganisms failing to form colonies, will not be counted.

The SPC does not indicate the source(s) of bacterial contamination or the identity of production deficiencies leading to high counts. Its sole value is to indicate changes in the production, collection, handling, and storage environment.

The genera associated with the Coliform group include *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia, and Citrobacter* (Guentzel, 2007). Testing for these non psychrotrophic bacteria is used as a general indicator of contamination. As some of the coliforms are associated with animal and human feces, a coliform test may also indicate fecal contamination. *Escherichia coli* is the primary pathogen associated with the coliform group.

2.4.2 Effects of Microbial contamination on Milk Quality

The presence and growth of bacteria in milk affects milk quality. Chemical components of milk can be degraded by bacterial metabolism and various enzymes secreted by bacteria. Products of these degradation reactions can have undesirable effects on milk structure, smell, and taste.

Lactose present in milk is readily fermented by lactic acid bacteria, resulting in sour flavor notes and, if the pH of milk drops below 4.6, precipitation of casein proteins (Bylund, 1995; Jay, 2000). Fermentative metabolism of lactose by a variety of bacteria can also produce numerous volatile compounds, including acetic and butyric acids, carbon dioxide and hydrogen gas, and various alcohols that can adversely affect milk odor and flavor.

Proteins are also subject to degradation by bacteria and their secreted enzymes. Digestion of proteins by extracellular proteases can create bitter-tasting peptides; cause curdling and clotting of the milk; result in production of ammonia and hydrogen sulfide; and ultimately cause gelation of the milk. Lipase, which breaks down triglycerides, creates short chain fatty acids that give milk a rancid smell and taste.

A malty flavor or odor can occur in milk if *Streptococcus lactis var. maltigenes* grows and metabolizes amino acids in milk to aldehyde and alcohols. Growth of molds, yeasts, coliforms, *Pseudomonas spp.*, *Actinomyces spp*., and *Lactococcuslactis ssp.lactis biovar. maltigenes* can give milk musty, fruity, cow like, fishy, earthy, or malty odors, respectively.

2.4.3 Control of microorganisms in milk

- **Refrigeration**

Ideally, microbial contamination of raw milk and milk products should be addressed primarily through preventive measures on the farm and throughout processing. Reduced temperatures inhibit growth of mesophils and thermophils and reduce the activity of degradative enzymes. Modern dairy farms use refrigerated bulk storage tanks which maintain milk at 4°C or below. As bulk tank milk pick-up typically occurs daily or every other day, product from multiple milking is frequently mixed and stored in the same tank.

- **Heat Treatment**

Heat treatment plays a critical role in controlling bacterial numbers in processed milk products. The three basic approaches to heat treatment of raw milk, pasteurization, ultra-pasteurization and UHT, differ primarily in their underlying purpose. Pasteurization aims to eliminate the non-spore-forming pathogen most resistant to thermal destruction, currently recognized as being *Coxiella burnetii*, and concurrently reduce nonpathogenic bacterial numbers in milk. Ultra-pasteurization adds the additional goal of increasing product shelf life through further reduction in total bacterial numbers. UHT processing aims to achieve microbial sterility to create a shelf-stable fluid milk product.

In low-temperature long-time (LTLT) or ‘‘vat,’’ pasteurization, which is commonly used for milk intended for manufactured products such as cheese and yogurt, milk is held at a minimum of 63°C for 30 min. In high-temperature short-time (HTST) pasteurization, is commonly used for fluid

milk products, milk is held at a minimum of 72°Cfor 15 second. In ultra-pasteurization, milk is held at a minimum of 138°C for at least 2 s, and in UHT processing, milk is held at 140–150°C for a few seconds (Bylund, 1995; U.S. Public Health Service, 1995). UHT processing involves the additional step of aseptic packaging in which heat-treated milk is cooled and packaged directly into sterilized containers under aseptic conditions. Typical shelf lives for heat-treated fluid milk are 14–21 days for HTST; 40–60 days for ultra-pasteurized (Boor and Nakimbugwe, 1998); and up to 6 months for UHT (Dunkley and Stevenson, 1987). Whereas HTST and ultra-pasteurized products require refrigeration at 4°C or less during storage, UHT products can be stored at 25°C.

- **Centrifugation**

Two techniques known as clarification and Bactofugation rely on the greater relative densities of bacterial cells and of other foreign particles to separate milk from contaminants. Centrifugation of milk causes denser bacteria, dirt particles, somatic cells, animal hairs, and bacterial spores to migrate outward, whereas lighter fat globules and casein micelles migrate inward. Appropriately designed outlet nozzles allow for separation of milk from contaminant sludge. Clarification is primarily designed to remove dirt particles, somatic cells, and animal hairs, whereas Bactofugation is a kind of high speed (up to 20,000 rpm) clarifier provided with discharge nozzeles in the bowl wall. Bactofugation is specially designed to remove bacterial spores from milk (Spreer, 1998). Using high-force centrifugation, the spore load of raw milk can be reduced by greater than 99% (Olesen, 1989; Torres-Anjel and Hedrick, 1971).

- **Filtration**

Microfiltration and ultrafiltration utilize the larger relative size of bacterial cells to separate out microbial contaminants. Filters with very small pores allow milk components to pass through while blocking bacteria, thus separating contaminants (Olesen, 1989). Typically rated in terms of pore diameter, microfiltration filters range from 0.2 to 5.0μm. Using microfiltration, lactose, minerals, and small proteins pass through into the permeate, whereas fat, very large proteins, and bacteria are retained. Typically rated in terms of the largest molecular weight molecule that can pass through the pores, ultrafiltration filters range from 10^3 to 10^5Da. Using ultrafiltration, minerals and lactose pass through into the permeate, whereas proteins, fats, and bacteria are retained (Smith, 2000).

- **Antimicrobial Constituents**

There are some naturally occurring antimicrobial systems present in raw milk like lactoferrin, lactoperoxidase, lysozyme etc. that might improve its shelf life (Yassin et al, 2015).

2.4.4 Aflatoxin in milk

Mycotoxins are produced by fungi through their secondary metabolism. The term mycotoxin is derived from the Greek word "*mycos*" meaning fungus, and the Latin word "*toxicum*", which means poison (Jouany et al., 2009). Aflatoxins belong to the class of mycotoxins and it is a group of approximately twenty related fungal metabolites generally produced by *Aspergillus* species, namely *A. flavus*, *A. parasiticus*, *A. ochraceoroseus*, *A. bombycis*, *A. nomius*, *A. fumigitus* and *A. pseudotamari* (Cheraghali et al., 2007). Aflatoxins are considered an important public health concern in the developing world and can seriously affect people's health and livelihoods. Recent estimates suggest that there are more than five billion people worldwide at risk of chronic exposure to aflatoxins (Yosef et al., 2014).

For mycotoxins to produce aflatoxin it depends on certain conditions like drought stress and rainfall, suitability of crop genotype for its climate, insect damage, and agricultural practices (Yitbarek & Tamir, 2014). Mycotoxin can also produce aflatoxin in "postharvest" conditions like storage, transportation, and food processing. Aflatoxin contamination is a particular problem in maize, oilseeds, spices, peanuts, tree nuts (almonds, pistachios, hazelnuts, pecans, Brazil nuts, and walnuts), milk (in the form of aflatoxin B1"s metabolite aflatoxin M1), and dried fruit (Shephard, 2008).

Many types of naturally occurring aflatoxins are known, the molds *Aspergillus flavus* and *A. parasiticus* produce exclusively aflatoxin B1, B2, G1 and G2, and all the other aflatoxins are derivates of these four. "B" and "G" refer to the blue and green fluorescent colors produced under UV light on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds, respectively (Felicia & Wu, 2011). Aflatoxin B1, is the most toxic of all the aflatoxins. Aflatoxin B1 present in feed of lactating animals gets transformed to 4-hydroxylated metabolite in liver and is excreted in milk as aflatoxin M1 (AFM1).

The AFM1 could be detected in milk 12-24 h after the first AFB1 ingestion, reaching a high level after a few days. When the intake of AFB1 is completed, the AFM1 concentration in the milk decreases to an undetectable level after 72 h (Hampikyan et al, 2010).

Milk is a very nutritious so it's highly perishable if it is not treated. Since milk may be processed in many ways, the effects of storage and processing on stability and distribution of AFM1 are of great concern. The occurrence of AFM1 in milk and milk products is a serious problem of food hygiene. AFM1 distribution in milk is not homogeneous. Cream separation can affect AFM1 distribution, since 80% is partitioned in the skim milk portion (Grant & Carlson, 1971) because of AFM1 binding to casein. Although AFM1 affinity for milk caseins is well known, very little information is available on AFM1ewhey proteins interaction.

Currently the limits of AFM1 are highly variable depending upon the degree of development and economic standing of the countries. These levels are variable and depend on economic and developing status of the countries (Yitbarek & Tamir, 2014). In US, the FDA has permitted a total amount of 20 ng/g in livestock feed and 0.5 g/kg or 50 ng/l in milk (Ellis et al., 1995). In European countries, permitted levels of aflatoxin M1 in milk, milk products and baby food are 0.005 mg/kg (Creppy, 2002).

2.5 Bioactive proteins in camel milk

Bioactive components in camel milk comprise immunoglobulin, lactoferrin, lactoperoxidase, lysozyme and insulin like protein. These components have paramount effect on human health and wellbeing (El-Agamy, 2009).

2.5.1 Lactoferrin

Lactoferrin (Lf) is an iron binding ,78–80 kDa glycoprotein of the transferrin family found to be widely distributed in mammalian milk and most other exocrine secretions such as tears, nasal and bronchial mucous, saliva etc. (Luna-castro et al., 2017).

Lactoferrin is non-heme iron binding glycoprotein that contains around 690-702 amino acids residues, folded into two symmetrical globular lobes - N and C lobes as shown in Figure 2-1. The common property of this protein family is the binding of two metal cations, preferably Fe^{3+}, at structurally closely related binding sites. Each lobe has one binding site for iron ions (Fe^{+2} or Fe^{+3}), and one or more potential glycosylation sites, depending on the species from which LF is isolated. Depending on its form, the molecular weight of lactoferrin varies between 76 and 80 kD. Iron binding was initially considered to be the major mechanism responsible for the bacteriostatic activity of lactoferrin (Saima et al., 2017). Its high affinity for iron, together with its presence in

an iron-free form in body secretions, allows lactoferrin to produce an iron-deficient that limits bacterial growth. Most lactoferrins are needed for storage or transport of iron (Ibrahim et al., 2017).

The antimicrobial activity of lactoferrin is primarily due to its ability to bind with iron, resulting in nutritional deprivation against iron in pathogens. The carbohydrate content of camel lactoferrin from end-lactation milk is 6.2 – 6.8% of total protein mass. Lactoferrin of colostral camel milk has a low iron saturation of 9% similar to lactoferrin of bovine colostral milk. This variation is mainly due to differences in lactation period, feeding regimen, number of analyzed samples, breeds, and methods of analysis (El-Agamy, 2009).

