Isolation of Lactoferrin from Raw and Commercial Milk Samples Followed by the Observation of Antimicrobial Activity against Pathogens

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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DECLARATION

This is to declare that the research work embodying the results reported in this thesis entitled "**Isolation of Lactoferrin from Raw and Commercial Milk Samples Followed by the Observation of Antimicrobial Activity against Pathogens"** has been carried out by the undersigned under supervision of Kashmery Khan, Lecturer, Biotechnology program, Department of Mathematics and Natural Sciences, BRAC University. It is further declared that the research work presented here is original and submitted in the partial fulfilment for the degree of Bachelors of Science in Biotechnology, BRAC University, Bangladesh and has not been submitted anywhere else for a degree or diploma.

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Certified

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Dedicated to

My beloved parents and brother

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Abstract

Lf is a multifunctional glycoprotein with 80 kDa size found in the milk of most mammals. In addition to its well-known role of binding iron, Lf carries many important biological functions, including the promotion of cell proliferation and differentiation, and as an anti-bacterial, anti-viral, and anti-parasitic protein. Milk is the highest quality source of well-balanced nutrients and also displays a range of biological activities that affects digestion, metabolic responses to absorbed nutrients, growth & development of specific organs, and resistance to disease. The purpose of this study is to isolate Lf from different milk samples like raw and commercial milk samples and observe the antimicrobial activity of them. Along with this, the quantity of Lf was differentiated between raw and commercial milk samples. Firstly, Lf was extracted from both raw and commercial milk by acid-base adjustments. Then the concentration of isolated Lf protein was measured by NanoDrop technology and the quantities were compared between the raw and commercial ones. Finally, Lf was identified by SDS-PAGE where 5 bands of Lf were observed among eleven samples. After that, all the samples were tested for their antimicrobial activity against 18 pathogens collected from laboratory stocks. Well diffusion method was used to conduct this test. Lf containing samples showed ZOI against some of the pathogens but some other samples those do not contain Lf also gave ZOI against some of the pathogens. The reason could be, though they do not contain Lf but they contain such proteins that can reduce the microbial growth. All these results are collected and discussed in this study. The raw milk contain higher quantity of Lf than the commercial milk and Lf showed effective results against pathogens that can cause infectious disease in human body. This study suggested that Lf can be used as the potential alternative of antibiotics for many diseases and also can be used to reduce microbial deterioration in food and feed industry.

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LIST OF ABBREVIATION

Lf	Lactoferrin
BRAC	Bangladesh Rural Advancement Committee
et al	And Others
G	Gram
Ml	Milliliter
Mm	Millimeter
PBS	Phosphate Buffer Saline
NaCl	Sodium Chloride
Na_2HPO_4	Disodium Phosphate
KH_2PO_4	Monosodium Phosphate
KCl	Potassium Chloride
dH_2O	Distilled Water
HCl	Hydrochloric Acid
NaOH	Sodium Hydroxide
$(NH_4)_2SO_4$	Ammonium Sulphate
bLf	Bovine Lactoferrin
hLf	Human Lactoferrin
rhLf	Recombinant Human Lactoferrin
IL-18	Interleukin 18
Rpm	Revolutions Per Minute
BSA	Bovine Serum Albumin
TGS	Tris-Glycine-SDS Buffer
NA	Nutrient Agar
MHA	Mueller Hinton Agar
V	Volt
ZOI	Zone of Inhibition
STEC	Shiga Toxin-producing *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
Std	Standard
°C	Degree Celsius

N	Normality
M	Molarity
APS	Ammonium Per Sulphate
SDS	Sodium Dodecyl Sulphate
PAGE	Polyacrylamide Gel Electrophoresis
L	Litre

Chapter One

Introduction

1.1 Introduction:

Lf is an iron binding glycoprotein of the transferring family which is of 80 kDa in size (Sharbafi and Rafiei *et al.*, 2014). Lf is a noteworthy component of milk and furthermore, is introduced in neutrophil granules or other exocrine discharges, for example, tears, salivation and the cervical bodily fluid (Sharbafi and Rafiei *et al*., 2014). Milk is by far the most abundant source of Lf with human colostrum, the early milk, containing up to 7g/l (Farnaud & Evans *et al.*, 2003). The concentration in tears is as high as 2mg/ml while that in blood is typically just as high as 1g/ml, in spite of the fact that it can ascend as high as 200g/ml in the inflammatory circumstance (Farnaud & Evans *et al.*, 2003). Lf, highly conserved among human, bovine, mouse, and porcine species, is of about 690 amino acid residues and is able to reversibly chelate two Fe(III) per molecule with high affinity (Kd ~ 10−20 M) retaining ferric iron to pH values as low as 3.0, whereas transferrin retains iron at pH of about 5.5 (Berlutti & Pantanella *et al.*,2011).The most astounding substance among amino acids build ups has alanine (~10 %), leucine (~9 %) and glycine (~7 %), the minimal tryptophan (~1.5 %), histidine (~1.3 %) and methionine (~0.6 %) (Adam *et al.*, 2008). The iron-restricting affinity is sufficiently high that, within the sight of lactoferrin or transferrin, the grouping of free iron in body liquids can't surpass 10–18 M, consequently keeping the precipitation of this metal as insoluble hydroxides, restraining microbial development and blocking arrangement of reactive oxygen species (Berlutti & Pantanella *et al.*, 2011). Lf is now known to be a multifunctional or multitasking protein. As of late it has been perceived that oral organization of Lf applies different wellbeing helpful impacts, for example, against infective exercises not just in children additionally in grown-up creatures and human (Sharbafi and Rafiei *et al.*, 2014).It is a major component of innate immune system of mammals. Its protective effects range from direct antimicrobial activities against a large panel of microorganisms including bacteria, viruses, fungi and parasites, to anti-inflammatory and anticancer activities.

1.2 Structure :

1.2.1 Iron-Binding Sites

The two lobes of Lf are additionally separated into two domains (N1 and N2, C1 and C2) and each projection ties one Fe (III) ion in a profound split between two areas

(Berlutti & Pantanella *et al.*, 2011).The iron locales are exceedingly conserved in all iron-binding proteins, recommending a typical transformative source. The ligands for Fe (III) are the same in both projections: one aspartic corrosive, two tyrosines, and one histidine (Asp-60, Tyr-92, Tyr-192, and His-253 in the N-projection and Asp-395, Tyr-433, Tyr-526, and His-595 in the C-projection), together with two oxygen from the CO_3^{2-} anion (Steijns and Hooijdonk *et al.*, 2000).

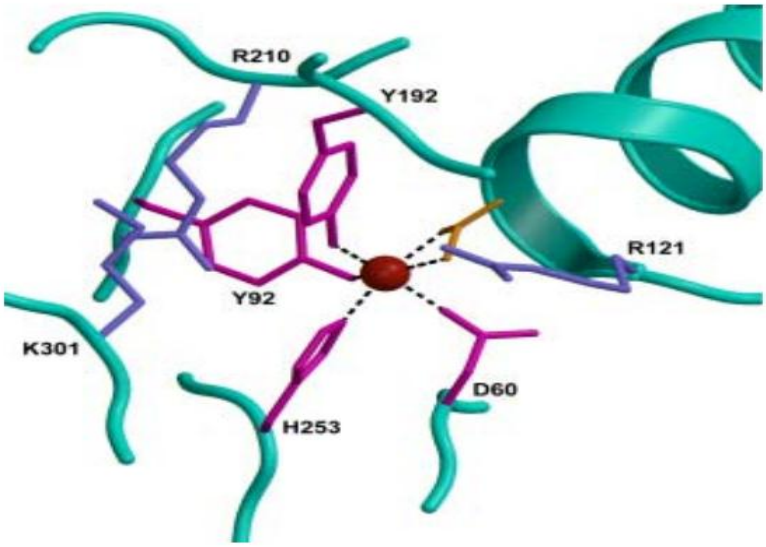


Figure 1: Iron-binding site in the N-lobe of Lf (Adapted from Baker & Baker, 2005)

Spectroscopic reviews and the 3D structure recommend that the CO_3^{2-} ion ties to start with, along these lines neutralizing the positive charge of the arginine build-up (Arg-121 in the N-lobe and Arg-465 in the C-lobe) (Berlutti & Pantanella *et al.*,2011). The participation of the CO_3^{2-} particle in the iron coordination binding has all the earmarks of being perfect for iron reversible binding since the protonation of CO_3^{2-} ion is a possible initial phase in the separation of the iron site at low pH (Adam and Zitka *et al.*, 2008).