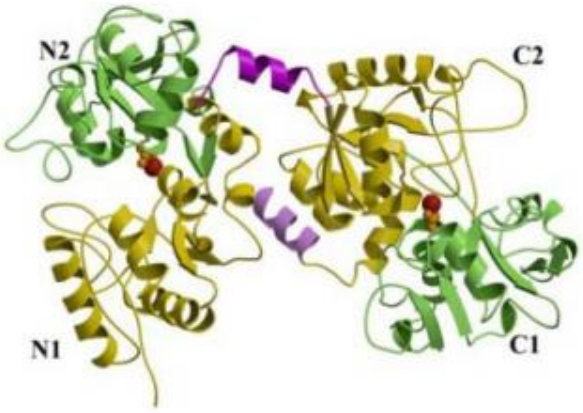

Figure 2- 1 Structure of lactoferrin (According to Berlutti et al., 2011)

Camel milk is rich in lactoferrin with potent antimicrobial and anti-inflammatory properties. Lactoferrin is also known to damage the outer membrane of gram-negative bacteria, causing the release of lipopolysaccharides that sensitize the cell to antibiotic action (Yassin et al., 2015). Lactoferrin binding molecules have been characterized in many types of microorganisms, including bacterial inhibition (*Staphylococcus aureus, Escherichia coli, clostridium* and *Helicobacter pylori*), antiviral effects (HBC, CMV, herpes simplex virus-1, and human immunodeficiency virus (HIV), antifungal effects (*Candida albicans*),immune supportive and immune modulating functions (regulates the maturation and activation of neutrophils and macrophages), the maturation and function of lymphocytes (antioxidant and anti-inflammatory) and anti-cancer actions (El-Agamy, 2009).

2.5.2 Lactoperoxidase

Lactoperoxidase is one of the most abundant and heat stable enzymes in many mammalian milk systems. Lactoperoxidase has no antibacterial activity by itself but in the presence of specific co-factors, they act together as an important defense system in liquid solutions (Perez, 2015). The main co-factors are hydrogen peroxide and a halide or pseudo halide (thiocyanates). It contributes to the non-immune host defense system, exerting bactericidal activity mainly on Gram - negative bacteria. It is supposed that the main function in milk is the protection of the udder from microbial infections. In milk, lactoperoxidase catalyzes and inactivates a wide range of microbes and acts as a natural preservative. This property is extremely useful when storage conditions are limited. Camel milk lactoperoxidase is a monomeric protein, which has 79.3% sequence similarity to human myeloperoxidase, and 79.2% sequence similarity to human eosinophil peroxidase (El-Agamy, 2009).

2.5.3 Immunoglobulins

Immunoglobulins are gamma globulin proteins that are found in blood and other body fluids of human, bovine, camel and all other lactating species. These are used in the immune system to identify and neutralize foreign objects such as bacteria and viruses, which are generally called antigens. Immunoglobulins are classified into five classes: immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin D (IgD), and immunoglobulin E (IgE). Three classes as IgG, IgA, and IgM are recognized in camel milk. IgG class is found to have three different subclasses: IgG_1, IgG_2 and IgG_3 (El-Agamy, 2009).

2.5.4 Lysozyme

Lysozyme is found also in secretions of milk, tears, nasal secretions, urine, etc. Lysozymes in human, goat, mare, and camel milk are considered type c lysozymes; however, it is unclear whether bovine milk lysozyme is a type c or g lysozyme. Camel milk lysozymes have no antigenic similarities between camel and bovine milk lysozyme, suggesting different structures. Camel milk contains 228, 288, and 500 μg/100 mL of lysozyme (El-Agamy, 2009).

2.6 Antimicrobial properties of lactoferrin from camel milk

In the beginning, the antimicrobial effect of lactoferrin was considered to be a function of its ability to chelate iron, with the protein inhibiting microbial growth through nutritional deprivation of iron

(Holley, 2006). Lactoferrin plays an important role for preservation of food products due to its iron binding capability. Several studies have proven the bacteriostatic and bactericidal effect of Lactoferrin, against a wide range of Gram-positive and negative bacteria (Mohanty et al., 2016). Iron deprivation induced by lactoferrin may only delay bacterial growth and further studies have demonstrated that lactoferrin exhibits bactericidal activity distinct from its iron-with holding capacity. Further mechanisms other than iron holding can also be involved in the antibacterial activity of lactoferrin, such as blocking microbial metabolism of carbohydrates or destabilizing the bacterial cell wall (Niaz et al., 2017).

The cationic molecule lactoferrin interacts through its positively charged cluster in the N-terminal region of N-lobe with the anionic part of lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria. This interaction damages the bacterial membrane, alters its permeability and results in lipopolysaccharide release. And this enhances bacterial susceptibility to hydrophobic antibiotics. Remarkably, it has been suggested that by the ability to bind Ca^{2+}, lactoferrin could influence the release of lipopolysaccharide by either displacing or chelating the divalent cations from lipopolysaccharide (Balcão et al., 2013). In the case of Gram-positive bacteria, lactoferrin acts by binding through electrostatic interactions to the negatively charged lipid matrix of the bacterial membrane.

The inhibition effect of lactoferrin depends mainly on iron requirements of microorganisms. Lactoferrin can block bacteria's carbohydrate metabolism, destabilize their cell walls, or interact with lysozymes in milk to stop bacteria. For example, *E. coli* is much more sensitive than lactic acid bacteria (M. Shafiur Rahman, 2011). Camel milk lactoferrin is effective against *Salmonella typhimurium*, and the clearance inhibition zones were 18.2 and 17.4 mm (El-Agamy, 2009).

The inhibition rate of camel lactoferrin against such microorganisms was detected in synthetic media, this effect is probably different when liquid media such as milk are used, because citrate ions can counteract the bacteriostatic activity of lactoferrin, that is compete for iron, unless the bicarbonate concentration is high. Therefore, lactoferrin activity in camel milk will be higher due to the lower concentration of citrate ions (Gopal et al., 2017). It can be assumed that the inhibition effect of lactoferrin in camel milk, when ingested by the nomads in the desert, is due to two main factors, these are the low content of citrate in camel milk and the high bicarbonate concentration in the intestinal fluid, where bicarbonate is the main buffer (Streicher, 2010). These two factors

will provide the proper conditions for lactoferrin to bind iron and inhibit sensitive microorganisms such as *E. coli*.

2.7 Extraction and isolation technology for milk bioactive compounds

Generally, protein isolation or separation is performed based on the protein native properties such as charge, size and hydrophobicity (Duellman & Burgess, 2008). There is a wide range of technologies available for protein separation based on end user applications and commercial viability. Most popular separation techniques for extraction of proteins from crude mixtures are salt or solvent precipitation and adsorption chromatography methods.

However, the processes available for commercial-scale and industrial-scale extraction of proteins are limited. The industrial-scale methods are divided into mainly three categories: selective precipitation, adsorption and membrane filtration. Most of these process steps are used either individually or in combination with several individual methods to isolate protein of interest from complex mixtures depending on the end user applications.

2.7.1. Milk whey protein isolation

There are wide ranges of methods used recently to extract the whey proteins of milk. Most of the literatures on minor protein of milk starts from converting the milk into acidic whey and precipitation then followed by dialysis and electrophoresis analysis.

- **Acidic whey**

Caseins are polyelectrolytes (polymers with charged side groups) and they are very susceptible to changes in pH. Solubility of hydrophilic protein depends on its charge and hydrogen bonding with water molecule. Caseins, in general, have an isoelectric point (no net charge) in the region of pH 4.6. Changing the pH of skimmed milk from ~ 6.7 (natural pH) to 4.6 causes the caseins to self-aggregate on a macroscopic scale. This pH-related behavior of the casein component in milk allows for a concentration of the casein and elimination of the other components in the milk (lactose, whey proteins and soluble minerals). Hydrochloric acid is often used to change the pH of milk to the isoelectric point resulting in the formation of a coarse precipitate. Nevertheless, sulphuric acid has been used and natural fermentation of the milk with *Lactobacillus* sp. to produce lactic acid is often the method of choice. The whey is separated from the curd using decanter centrifuges and this is generally termed acid whey.

2.7.2. Precipitation

Precipitation has been widely used in downstream processing for recovery of biological products such as proteins, enzymes and other bioactive compounds. The selective precipitation technique involves adjusting physical properties of desired components in crude solutions by addition of organic solvents, salts, pH changes, and/or heating to promote selective insolubility or aggregation.

While numerous diverse precipitation methods have been used over the last decades, ammonium sulfate has remained the most widely used especially for acidic proteins. While several salts can be used as precipitants, ammonium sulfate has several properties that make it the most useful. It is very stabilizing to protein structure, very soluble (low heat of solubilization, prevents denaturation of proteins), relatively inexpensive, concentrated solutions prevent microbial growth, pure material is readily available, and the density of a saturated solution (4.1 *M*) at 25°C (1.235 g/cm^3) which creates possibility for protein to be collected in pellets by centrifugation.

The most common process of protein precipitation is salting-out at high concentrations of salt, ammonium sulphate ($(NH_4)_2SO_4$). As the salt concentration is increased, a point of maximum protein solubility is reached. Further increase in the salt concentration implies that there is less water available to solubilize protein. Finally, protein starts to precipitate when there are no sufficient water molecules to interact with the protein molecules.

Selective precipitation has been the most commonly used method in dairy industry for making cheese by adjusting milk pH (Acid whey) at 40°C, which leads to aggregate casein proteins. Apart from this, precipitation methods have also been applied for selective extraction of whey proteins from whey.

2.7.3 Dialysis

After an ammonium sulfate precipitation step, or an ion exchange chromatography step, the whey protein may be in a high salt buffer; salt may hinder the next purification step. One of the most common methods to remove salt is dialysis.

The method of dialysis makes use of semi-permeable membranes. The main feature of this membrane is that it is porous - the pore size is such that small salt ions can freely pass through the membrane, larger protein molecules cannot (i.e. they are retained). Thus, dialysis membranes are characterized by the molecular mass of the smallest typical globular protein (lactoferrin) which it

will retain. Dialysis proceeds by placing a high salt (ammonium sulfate) sample in dialysis tubing (i.e. the dialysis "bag") and putting it into the desired low salt buffer (phosphate buffer).

2.7.4 Gel Electrophoresis of Proteins

Gel electrophoresis is a widely known group of techniques used to separate and identify macromolecules as DNA, RNA, or proteins based on size, form, or isoelectric point. The separation of molecules by electrophoresis is based on the fact that charged molecules migrate through a gel matrix upon application of an electric field.