1.2.2 Binding with other Metals

Lf is delegated as an iron binding protein, however can likewise tie other metal particles including Cu^{2+}, Mn^{2+}, Zn^{2+}, regardless of the possibility that with lower affinity. Metal binding can be tested by an expansion in adsorption at 240–280 nm as outcome of ionization of the tyrosine ligands which tie to the metal ions. The crystal

structures of Lf immersed with Mn^{2+} or Zn^{2+} have all indicated shut shapes, in this manner recommending that Lf could have a part in binding other metal particles (Farnaud & Evans *et al.*, 2003). In addition, it has been exhibited that Mn2+-or Zn2+-soaked structures keep up some physiological elements of Lf, disconnected to its iron binding ability yet presumably identified with its three striking groupings of positive charge: Residues 1–7, 13–30 and between lobe area, near the connecting helix (Berlutti & Pantanella *et al.*, 2011;Brock *et al.*, 2009).

1.2.3 Genes of Lf

At least 60 gene sequences of Lf have been characterized in 11 species of mammals. In most species, stop codon is TAA and TGA in *Mus musculus*. Deletions, insertions and mutations of stop codons affect the coding part and its length varies between 2,055 and 2,190 nucleotide pairs (Baker &Baker *et al.*, 2005).In human, Lf gene LTF is situated on the third chromosome in the locus 3q21-q23. In oxen, the coding grouping comprises of 17 exons and has a length of around 34,500 nucleotide sets. Exons of the Lf quality in bulls have a comparable size to the exons of different qualities of the transferrin family, while the sizes of introns contrast inside the family (Kruzel & Bacsi *et al.*, 2006). Comparability in the measure of exons and their circulation in the areas of the protein atom demonstrates that the transformative improvement of Lf quality happened by duplication (Baker & Baker *et al.*, 2005; Berlutti & Pantanella *et al*., 2011).

1.2.4 Glycosylation

Lf is a glycosylated protein, having distinctive number and area of putative glycosylation destinations, as per distinctive species (Berlutti & Pantanella*et al.*, 2011). Human Lf contains three potential *N*-glycosylation sites: asparagine (Asn) 138, Asn479, Asn624; caprine, bovine and ovine Lf have five sites: Asn233, 281, 368, 476 and 545 whereas murine Lf has only one potential *N*-glycosylation site: Asn476 (Karav & German *et al*., 2017**)**. Among these glycosylation sites, only two sites are commonly glycosylated in hLF: Asn138 and Asn479, and four sites are glycosylated in blF: Asn233, Asn368, Asn476, and Asn545 (Karav & German *et al.*, 2017**)**.The nature and the area of the glycosylation destinations don't impact the polypeptide folding or iron and different molecule binding properties. On the other hand, the loss

of carbohydrate or sialic acid builds its affectability to proteolysis or impacts some physiological capacities (Sharbafi and Rafiei, 2014).

1.3 Biological Functions of Lf

The biological significance of Lf is as yet not completely clear. Its antiphlogistic, bacteriostatic and bactericidal impacts are assumed (Adam *et al.*, 2008). Numerous parts have been proposed, and keep on being proposed for Lf. Although some of these are unmistakably identified with its iron-restricting properties, for instance its ability to provide bacteria with a source of iron and therefore act as a "promicrobial" whereas others appear to be independent of iron binding (Farnaud *et al.*, 2003).