It is influenced by the type, concentration and pH of the buffer, and by the temperature and field strength. This technique is used chiefly for the analysis and purification of large molecules, such as proteins and nucleic acids, as well as for simpler charged molecules, including charged sugars, amino acids, peptides, nucleotides and simple ions (Westermeier, 2001). The electrophoresis of macromolecules is carried out by of a sample to a porous matrix. Under the applying a thin layer influence of an applied voltage, different molecules in the sample move through the matrix at different velocities. The matrix can be composed of different materials, including paper, cellulose acetate or gels made of polyacrylamide. Polyacrylamide is the most common matrix for separating proteins and small proteins (Westermeier, 2001).

The main fields of the application of electrophoresis are biological and biochemical research, protein chemistry, food control as well as molecular biology, pharmacology, clinical investigations, and veterinary science and forensic medicine.

- **Electrophoresis system**

Different equipment is available for the operation of polyacrylamide gels, each with has characteristics specifically adapted for limited applications. Vertical systems are widely used and recommend a great deal of flexibility with accessories.

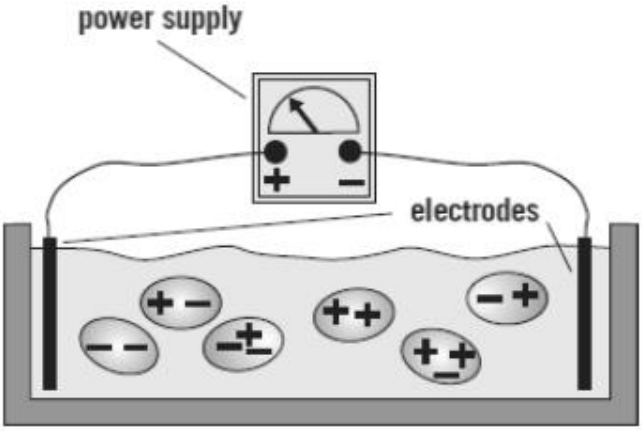

Figure 2- 2 The basic principle of electrophoresis

Source (Sameh Magdeldin, 2012)

- **Electrophoresis separation methods**

Generally, two different electrophoresis separation methods are employed in practices which are Isoelectric focusing (IEF) and Polyacrylamide gel electrophoresis (SDS-PAGE). But this paper focuses on Polyacrylamide gel electrophoresis (SDS-PAGE).

- **Polyacrylamide gel electrophoresis (SDS-PAGE)**

Gel electrophoresis of proteins with a polyacrylamide matrix, commonly called polyacrylamide gel electrophoresis is undoubtedly one of the most widely used techniques to characterize complex protein mixtures (Nison, 2011). It is a convenient, fast and inexpensive method because they require only the order of micrograms quantities of protein.

The proteins have a net electrical charge if they are in a medium having a pH different from their isoelectric point and therefore have the ability to move when subjected to an electric field. The migration velocity is proportional to the ratio between the charges of the protein and it's mass. The higher charge per unit of mass the faster the migration.

Proteins do not have a predictable structure as nucleic acids, and thus their rates of migration are not similar to each other. They can even not migrate when applying an electromotive force (when they are in their isoelectric point). In these cases, the proteins are denatured by adding a detergent such as sodium dodecyl sulfate (SDS) to separate them exclusively according to molecular weight.

SDS is an anionic detergent and, in solution forms, globular micelles are composed of 70-80 molecules with the dodecyl hydrocarbon moiety in the core and the sulphate head groups in the

hydrophilic shell. SDS masks the charge of the proteins and the formed anionic complexes have a roughly constant net negative charge per unit mass.

SDS is a reducing agent that breaks disulfide bonds, separating the protein into its sub-units and also gives a net negative charge which allows them to migrate through the gel in direct relation to their size. In addition, denaturation makes them lose their tertiary structure and therefore migration velocity is proportional to the size and not to tertiary structure. There is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of the migration of the SDS-protein complex (Garfin, 2009; Jacob & Maizel, 2000).

- **Polyacrylamide Gels**

Polyacrylamide is stable, chemically inert, electrically neutral, hydrophilic, and transparent for optical detection at wavelengths greater than 250 nm. These characteristics make polyacrylamide ideal for protein separations because the matrix does not interact with the solutes and has a low affinity for common protein stains (Garfin, 2009). Polyacrylamide which makes a small pore gel which is used to separate most proteins with a molecular weight of between 5,000 and 200,000 Da in size (Righetti, 1995).

- **Polymerization**

Polyacrylamide gels are prepared by free radical polymerization of acrylamide and a comonomer cross-linker such as bis-acrylamide. Polymerization is initiated by ammonium per sulfate (APS) with tetramethylethylenediamine (TEMED) acting as a catalyst. Polymerization speed depends on various factors (monomer and catalyst concentration, temperature, and purity of reagents) and must be carefully controlled because it generates heat and may lead to non-uniform pore structures if it is too rapid.

- **Buffer Systems and Gel Chemistries**

The pH and ionic composition of the buffer system determine the power requirements and heavily influence the separation characteristics of a polyacrylamide gel. Buffer systems include the buffers used to:

- ✓ Cast the gel
- ✓ Prepare the sample (sample buffer)
- ✓ Fill the electrode reservoirs (running buffer)

Proteins have an intermediate mobility, making them stack, or concentrate, into a narrow zone at the beginning of electrophoresis. As that zone moves through the gel, the sieving effect of the gel matrix causes proteins of different molecular weights to move at different rates**.** The most commonly-used buffer system for SDS-PAGE is the tris-glycine system. This buffer system separates proteins at a high pH, which confers the advantage of minimal protein aggregation and clean separation, even at relatively heavy protein loads (Garfin, 2009; Jacob & Maizel, 2000).

2.7.5 Detection of proteins in gels

Proteins separated on a polyacrylamide gel can be detected by various methods, for instance dyes and silver staining. Once the gel is stained, it can be photographed, scanned or dried on a transparent backing or filter paper for a record of the position and intensity of each band (Merril, 1990; Steinberg, 2009).

- **Dyes**

Protein is usually stained with dye. The Coomassie blue staining allows detecting up to 0.2 to 0.6 µg of protein and is quantitative (linear) up to 15 to 20 µg. For most gels, separated proteins can be simultaneously fixed and stained in the same solution. The gel is then destained to remove the background. The proteins are detected as blue bands on a clear background.

- **Silver staining**

It is an alternative to routine staining protein gels because its ease use and high sensitivity (50 to 100 times more sensitive than Coomassie blue staining). This staining technique is particularly suitable for two-dimensional gels.

2.7.6 Molecular weight estimation

SDS-PAGE is a reliable method for estimating the molecular weight (MW) of an unknown protein, since the migration rate of a protein coated with SDS is inversely proportional to the logarithm of its MW. The key to accurate MW determination is selecting separation conditions that produce a linear relationship between log MW and migration within the likely MW range of the unknown protein.

After separation, determine the relative migration distance (Rf) of the protein standards and of the unknown protein. Rf is defined as the mobility of a protein divided by the mobility of the ion front.

Because the ion front can be difficult to locate, mobilities are normalized to the tracking dye that migrates only slightly behind the ion front

$$Rf = (distance\ to\ band)/(distance\ to\ dye\ front)$$

2.8 Application of Lactoferrin

The most important known antimicrobial property of lactoferrin is to inhibit pathogenic bacteria by binding the iron ions. Lactoferrin is inhibitory for the high-iron-requiring bacteria whereas it has a low effect on low-iron-requiring bacteria (Ibrahim et al., 2017).

Fungicidal activity of lactoferrin significantly decreases under anaerobic growth conditions, in the presence of mitochondrial inhibitors and low extracellular Na^{+2}, K, Ca^{+2} and Mg^{+2} conditions. Lactoferrin has been reported to have lower antifungal than commercially available drugs (Balcão et al., 2013).

Lactoferrin is generally used in the industry of infant dairy formulas as a substitute to breast milk to provide protection against pathogens in infants, in medicines to increase iron absorption and to strengthen the immune system during pregnancy, in functional foods to increase iron absorption, in cosmetics as antioxidants, in oral care products to provide oral hygiene, and in probiotic foods that enhance the beneficial intestinal flora.

Lactoferrin has applications in food preservation and safety, either by retarding lipid oxidation or by limiting the growth of microbes. In vegetable oil industry, lactoferrin has extended the shelf life of soybean oil powder by preventing the oxidation of unsaturated fatty acids that are present in this product. It can also be used as a shelf-life extension agent in many natural food products like meat (Taylor et al. 2004) to store for longer periods.

In dairy industry (Mohanty et al., 2016), in their study on beneficial active packaging to control *Pseudomonas* which causes deterioration in cheese, focused on coating using a plasma functionalized with lactoferricin B. Functional coatings immobilized with lactoferricin B was found to be a promising tool to control microorganisms that cause deterioration and to extend the shelf life of cheeses.

Alternatively**,** an anti-infective, lactoferrin, is minor but mighty bioactive component of milk playing amazing role, not only in weight management but also as a natural alternative to synthetic

antibiotics (Ramesh, 2008). Food and Drug Administration (FDA) certified the lactoferrin as "Generally Recognized as Safe (GRAS)".

2.9 Concluding Remarks

Camel milk is an important source of protein for people living in the arid lands of the world. Camel milk is a rich source of biologically active compounds, which could be used for the production of different products including natural antimicrobial. In addition, camel milk is known for its medicinal properties, which are widely exploited for human health. Camel milk lactoferrin found in milk protein are associated with beneficial health attributes and has many interesting biological properties. The role of this natural antimicrobial needs further research regarding its isolation process and application.

Chapter Three

3. Methodology

3.1 Sample collection, preparation and storage

Whole camel milk was collected from local market in Addis Ababa in a cool container. The milk was kept in airtight bottles up until used for further analysis in food laboratory.