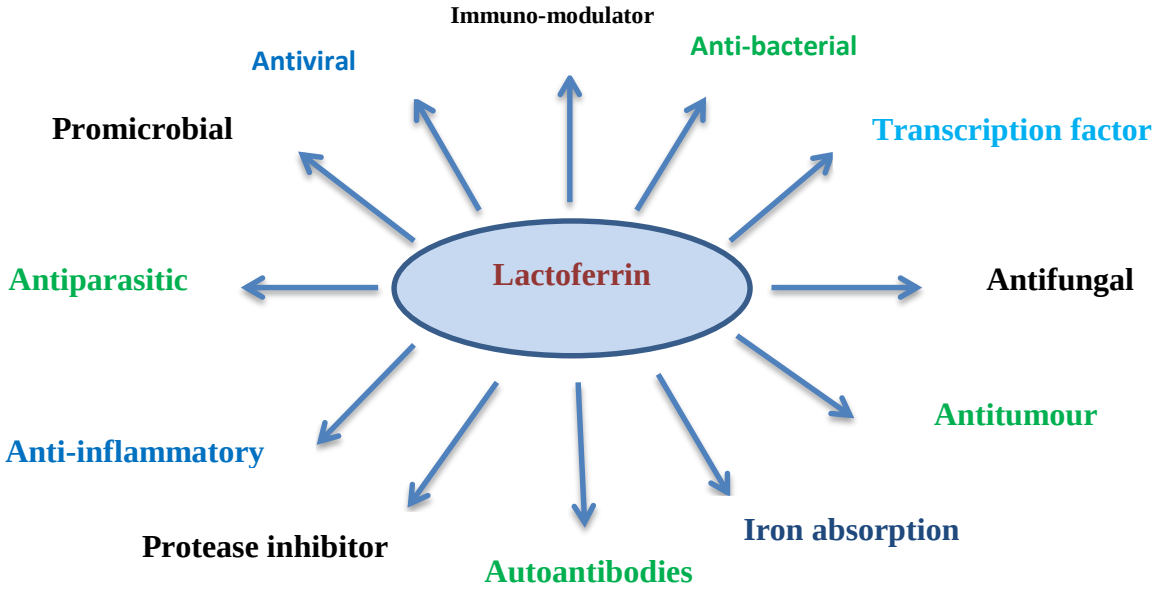


Figure 2: Proposed roles of Lf (Adapted from Brock, 2002)

It has been widely accepted for many years that Lf displays antimicrobial activity against many different infectious agents. This activity was originally attributed to its ability, in common with transferrin, to sequester iron with a high affinity and unlike transferrin, retain its bound iron under acidic conditions (Griffiths and Williams *et al.*, 2001). More recently it has become apparent that some of the antimicrobial properties of Lf are independent of iron binding.

1.3.1 Antibacterial Activity

The anti-bacterial activity of Lf was initially ascribed to its ability to bind and sequester environmental iron, thereby depriving potential pathogens of this essential nutrient(Arnold *et al.*,1977).The capacity of Lf to restrain bacterial development in vitro was without a doubt one of the soonest capacities depicted for the protein (Farnaud *et al.*, 2003). The antimicrobial action of Lf was exhibited towards a number of microscopic organisms and Lf was observed to be bactericidal for *Streptococcus mutans* and *Vibrio cholerae*, however not for *Escherichia coli* (Arnold *et al.*, 1977). Additionally examines have demonstrated that Lf was bactericidal just when in its sans iron state and that iron-immersed Lf has a diminished antimicrobial movement (Arnold *et al*., 1980; Kalmar what's more, Arnold, 1988; Yamauchi *et al*., 1993).

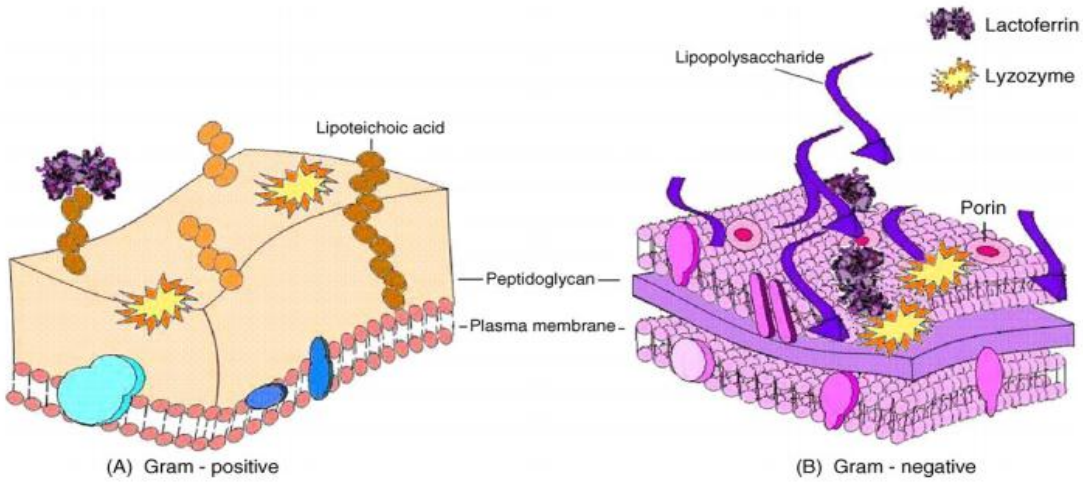


Figure 3: Mechanism of antibacterial action of Lf. (A) Gram-positive bacteria: Lf is bound to negatively charged molecules of the cell membrane such as lipoteichoic acid, neutralising wall charge and allowing the action of other antibacterial compounds such as lysozyme. (B) Gram-negative bacteria: Lf can bind to lipid A of lipopolysaccharide, causing liberation of this lipid with consequent damage to the cell membrane (Chávez *et al*., 2008).

1.3.2 Antiviral Activity

The antiviral activity of Lf has been investigated in great detail. Pioneer work demonstrated that only enveloped viruses were affected, and that this activity was due to either inhibition of virus host interaction e.g. hepatitis B virus (HBV), herpes simplex virus (HSV) and human cytomegalovirus (HCMV) or direct interaction

between Lf and the viral particle e.g.; feline herpes virus (FHV-1), hepatitis C virus (HCV), hepatitis G virus (HGV) and human immunodeficiency virus (HIV) (Jenssen *et al.*, 2008). However, recently it has also been demonstrated that naked viruses like rota-, polio-, adeno- and entero virus are susceptible to inhibition by Lf. In all cases studied, it appears that Lf exhibits its antiviral activity at an early phase of the infection process (Karav *et al.*, 2011). In vitro studies also demonstrated that Lf exhibits synergy, in combination with zidovudine, against HIV-1. A synergistic antiviral activity was also observed for HSV-1 and HSV-2 when acyclovir was used in combination with Lf (Karav *et al.*, 2011). In clinical trials on a limited set of HCV patients, it was demonstrated that Lf significantly reduces the HCV RNA titre, and contributes to the effectiveness of a combined therapy with interferon and ribavirin. Oral administration of Lf has also led to promising improvement in the immune responses of antiretroviral therapy-naive children suffering from HIV (Jenssen *et al.*, 2008).

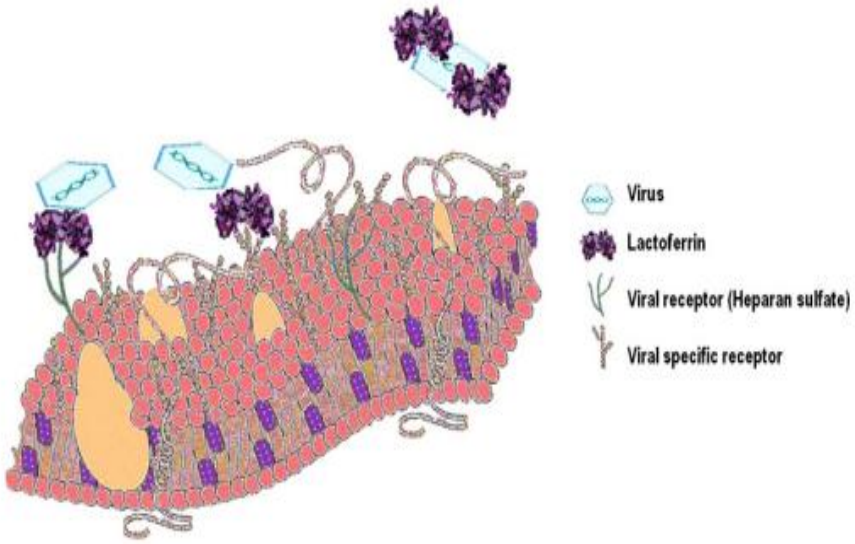


Figure 4: Mechanism of antiviral action of Lf. LFf can be linked to the viral particle and to glycosaminoglycans, specific viral receptors or heparan sulfate to prevent internalisation of the virus into the host cell (Chávez *et al.*, 2008).