3.2 Experimental framework of the thesis

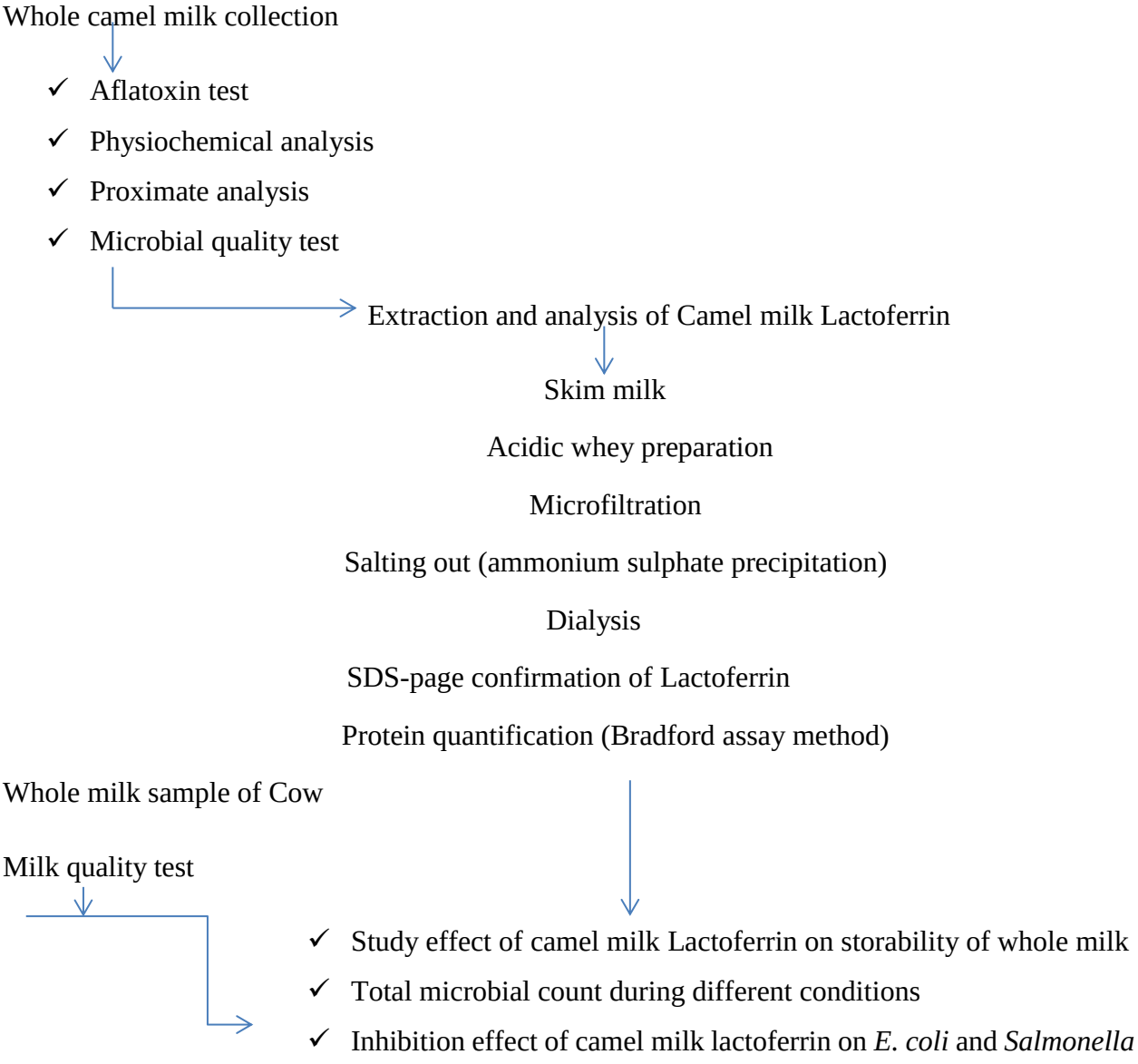

Figure 3- 1 Experimental frame work of the thesis

3.3 Processing methods and their combination

- **Extraction of Lactoferrin**

3.3.1 Acidic whey preparation and precipitation

The extraction process of lactoferrin started from removing the milk fat by centrifugation at 2500 x g for 30 min at 4°C and acidifying skim milk of pH 6.53 by adding 1N HCl till it reached the milk isoelectric point that is 4.6. Aggregation of casein occurred into macroscopic level, it was then centrifuged again which resulted in acidic whey. Then, whey was filtered using Millipore filter (Φ = 0.45µm), to remove the casein precipitate. Native whey protein was obtained by the microfiltration and subsequent separation of whey proteins from skim milk (Niaz et al., 2017). The filtrate (whey sample) was frozen at -20 ^{o}C to avoid microbial spoilage during the experimental period and prior to proceeding for further analysis (Moradian et al., 2014).

3.3.2 Ammonium sulfate precipitation (salting out)

For whey protein precipitation, ammonium sulfate at different concentration of six level (20-70%) at 4°C was added to concentrate the whey (Appendix I). The salting out process resulted in small precipitated pellets which were collected into 100mM phosphate buffer (Niaz et al., 2017). The protein was precipitated from crude extract using 20% (w/v) ammonium sulfate salt $(NH_4)_2SO_4$ while saturating at 4^{o}C and centrifuge at 10,000 x g for 20 min**.** The pellet and the supernatant were separated and the pellet was preserved in 50 mM phosphate buffer (pH 7.0). Then, it was re-suspended in 50 mM phosphate buffer (7.0) and dialyzed against the phosphate buffer for 12hr (Niaz et al., 2017).

3.3.3 Dialysis

Dialysis of the sample after ammonium sulfate precipitation was done (Niaz et al., 2017). At the outset the dialysis bag was prepared by cutting dialysis tube into the required length, and placed in 2% sodium carbonate solution (pH 8.0) and boiled in a hot water bath for 10 min. Sodium carbonate solution was then decanted. The dialysis bag was rinsed thrice with distilled water by keeping it in boiling water bath for 10min. After the third time, the dialysis bag was boiled in 10 mM EDTA (pH 8.0) in the water bath for 10 min. The dialysis bag was allowed to cool down at room temperature and stored at 4°C; water was added to the dialysis bag to check leakage.

The sample obtained after ammonium sulfate-precipitation was then poured into the bag and placed in a solution of 100 mM phosphate buffer (7.0) at 4°C for 12 h. To further purify that is remove the salt the protein was dialyzed again by the same manner for another 12h. Molecules having dimensions significantly greater than the pore diameter are recollected inside the dialysis bag, whereas smaller molecules and ions traverse the pores of such a membrane and emerge in the dialysate outside the bag. The buffer was replaced after every 3 h respectively.

3.3.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel-electrophoresis (SDS-PAGE)

Proteins can be separated largely on the basis of mass by electrophoresis in a polyacrylamide gel under denaturing conditions. The purity of camel milk lactoferrin was checked through electrophoresis using SDS-PAGE. The molecular mass was determined by applying the partially purified lactoferrin on 12% SDS-PAGE.

The resolving gel mixture according to Appendix IV was poured into the gel apparatus already assembled. After pouring, isopropanol was layered on the top of the resolving gel to get an even surface. Then isopropanol was removed and top of the gel surface was washed 3 times with distilled water. The stacking gel mixture according to Appendix IV was then poured on top of the polymerized resolving gel. The comb was immediately inserted and stacking gel was allowed to polymerize at refrigerated temperature. Dialyzed fractions of ammonium sulfate precipitated whey were mixed in 2X sample loading buffer according to Appendix IV and boiled for 3 min for the binding of SDS to the protein prior to loading on the gel in order to get the protein in its primary structure. Protein marker with molecular mass ranging from 10-200 kDa also runs as the standard. Protein ladder was applied directly to SDS-PAGE. The 5X running buffer was prepared according to Appendix IV and added to the tanker. The SDS-PAGE was initially run at 80 volts and then brought up to 120 volts after 30 min. The PAGE was stopped when tracking dye front reached the bottom of the gel.

3.3.5 Quantitative analysis

Bradford assay was performed for the quantification of CLf in the samples (Moradian et al., 2014; Bradford, 1976). Bovine serum albumin (BSA) was prepared for standard curve Appendix VI.

3.4 Compositional analysis methods of whole camel milk

Proximate analyses for moisture, crude fat, crude protein, crude fiber and ash were carried out in accordance with the official methods of the Association of Official Analytical Chemists (AOAC, 2000).

3.4.1 Total solids

Total solids were determined by (AOAC, 2000), heating 5 mL sample in oven at 100 ºC for 3 hrs. A total solid was calculated by the formula:

$$Total\ solids\ (\%) = \frac{Wt.of\ residue\ after\ drying}{Wt.of\ sample} \times 100$$ ..... Equation 3.1

3.4.2 Crude fat

Fat in milk was determined by (AOAC, 2000), method No. 905.02. The milk sample was treated with ammonium to dissolve the protein and ethyl alcohol to precipitate the protein. Fat was extracted using diethyl ether and petroleum ether. Then the mixed ethers were evaporated and the residue was weighed.

3.4.3 Solids-not-fat

Solids not fat (SNF) content was determined by difference as reported by Harding (1995), using the following formula:

$$SNF\ content\ (\%) = TS\ (\%) - Fat\ (\%)$$ .....Equation 3.2

3.4.4 Total protein

Total protein in the milk was determined by the international dairy federation method, IDF 20-1 (2001). Nitrogen from protein and other nitrogenous sources was converted into ammonium sulfate, and then ammonium was distilled in boric acid solution and titrated against known normality acid.

Calculation

Total nitrogen % was calculated by formula mentioned below and the value was multiplied by the conversion factor of 6.38 to get total protein.

$$\%\ Nitrogen = \frac{Vol.of\ H2SO4(ml) \times 250 \times 0.0014}{Vol.used\ for\ digestion \times vol.of\ digested\ sample} \times 100$$ .....Equation 3- *3*

$$\textbf{Total protein}\ \% = \%\ \textbf{Nitrogen} \times \mathbf{6.38}$$

3.4.5 Ash

Ash content of milk was estimated by gravimetric method using the method No. 945.46 as given in (AOAC, 1990).

$$Ash\ \% = \frac{Wt.of\ ash}{Wt.of\ sample} \times 100$$ ..... Equation 3.4

3.4.6 Lactose

Lactose content was determined by method given in (AOAC, 2003).

Lactose % = TS % - (Total protein % + Fat % + Ash %)

3.4.7 Physicochemical Analysis of whole camel milk

- **pH**

The pH of milk was measured with digital pH meter. 4 and 7 pH buffers were used for the calibration of pH meter. After calibration, 20 mL of milk was taken in a beaker and then electrode was immersed in the milk until constant reading was attained.

- **Acidity**

Acidity of milk was determined by titration method No. 947.05 given in (AOAC, 2000). Acidity was determined by taking 10 mL of milk in a titration flask and adding 3 drops of phenolphthalein, it was titrated against 0.1N NaOH until light pink color appeared persistently. The percent acidity was calculated by following formula:

$$Acidity\ \% = \frac{0.009 \times Vol.of\ NaOH\ used(ml)}{Wt.of\ sample} \times 100$$ .....Equation 3.5

3.4.8 Aflatoxin determination

The amount of aflatoxin in the whole camel milk was determined by using HPLC according to the official method given in (AOAC 2000.08). The experimental analysis was conducted at Bless laboratory.

3.4.9 Microbiological quality Analysis of whole camel milk

For the bacteriological quality analysis, total bacterial count (TBC) using plate count agar and coliform count were performed using Violet Red Bile Agar (NMKL, 2006).

3.5 Applying lactoferrin to whole milk for storability

Microbiological parameters of experimental samples that is, the whole milk and the whole milk with activated lactoferrin, were determined by total plate count method using nutrient agar.

3.6 Antibacterial Effect of lactoferrin on pathogenic milk bacteria

The inhibition effects of camel milk lactoferrin on *E. coli* and *Salmonella Typhi.* were tested by disc diffusion method using Mueller Hinton Agar according to the recommendation of the Clinical Laboratory Standards Institute (CLSI, 2011).

3.7 Design of experiments and statistical analysis

The experiments were completely randomized designs and replicated three times. The design of experiment for this work was conducted by ANOVA method using Design – Expert ® version 6.8.0. software. The differences between means were analyzed using least-significant difference procedures. The significance level was defined as $P < 0.05$.

Chapter Four

4. Results and Discussion

The very intent of this particular work was to extract lactoferrin from camel's milk, and thereby applying the partially purified lactoferrin as an alternative natural antimicrobial to extend storability of whole milk before processing. In order to get an effective product, the quality of whole camel milk was analyzed through its proximate composition, physicochemical properties and microbial quality including its aflatoxin content. The results of samples analyzed in this work are presented in different tables and figures.

4.1 Compositional values of whole camel milk

The values of the main components of whole camel milk are presented in Table 4-1 which is done in dry basis. There is a wide variation in the total solids content of whole camel milk. The values varied between 8.01 to 11.9 g per 100 g with an average of 10.23 ± 1.49 g per 100 g. The variation in total solids of camel milk is mainly due to the changes in fat, lactose, minerals and protein content of camel milk. These results were in line with the values reported by different workers (Farag & Kebary, 1992; Al-Kanhal, 1993).

Solid not fat is the portion of milk other than fat. SNF also varies when total solids in milk increased or decreased. Table 4-1 shows that solid not fat contents were found minimum 7.1 and maximum 9.39 percent. The mean value for SNF was 7.99 ± 0.916 %.

Table 4-1 Composition of camel milk

Components	**Mean ± SD**
Total solids (g/100g)	10.23 ± 1.49
Fat (g/100g)	2.92 ± 0.530
SNF (g/100g)	7.99 ± 0.916
Protein (g/100g)	3.09 ± 0.291
Ash (%)	0.77 ± 0.048
Lactose (%)	4.44 ± 0.609

The percentage of fat content in whole camel milk varied between the values 2.3 to 3.7% with an average of 2.92 ± 0.530% as shown in Table 4-1. These values fall just within the range of 2.90 to 5.40% often quoted in the literature (Farah & Fischer, 2004). This low percentage is certainly due to a dietary difference reflecting the desert nature of Afar region, can relate to thirstiness of the camels (Konuspayeva et al., 2009).

The protein content of whole camel milk showed an average of 3.09 ± 0.10%. These value falls between the mean of 3 to 3.9% reported in many camel milk studies (Yagil & Etzion, 1980; Knoess, 1982).

The total amount of minerals is commonly presented as total ash and in case of camel milk this value ranged between 0.71 to 0.83 percent. The rise and fall in mineral level were proposed to be due to the differences in feeding, breed, water intake and analytical procedures (Haddadin et al., 2008).

Lactose is the major carbohydrate in milk. The whole camel milk lactose contents ranged between 3.66 to 5.16 percent. The average content of lactose was 4.44 ± 0.609. The nature of vegetation eaten by the camels in deserts areas could be a significant factor for extensive variation in lactose contents (Konuspayeva et al., 2009).

4.1.1 Physicochemical composition of whole camel milk

The acidic and bitter taste is caused due to the pH that is the non-dissociation of the acids. In the dairy processing industries pH plays an important role to determine the end product quality of the dairy products. Table 4-2 shows that the pH of whole camel varied from 6.52 to 6.77 with an average of 6.61 ± 0.113. These values were in line with the values reported by other researcher that is the pH of whole camel milk pH is ranges from 6.5 to 6.7 (Khaskheli et al., 2005). These results indicate that the milk samples have the expected quality.

The titrateable acidity of camel milk is the measure of lactic acid formed in whole camel milk. Table 4.2 shows that camel milk acidity varied from 0.12 to 0.17%, with an average value of acidity was 0.14 ± 0.022. These results shows a slight difference between the reported value of titratable acidity of other researchers which were equivalents of 0.13-0.16% lactic acid in fresh camel milk (Seher et al., 2013).

Table 4-2 Physicochemical properties of camel milk

Components	Mean ± SE
pH value	6.61 ± 0.113
Acidity %	0.14 ± 0.022

4.1.2 Aflatoxin test result for whole camel milk

The result of the whole camel milk tested for aflatoxin M1 contamination level was < 0.02 µg /L as shown in Fig 4-1, which is less than the limit of the standard given by Ethiopia, 0.05 µg/L according to ES ISO 14501. So, this result implies that whole camel milk is not contaminated by aflatoxin M1. Barely any published data are available on the occurrence of aflatoxin M1 in whole camel milk.

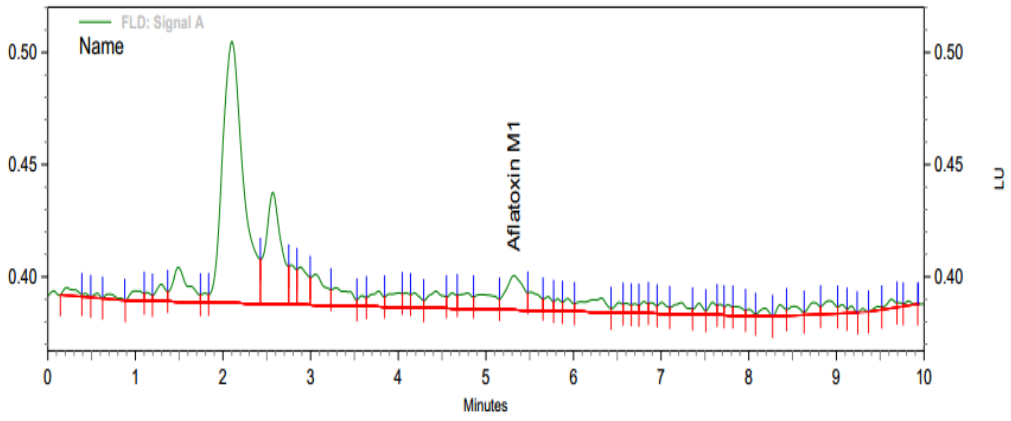

Figure 4- 1 HPLC output of aflatoxin

4.1.3 Microbiological Quality result of whole camel milk

The total bacterial count of whole camel milk varied between 4.9 to 5.9 log cfu/ml with an average values of 5.6 ± 0.497 log cfu/ml. Apparently there is no official standard stated for the quality of camel milk. Therefore, the microbiological limit value for cow milk was used to assess the quality of camel milk. When these values are compared with the works of other researchers the results were found to be similar (5.6 log cfu/ml in average) (Semereab and Molla, 2001) in Afar Region of Ethiopia and also (5.4 log cfu/ml in average) (Al-Mohizea, 1994) in Riyadh, Saudi Arabia.

Coliform bacteria are a commonly used as indicators of sanitary quality of foods and water. Coliforms can be found in the aquatic environment, in soil and on vegetation. The coliform group

of bacteria comprises all aerobic and facultative anaerobic, Gram-negative, non-spore-forming rods able to ferment lactose with the production of acid and gas at 32 or 35°C within 48 h (Christen et al., 1992).

The VRB agar and sample mixture were allowed to solidify on a flat surface 10 min. The plates were incubated in an inverted position for 24 h at 32°C. Dark-red colonies on un-crowded plates were counted. The present results indicated an overall mean coliform count of 1.82 ± 0.119 log cfu/ml. When this value was compared to the European Union standard and Ethiopian standard ES ISO 5541-1 and ES ISO 5541-2 it has an acceptable level of coliform count (1.69-2.00).

4.2 Camel's milk Lactoferrin yield

4.2.1 Effect of dialysis time and desalting concentration on the yield of camel milk lactoferrin

Dialysis is the movement of molecules by diffusion from high concentration to low concentration through a semi-permeable membrane. The dialysis in the present study is used in order to remove ionic molecules especially for sample desalting so that it improves the final resolution and fractionation of the sample. The yield of lactoferrin therefore, depends on concentration of the dialysis buffer and the dialysis time of the purification.

In this study, the yield of lactoferrin were affected by the concentration of the dialysis buffer and the dialysis time. The yields for 11 lactoferrins run are indicated below in Table 4-3.

Table 4- 3 Variation of desalting concentration and dialysis time on the yield of lactoferrin

	Coded factors		**Factor 1**	**Factor 2**	**Response**
Run	A: Desalting concentration	B: Dialysis time	A: Desalting concentration (mM)	B: Dialysis time (hr)	Yield Lf (mg/ml)
7	-1	-1	50	12	0.24
3	1	-1	100	12	0.36
4	-1	1	50	36	0.38
5	1	1	100	36	0.93
11	-1	0	50	24	0.35
10	1	0	100	24	0.71
1	0	-1	75	12	0.53
8	0	1	75	36	0.75
9	0	0	75	24	0.62
2	0	0	75	24	0.67
6	0	0	75	24	0.64

It follows that, the yield of lactoferrin ranged from 0.24 to 0.93 with an average value of 0.56. The maximum yield at coded point (+1, +1) was about 3.88 times more than the minimum yield at the coded point of (-1, -1) table. The present findings seem to be consistent with other research which found the lactoferrin content in milk ranges from 0.03-2.3 mg/ml (Kawakami et al., 1987).

Generally, it is interesting to note that only those protein molecules that are small enough to fit through the membrane pores were able move through the membrane and reach equilibrium with the entire volume of solution in the system. When the concentration of the desalting buffer is decreased (50mM phosphate buffer) and the dialysis time is also decreased (12h) the yield of lactoferrin is very less as shown in Table 4-3. The reason behind this effect is once equilibrium is reached, there is no further net movement of the substance because molecules will be moving through the pores into and out of the dialysis unit at the same rate (Mirica et al., 2012).

The ANOVA of the yield response is given in Appendix III. Thus, the Model F-value of 47.19 indicates that the model was significant ($P < 0.05$). R-squared (0.9792) and Adjusted R-squared (0.9585) were in reasonable agreement with each other. Moreover, the adequate precision (20.76) is greater than 4, indicating a good fit of experimental data and the acceptability of the model for prediction purposes. Considering these criteria, the following response model was selected for representing the variation of yield for further analysis.

Yield of LF = $+0.65 + 0.17* A+ 0.16*B - 0.14 *A^2- 0.029*B^2 +0.11*A*B$……Equation 4-1

From the above equation (Equation 4.3), it can be seen that the coefficients of A (concentration of the desalting buffer) and B (dialysis time) were positive. Hence, increase in concentration of the desalting buffer, and dialysis time may increase yield of lactoferrin. Figure 4-2 dictates this fact as well, in which the yield has increased upon increment of concentration of the desalting buffer and dialysis time. From the response surface plot, it can be seen that decrease in yield of LF was displayed at lower treatments of concentration of the desalting buffer and dialysis time. In order to remove additional unwanted substance, it is necessary to replace the dialysis buffer so that a less salt concentrated lactoferrin is to be found (Mirica et al., 2012).

The interaction model term AB had increasing effect on the final yield of lactoferrin. Whereas, the quadratic model terms B^2 showed a decreasing quadratic effect on the yield of lactoferrin.