1.3.3 Antifungal Activity

Lf was first reported to have anti-fungal activity by Kirkpatrick (1971), (Farnaud *et al.*, 2003). Lf restrains *in vitro* development of *Trichophyton mentagrophytes*, which are in charge of a few skin maladies, for example, ringworm (Laquerre *et al*., 1998). Lf likewise acts against the *Candida albicans* – a diploid organism (a type of yeast) that causes crafty oral and genital diseases in humans. Fluconazole has been utilized against *Candida albicans*, which brought about development of strains impervious to this medication (Hwang *et al.*, 2009). Be that as it may, a blend of Lf with fluconazole can act against fluconazole-safe strains of *Candida albicans* and additionally different sorts of *Candida: C. glabrata, C. krusei, C. parapsilosis* and *C. tropicalis* (Hwang *et al.*, 2009). Antifungal action is watched for successive brooding of *Candida* with Lf and after that with fluconazole, however not the other way around. Organization of Lf through drinking water to mice with debilitated insusceptible frameworks and side effects of aphthous ulcer lessened the quantity of *Candida albicans* strains in the mouth and the span of the harmed ranges in the tongue (Jenssen *et al.*, 2008).Oral organization of Lf to creatures additionally diminished the quantity of pathogenic living beings in the tissues near the gastrointestinal tract (Tachezy *et al.*, 1996).

1.4 Applications of Lf

1.4.1 Lf and intestine inflammation

Endoscopy coupled with biopsy represents the standard of intestine inflammation detection (Adam *et al.*, 2008). This technique is however very expensive and invasive. Because of these facts the new techniques and markers for intestine inflammation detection have been proposing (Adam *et al.*, 2008). Their advantage bases in the facts that they enable us to predict relapse of the inflammation and to monitor effect of the treatment (Adam *et al.*, 2008). Many of inflammatory mediators such as leucocytes, cytokines and proteins from neutrophil activation have been analysed in faeces (Buttner *et al.*, 2002). It was ascertained that Lf level has diagnostic signification especially in the cases of inflammations at colon area (colitis) (Chávez *et al.*, 2008). The Lf level is significantly increased in the case of Crohn´s disease and may be excellent indicator of inflammation at colon area determined from faecal samples (Buttner *et al*., 2002). In addition the increase in Lf level was observed at more than

half of patients with colorectal adenocarcinoma (Buttner *et al.*, 2002). The enhanced Lf level was determined immune histo-chemically at patients with malignant tumours of kidneys compared with healthy tissues, where the level of this protein was under the detection limit of the diagnostic technique used (Buttner *et al.*, 2002).

1.4.2 More Potential Applications

Table 1: Potential Applications of Lf (Weinberg, 2007)

Product	**Purpose**
Rice expressing Lf+LZ;fed to humans	Prevention of acute diarrhoea
Rice expression of hLf	Prevention of bacterial seedling blight
Pear expression of bLf	Prevention of bacterial fire blight
bLf added to food and drinks-stored products;e.g.,soy powder	Prevention of iron-induced oxidation during storage
bLf sprayed on meat products	Prevention of bacterial growth during storage
Topical rhLf	Enhancement of wound healing
Topical bLf	Promotion of bone repair
Oral rhLf	Enhancement of IL-18 in gut cells; Suppression of tumor cell growth.
Oral apo-Lf	Suppression of gut cell release of pro-inflammatory cytokines in ulcerative colitis
Oral apo-Lf+probiotic	Suppression of overgrowth of enteric pathogens
Vaginal activated Lf	Suppression of candidal growth
Cervical rhLf	Suppression of infection-induced pre-term delivery

1.5 Objective

Very few researches have been done with Lf so far in Bangladesh and this is the first work regarding Lf in our university laboratory. The main objective was to highlight the potential benefits of Lf. One of these benefits is its antimicrobial ability. As it shows antimicrobial activities, it can be used as the alternative of antibiotics for curing many diseases. The main objectives of the study are:

1. To isolate Lf from different milk samples available in our country.
2. To establish a standard protocol for the isolation of Lf.
3. To differentiate the quantity of Lf between the raw and commercial milk samples.
4. To observe the antimicrobial activity of Lf.