Analysis of variance of Equation 4-1 (Appendix III) shows that F-value for the model was 47.19 indicating the quadratic model selected was significant. p-values of 0.0003 and 0.0002 for the two linear factors of A and B respectively show that the model terms (concentration desalting buffer and dialysis time) were significant. The interaction model term AB, with p-value of 0.0039 was significant. It is apparent from Figure 4-2 also that there was sharp increase in yield with further increase in concentration of desalting buffer and dialysis time.

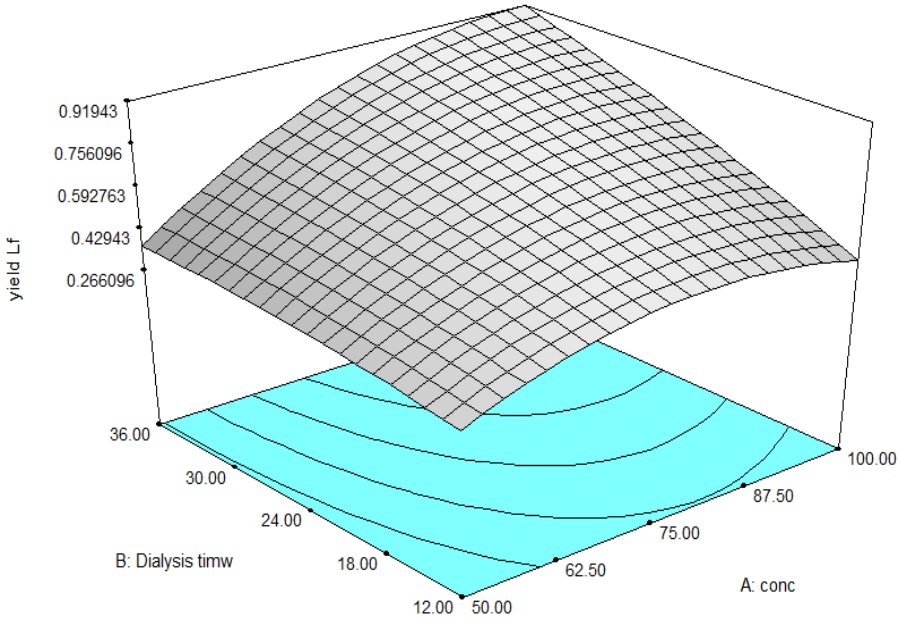

Figure 4- 2 Response surface plot for the effect of extraction variables on yield of lactoferrin

The dialysis buffer is changed with every 3h interval, after the third buffer change the sample was again dialyzed overnight at 4°C, which further purified the lactoferrin. Surprisingly, partially purified lactoferrin was obtained. The findings observed in this study mirror those of the previous studies that have examined the effect of dialysis by (Masson & Heremans 1967).

4.2.2 Molecular weight of lactoferrin using SDS-PAGE

Subsequently next to SDS-PAGE, molecular weight of the lactoferrin was calculated from drawing the relationship between the logarithms of the molecular weight for standard proteins compared to relative mobility. In this study protein ladder from 10-200 kDa was used as standard. After staining the gel, both the protein standard and sample bands were obtained which can be seen in Figure 4-2. By measuring the distance of bands relative to the top of the resolving gel, the relative migration distance (Rf) value of the standard protein ladder was determined. The standard protein calibration

curve was generated by plotting log (MW) versus relative migration distance (Rf) value. As given in Appendix V, the plot, had a strong linear relationship ($r^2 > 0.995$) between the proteins' MW and migration distances which demonstrated an exceptional reliability in predicting MW after separation.

Then the molecular weight of lactoferrin was determined by using the plotted standard protein ladder. The relative migration distance (Rf) of Lactoferrin was 0.445 (calculated as migration distance of the protein/ migration distance of the dye front) and its molecular weight was found from the plot by using the equation, y = -1.958x + 2.7491, when Rf value was substituted on the equation as x, the value of y or log(MW) gives 1.878, finally MW of lactoferrin was calculated as antilog (1.878).

Based on the calculation the molecular weight of lactoferrin was found to be 75.87 kDa and this value is in accordance to the values reported by other researchers (El-Agamy et al.1996). This result confirmed purity of the extracted lactoferrin by SDS-PAGE, and it is fair to say that the extraction procedure used in the present work gives pure lactoferrin which was somehow subjected to the presence of small traces of ammonium sulphate salt.

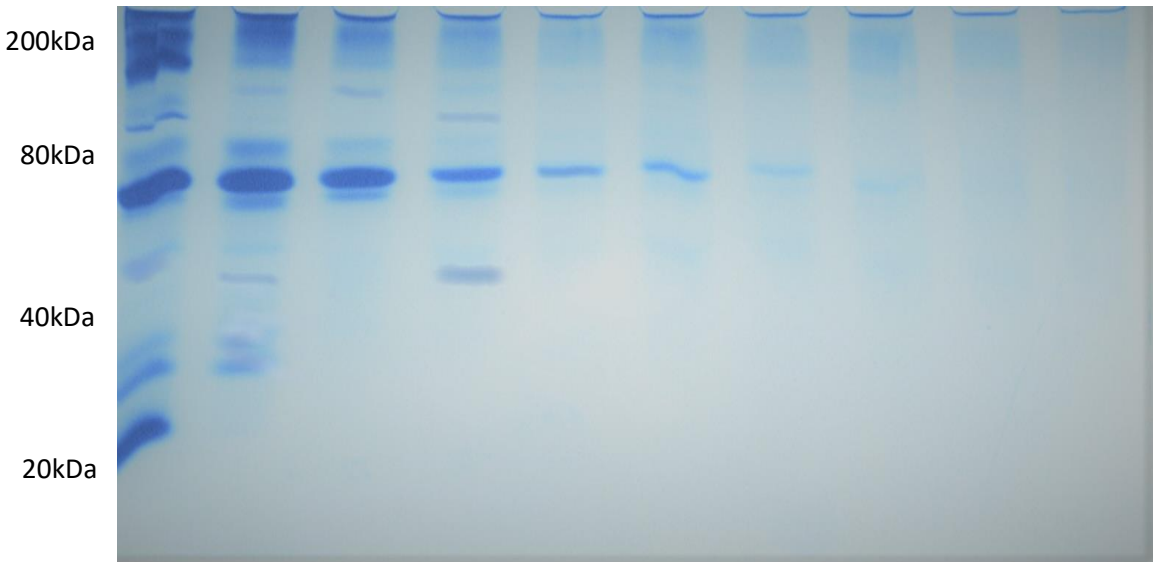

Figure 4- 3 SDS-PAGE of lactoferrin extracted from camel milk through protein dialysis

4.2.3 Quantitative Result of Lactoferrin (Bradford assay)

Protein samples (BSA) were mixed with Coomassie Brilliant Blue G 250 dye and allowed to react for at three minutes, and then the absorption at 595 nm was measured before one hour. On

performing Bradford's Assay, the following absorbance values at 595 nm were obtained using a spectrophotometer. Absorbance values are represented in Appendix VI.

By plotting the graph of Absorbance at 595 nm against the concentration of BSA (mg/mL), the resulting concentrations of the lactoferrin samples were recognized by extrapolating the standard graph which is illustrated in Figure 4-3.

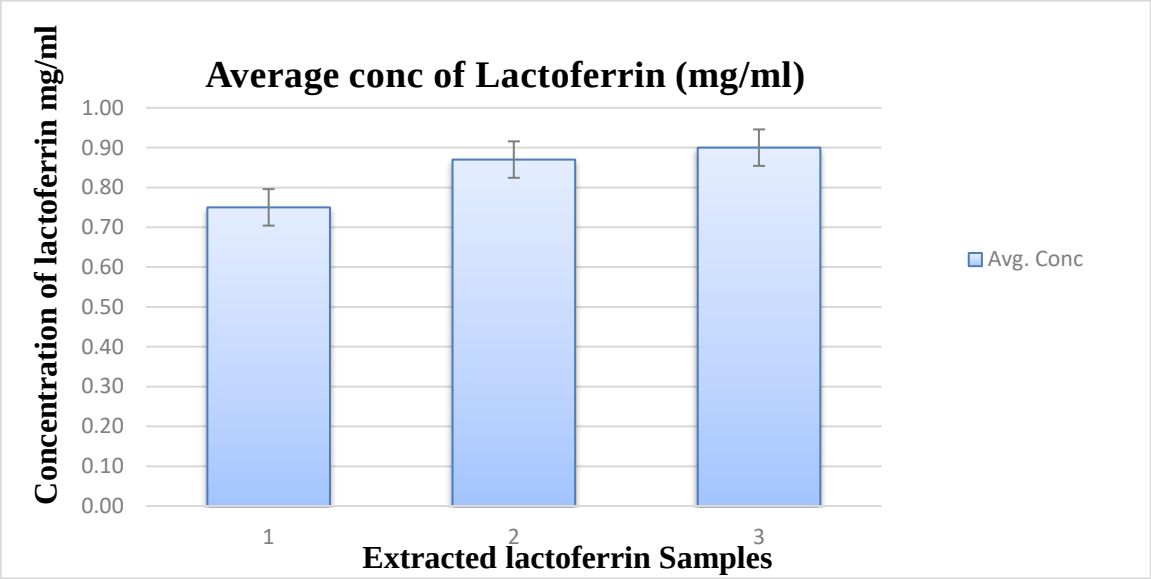

Figure 4- 4 Concentration of lactoferrin

The lactoferrin content in milk ranges from 0.03-2.3 mg/ml (Kawakami, Shinmoto, Dosako & Sogo, 1987).

4.3 Applying lactoferrin to whole milk and testing the Total microbial count

Milk contains natural inhibitors (Yassin et al., 2015). Some bacteria do not grow in milk despite the presence of sufficient nutrients and suitable conditions. The basically bacteriostatic nature of the LF, avoids the quick multiplication of the milk contaminant bacteria, by its iron binding mechanism which reduce the activity of Fe^{2+} ions, which results in lowering the deterioration of the initial quality (Jahani et al., 2015).

The temperature dependence of the growth rate has consequences for the keeping quality of milk. A low initial count and a low storage are essential. Whether the milk is kept at a low or at a higher temperature, a lower initial count always means that it takes more time for the milk to spoil. At high storage temperatures, the milk has a poor storing quality, even if its initial count is low; it should be processed within a few hours after production.

It is important to note that for raw milk to be kept for several days, the type of contamination may be of greater importance than the total count (Holley, 2006). In the present study the whole milk used for sampling was taken from the dairy farm of EIAR.

4.3.1 Effects of storability factors on total bacterial load

The experimental design selected for this study was RSM/FCD and the response measured was the total bacterial count per ml (logN cfu/ml). The three whole milk storability variables studied were milk storage temperature, milk storage time and concentration of LF. The Design Expert 6.8.0 software was used in analysis of variance (ANOVA).

The milk samples were kept at the temperature of 25, 27.5 and 30 °C based on the FCD design layout. Sampling was then conducted by the respective storage conditions of sample with the addition of different concentration of lactoferrin. The amount of lactoferrin added was determined based on the study of (Haddadin et al., 1996) which used the concentration of thiocyanate and hydrogen peroxide to activate lactoperoxidase system at 1.5 mg/ml with different dilution ratio to preserve the whole milk for about 8 h at room temperature.

Bearing the study in mind, here in this work the concentration of lactoferrin added ranges from 0 to10mg/L. and was applied by which the quality of the samples was checked within 8 h interval by conducting actual experiment of the total microbial count at the concentration of lactoferrin (0, 5 and 10), sample storage time (0, 8, 16 h) and storage temp (25, 27.5, 30 °C) as given in Appendix VII.

The response of the log cfu/ml was used to develop a mathematical model that correlates the storability of whole milk with different log cfu value. As it is evident from Table 4-4, the total bacterial count ranged from 4.29 cfu/ml to 6.57 cfu/ml with an average value of 5.52 cfu /ml. The maximum total bacterial count attained at coded point (1, -1, 1) was about 1.53 times more than the minimum total bacterial count at the coded point of (-1, 1, -1).

Table 4- 4 Variation of response the total bacterial count with three factors

Factors						Response
A: Storage time (hr)		B: Conc of LF(mg/L)		C: Milk storage temp(°C)		log(N) cfu/ml
Actual	Coded	Actual	Coded	Actual	Coded	
0	-1	0	-1	25	-1	5.32
16	1	0	-1	25	-1	6.38
0	-1	10	1	25	-1	4.29
16	1	10	1	25	-1	6
0	-1	0	-1	30	1	5.35
16	1	0	-1	30	1	6.57
0	-1	10	1	30	1	4.32
16	1	10	1	30	1	6.2
0	-1	5	0	27.5	0	4.41
16	1	5	0	27.5	0	6.31
8	0	0	-1	27.5	0	6.37
8	0	10	1	27.5	0	5.42
8	0	5	0	25	-1	5.63
8	0	5	0	30	1	5.71
8	0	5	0	27.5	0	5.2
8	0	5	0	27.5	0	5.31
8	0	5	0	27.5	0	5.1

The quadratic model F-value of 28.54 implies the model was significant ($p<0.05$). R-Squared and Adjusted R-Squared values of the model were 0.9448 and 0.9117, respectively. The "Lack of Fit F-value" of 5.07 implies the Lack of Fit is not significant relative to the pure error. There is a 17.50% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good because it is required the model to fit. Therefore, from these results the developed second order quadratic model provides an adequate approximation of the actual values. Bearing in mind these criteria, the following response model was selected for representing the variation of the total bacterial count for further analysis.

$$Log(N)(cfu/ml) = +5.44 + 0.78 * A - 0.38 * B + 0.053 * C - 0.19 * A2 + 0.34 * B2 + 0.16 * A * B \quad \text{.....Equation 4-2}$$

Where A, B and C are the coded values for milk storage time (hrs), concentration of LF (mg/L), and milk storage temperature (°C), respectively. While A^2, B^2, and AB, are interactions thereof.

In line to Equation 4-2 and Figure 4-5, the following observations were noted. Apparently, the coefficient of A was positive, and the coefficient of B was negative; indicating that decreasing in the milk storage time and increasing the concentration of lactoferrin may have a decreasing effect on the total bacterial count. However, even though the coefficient of C was positive the number is very small, which indicates that the storage temperature of the given range (25-30°C) did not show a significant effect on the storability of whole milk. As shown from the developed model equation the of interaction coefficient AB, that is the milk storage time and lactoferrin concentrations were significant model terms, with F-value 4.57 and p-value 0.04 respectively affected the storability of the whole milk significantly ($P<0.05$) and was supported with figure 4-5.

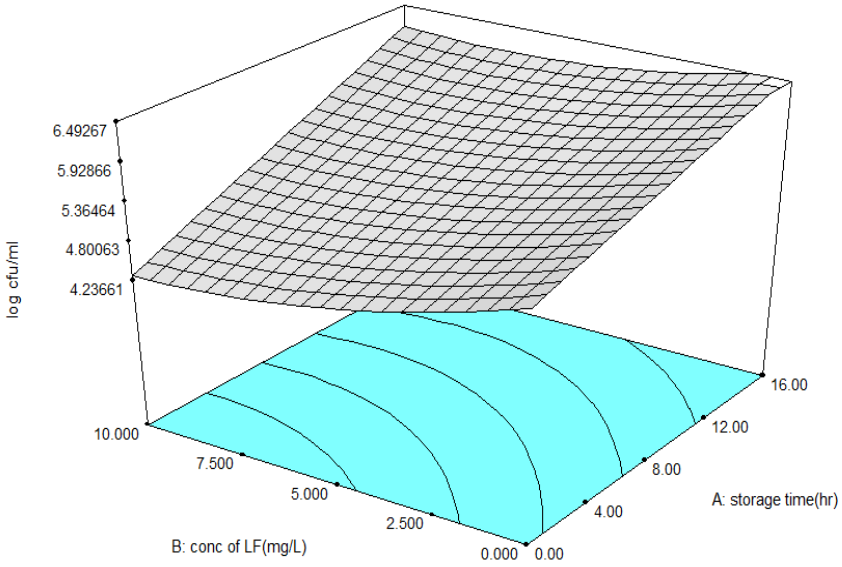

Figure 4- 5 Response surface plot for the effect of total bacterial count with three factors

The first mechanism of lactoferrin is that iron binding protein which scavenger free iron and reduce the microorganism present in the environment.Thus deficiency of iron prevents the growth of bacterias. The effect of lactoferrin was storage time dependent as illustrated in Figure 4-5. Lactoferrin extended the milk by 8 h, at a storage temprature of 30°C and the milk was unspoiled at the end of the experiment by having a total bacterial load of 5.71 cfu/ml.

4.4 Effect of lactoferrin on pathognes

The anti-bacterial activity of lactoferrin attributed to its iron sequestering properties. Lactoferrin can deplete the iron present in the system that is essential for bacterial growth and division (Kanwar

et al., 2015). The action of lactoferrin on the bacterial cells is by direct contact between lactoferrin and the bacteria; this mechanism leads to its bacteriostatic property. Iron deficiency can prevent the growth of bacteria such as *Escherichia coli* (Jahani et al., 2015).

In the present study the inhibtion effect of lactoferrin was seen on milk spoiling bacterias *E. coli* and *Salmonella typhi. Salmonella* are probably the most serious threat to consumers of milk and its products. Salmonella are commonly associated with raw milk (Balcão et al., 2013). Regarding the antibacterial effect of camel milk lactoferrin at different concentrations (0.01, 0.5, 1, 2 and 4 mg/ml) on growth, the results indicated that camel lactoferrin at concentration of 0.01 mg/ml had the least inhibitory effect whereas maximum inhibitory effect was recorded for 4 mg/ml against *E. coli* and *Salmonella typhi*. which have been presented in Table 4-5 .

Table 4- 5 Measurement of inhibition zone against tested microbes using lactoferrin

Targeted microrganisms	Conc. Of lactoferrin (mg/ml)	Inhibition zone (mm)
Escherchia coli	0.01	-
	0.5	16
	1	22
	2	25
	4	28
Salmonella typhi.	0.01	-
	0.5	10
	1	15
	2	17
	4	19

The values of zones of inhibition measured for all the concentrations of CLf against Salmonella typhi. revealed that 4 mg/ml concentration of CLf restricted the growth of pathogen upto 19 mm, although, CLf concentration ranging from 0.5-4 mg/ml was also effective against *Salmonella typhi.* As a general, CLf was found to be more effective against *E. coli* as compared to *Salmonella typhi*.

These findings are in parallel to those reported by Moradian et al. (2014), they concluded that bLF above 1mg/ml inhibited the bacterial growth especially for gram negative bacteria. As well Maria et al. (2014) stated that bLF at a concentration of more than 4 mg/ml, inhibit adherence of gram negative bacteria. Experimental studieshave demonstrated that the lactoferrin's bacterocidal effect depends on its concentration The inhibitory effect on microrganisms is obtained at a low dose (0.5–500 mg/mL) (Trybek et al., 2016). When compared to Salmonella, *E.coli* was susceptible to be inhibited at lower dose (2 mg/mL), whereas *Salmonella typhi.* needs more concentration (4 mg/mL) for its inhibition.

Chapter Five

5. Conclusion and Recommendation

5.1 Conclusion

Milk is prone to perish easily, since it has high nutrient composition, it's susceptible for growth of microorganisms and easily spoil during storage. The microorganisms that cause the spoiling of milk reduce the quality of raw milk, which results in significant economic losses.

For this study, the whole camel milk was analyzed for chemical composition, physical characteristics, and microbiological quality since, each parameter is a measure of the quality of the product. The test for aflatoxins M1 contamination in the whole camel milk was also less than the limit of the standard given by Ethiopia, 0.05 μg/l according to ES ISO 14501. Generally, the result of the analysis showed that the milk was of good quality.

In the present work, a simple isolation technique for the separation of the lactoferrin from camel milk was used. The isolation process begins from defatting the skim milk, followed by getting acidic whey, Millipore filtration, dialysis and characterize the purity by SDS-PAGE. The concentration of lactoferrin was quantified by Bradford assay method. Lactoferrin is the iron-binding protein, which is found mainly in milk and it is being widely explored for its biological properties such as antibacterial, antifungal, antioxidant, antiviral and anticancer activities. The isolated lactoferrin was used as antimicrobial agent for whole milk.

Camel's milk lactoferrin was extracted and used as antibacterial for whole milk. The milk protein lactoferrin, does have some antibacterial properties and it extended the storability of whole milk by 8hrs. Therefore, this work gives a baseline information on preserving whole milk using natural preservative and can have its own contributions in order to overcome the whole milk loss problems since, most lowland regions of Ethiopia, suffer by fast deterioration of whole milk quality due to high ambient storage temperature and lack of refrigeration system.

5.2 Recommendation

The isolation of specific bioactive proteins such as LF and Igs is still a challenge. A number of procedures are involved in the purification process of protein such as separation based on solubility, separation based on size and density, chromatography. Chromatographic techniques are widely used and can be operated as HPLC or FPLC. Using chromatographic separation techniques could increase the yield and activity of lactoferrin.

The antimicrobial property of lactoferrin on should be studied further with different techniques. A mere delay (or lag phase) in growth after addition of the bacteria to the milk is not proof of inhibition; the bacteria may not be adapted to milk in another words, they have to change their enzyme system before they can use the nutrients available. Therefore, this condition is also a challenge, which requires further study.

There are many researches done with regarding compositional properties of camel milk. Nevertheless, camel milk has been reported that it has many therapeutic properties which can be used for prevention, treatment and diagnosis many types of diseases.