Chapter Two

Materials and Methods

Materials and Methods

2.1 Place of Study

The present study was carried out in the Biotechnology and Microbiology Laboratory, Department of Mathematics and Natural Sciences, BRAC University.

2.2 Collection of samples

Raw and commercial milk samples were collected from different sources. There were both liquid and powder milk as commercial milk samples. All milk samples used are of Bangladesh except DUTCHLADY. A total number of 11 milk samples were collected in which 4 were raw milk samples and the other 7 were commercial ones. The commercial samples were collected from various departmental stores of Dhaka and Chittagong whereas raw milk samples were taken from dairy farms of Dhaka and cox's Bazar. In addition to these, breast milk was collected from a breast feeding mother.

Table 2: Name of the samples for Lf Isolation (Mentioned numbers were marked during experiment)

Number of samples	Raw and commercial Milk Samples
17	**Cow Milk**
07	**Goat Milk**
21	**Buffalo Milk**
20	**Breast Milk**
01	**Fresh**
08	**Red Cow**
09	**King**
10	**Dutch lady**
12	**Nido**
15	**Diploma**
19	**Olympic**

2.3 Preservation of collected samples

The raw milk (liquid) samples were stored at 0°C before performing the isolation procedure to prevent the deterioration of milk. The commercial milk samples were collected right before performing the isolation method for which there was no need of preservation. The collected commercial milks (powder) from Chittagong were stored at 4°C.

2.4 Isolation of Lf from milk samples

Lf can be isolated from milk samples by using centrifugation method followed by acidic-basic pH adjustment (Moradian & Sharbafi *et al.*, 2014). The isolation of Lf from milk samples were done in two steps. Initially casein was separated from milk and then the casein separated sample was treated to isolate Lf. The total days of three were needed to complete the full isolation process of Lf for each sample.

2.4.1 Separation of casein

40 mL of each sample was subjected to centrifugation for 10 minutes at 4000 rpm at 4^0 C. Then fat layer (top most) obtained was separated using a spatula and discarded. The volume of all defatted milk samples was noted and an equal volume of distilled water was added. After that, initial pH of each sample was recorded using the pH meter. 1N HCl was added slowly with constant stirring to each sample until pH was reached to 4.6 to precipitate casein, followed by centrifugation at 2000 rpm for 10 minutes at 4^0 C. Supernatants from each sample were stored in a refrigerator at 4^0 C for further analysis. Sometimes it was seen that only one time centrifugation was not enough to defat milk. Thus 2/3 times more centrifugation had to be done to obtain defat milk.

2.4.2 Lf extraction from stored supernatants and preservation

1N NaOH was added slowly with constant stirring to all the supernatants collected from the previous treatment till pH 6.0 was reached. Each sample's volume was noted and an equal volume of 45% ammonium sulphate solution was added to all samples with constant magnetic stirring at 100 rpm. Then stirring was gradually increased to 420 rpm after whole addition of 45% ammonium sulphate solution and was kept for 1

hour at room temperature. All samples were then subjected to the addition of 1N HCl slowly with constant stirring till pH 4.0 was reached, followed by addition of 1N NaOH slowly till pH 8.0. At pH 8.0, an equal volume of 80% ammonium sulphate solution was added with constant magnetic stirring at 100 rpm and gradually increased to 420 rpm for 1 hour after whole addition of ammonium sulphate solution. After these, all samples were incubated at 4^0 C overnight to precipitate Lf, followed by centrifugation at 4000 rpm for 10 minutes at 4^0 C. Lf precipitate obtained was then dissolved and re-suspended in 1mL 1x PBS buffer (pH 7.4) and stored in a refrigerator at 4^0 C in the respective tubes for further analysis.