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Appendices

Appendix I Final concentration of ammonium sulfate

Initial concentration of ammonium sulfate (percentage saturation at 0 °C)	Percentage saturation at 0 °C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (g) to be added to 1 l of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

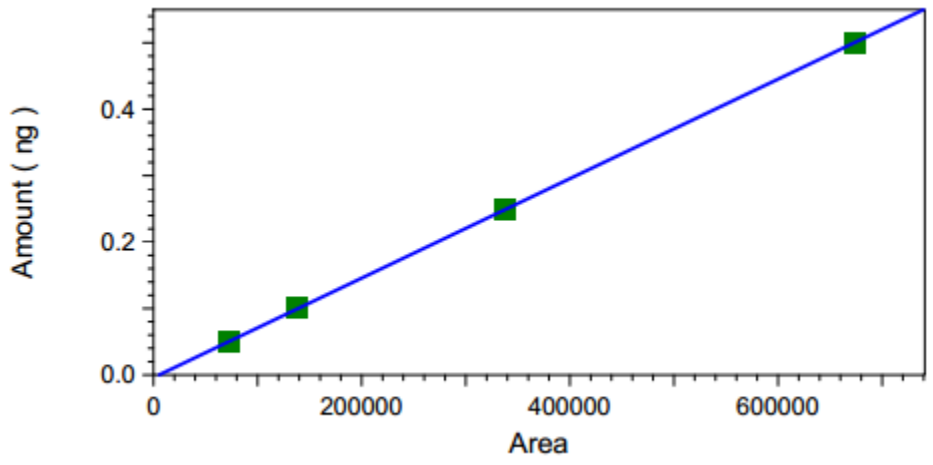
Source (Englard and Seifter, 1990)

Appendix II Chromatogram of raw camel milk AFM1 standard curve

Fit Type: Linear

y = 7.48935e-007x - 0.00403766

Goodness of fit (r^2): 0.999982



Chromatogram of raw camel milk AFM1 standard curve.

Appendix III ANOVA for second order quadratic model and its significance for yield of Lactoferrin

Source	Sum of square	DF	Mean square	F- value	P-value	Remark
Model	0.43	5	0.086	47.185025	0.0003	significant
A	0.18	1	0.18	97.400821	0.0002	
B	0.14	1	0.14	79.406137	0.0003	
A2	0.049	1	0.049	27.04431	0.0035	
B2	0.0021616	1	0.00216158	1.1907224	0.3250	
AB	0.046	1	0.046	25.463397	0.0039	
Residual	0.0090768	5	0.00181535			
Lack of Fit	0.0078101	3	0.00260336	4.1105725	0.2018	not significant
Pure Error	0.0012667	2	0.00063333			
Cor Total	0.4373636	10				

R-squared (0.9792), Adj. R-squared (0.9585), Pred. R-squared (0.9821), Adeq.precision (20.76), Mean (0.56). A and B are concentration of desalting buffer and dialysis time respectively.

Appendix IV SDS-PAGE solution preparation

The resolving gel

Distilled water	3.2 ml
Acrylamide/Bis-acrylamide (30/0.8% w/v)	4 ml
1.5M Tris(pH=8.8)	2.6 ml
10% (w/v) SDS	0.1 ml
10% (w/v) Ammonium persulfate (AP)	0.01 ml

TEMED	0.01 ml
The stacking gel	
0.5M Tris-HCl, pH 6.8	1.25 ml
Distilled water	2.975 ml
Acrylamide/Bis-acrylamide (30/0.8% w/v)	0.67 ml
10% (w/v) SDS	0.05 ml
10% (w/v) Ammonium persulfate (AP)	0.1 ml
TEMED	0.01 ml

2x sample loading buffer

0.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol (added fresh)

0.5 M Tris-HCl, pH 6.8 3.75 ml

50% Glycerol 15.0 ml

1.0% Bromophenol blue 0.3 ml

10% SDS 6.0 ml

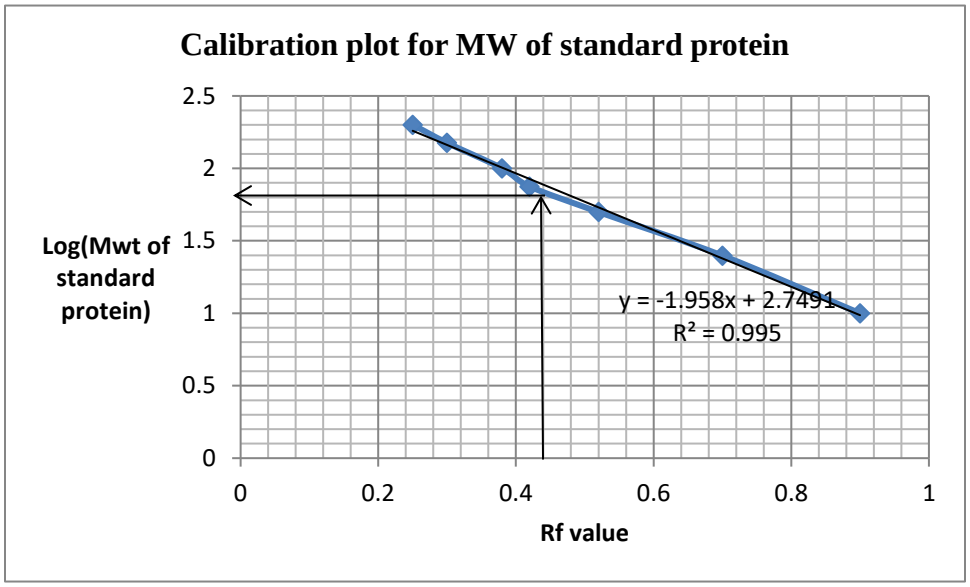
H_2O to 30 ml

β-mercaptoethanol (50 µl to 950 µl sample buffer) was added before use.

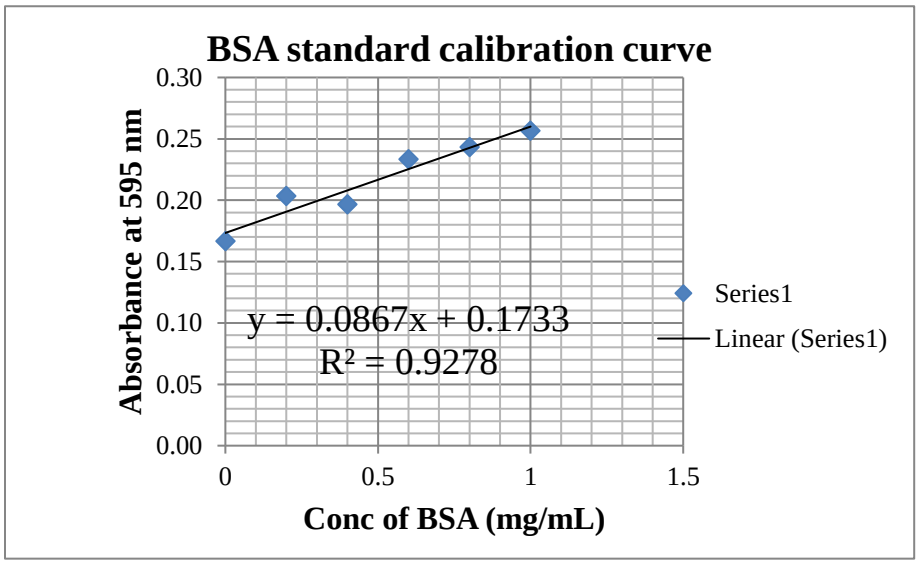
5x Running Buffer:

25 mM Tris-HCl, 200 mM Glycine, 0.1% (w/v) SDS, 0.1% (w/v) SDS, H_2O

Appendix V Calibration plots for molecular weight of standard protein 10-200kDa

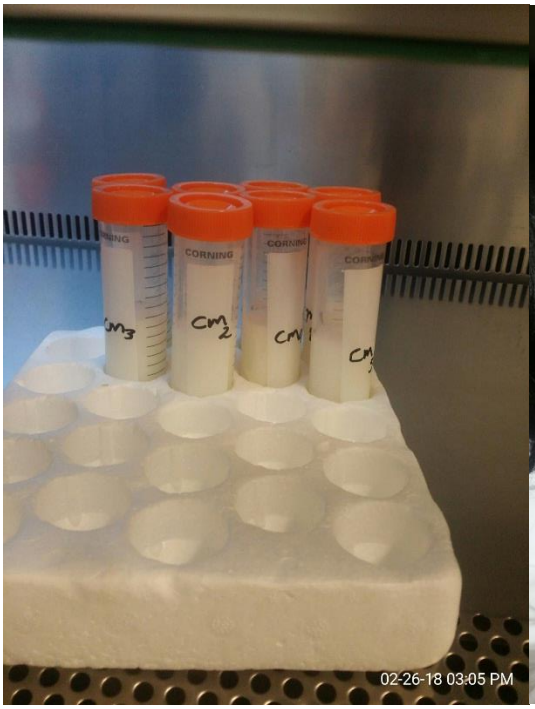


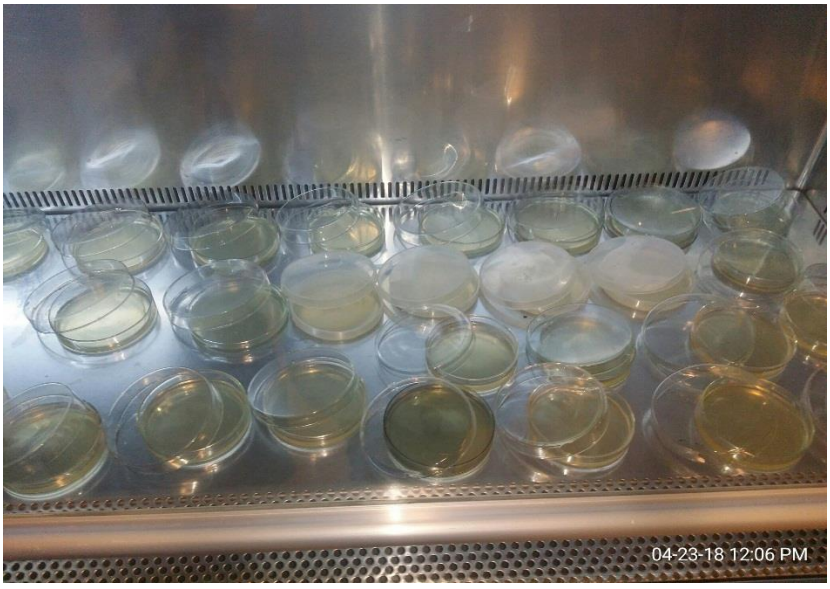
Appendix VI Bradford assay calibration curve



Glimpse on the events of laboratory analysis work

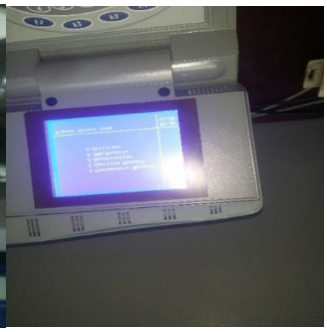
Microbial analysis





Ammonium sulfate precipitation and centrifugation


Dialysis and Bradford assay

Electrophoresis