Figure 5: pH adjustment

Figure 6: Mixing with magnetic stirrer

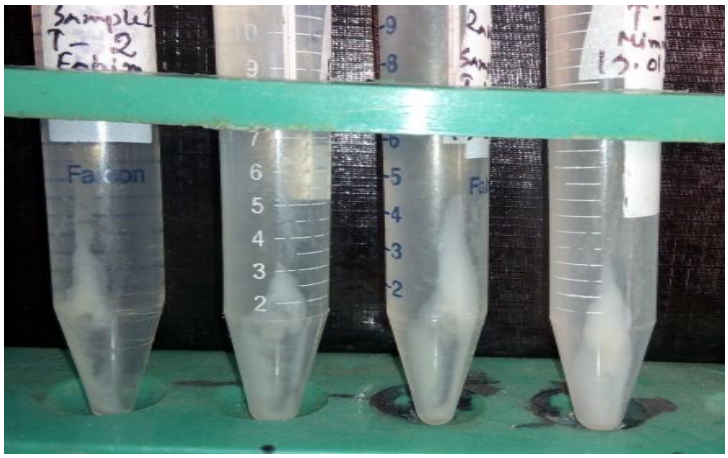


Figure 7: Lf precipitate

Figure 8: Lf ready for preservation

2.5 Biochemical tests for identification of Lf

According to the National Human Genome Research Institute, a biochemical test is performed for assessing the level of protein or enzyme activity in a sample. There are many experiments available for the identification of protein like Lf. Some of these are Bradford assay, nanodrop assay, SDS-PAGE, different chromatography techniques etc. (Adam *et al.*, 2008). All of these experiments could not be done due to laboratory limitations. Among these, nanodrop assay and SDS-PAGE were done to identify and quantify the protein Lf.

2.5.1 Protein quantification by using nanodrop technology

Nanodrop technology is used to quantify the amount of protein present in a sample. 1-2µL of sample can be quantified in seconds using this method (Kazemipoor *et al.*, 2012). The results are very accurate and reproducible. With the arm open, a sample is pipetted directly onto the pedestal. After the arm is closed, a sample column is formed. The pedestal then moves to automatically adjust for an optimal path length (0.05–1mm). When the measurement is complete, the surfaces are simply wiped with a lint-free lab wipe before going on to the next sample. This experiment was done by the help of INVENT technologies Ltd. BSA was used as standard protein for the measurement. Firstly, small amount of TE buffer was put onto the pedestal of nanodrop machine with the help of pipette to make it blank. Then BSA was quantified. The results were shown on the monitor connected to the machine. Thus proteins of all the 11 samples were quantified. The nanodrop well was cleared every time to prevent contamination. For better accuracy, each sample was quantified more than one time.

Figure 9: Step of NanoDrop technology

2.5.2 Lf identification by using SDS-PAGE

Sodium dodecyl sulphate (SDS)-Polyacrylamide gel electrophoresis (PAGE) is one of the most widely used laboratory methods to separate biological macromolecules such as proteins and nucleic acids. Macromolecules will be differentiated according to their electrophoresis mobility which is a function of the length, conformation and charge of the molecule (http://www.rockland-inc.com/SDS-Page-Electrophoresis.aspx). To separate molecules based on their lengths, samples are run in denaturing conditions. Sodium dodecyl sulphate (SDS) is used to linearize proteins and to negatively charge the proteins (Atlas *et al*., 2011). The binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass (Atlas *et al*., 2011). As a result, negatively charged proteins will migrate towards the positive electrode and will be fractionated by approximate size during electrophoresis. The gel which is used in SDS-PAGE is composed of two parts; stacking gel and resolving gel. Some of its benefits are like the molecular weight and purity of protein can be determined, small amount of sample is needed and it is highly sensitive test.

- Both the stacking and resolving gel were prepared according to the procedure of Bio-metra.
- The SDS-PAGE apparatus were assembled properly and the gel was set in the assembled glass. The resolving gel was poured first and left to set and then the stacking gel was poured followed by the addition of comb into it. Before

pouring the stacking gel absolute ethanol was poured on resolving gel to avoid bubble formation. When resolving gel is set properly, the absolute ethanol was soaked with filter paper before pouring stacking gel. 12.5% resolving gel and 5% (1.5mm) stacking gel was used.

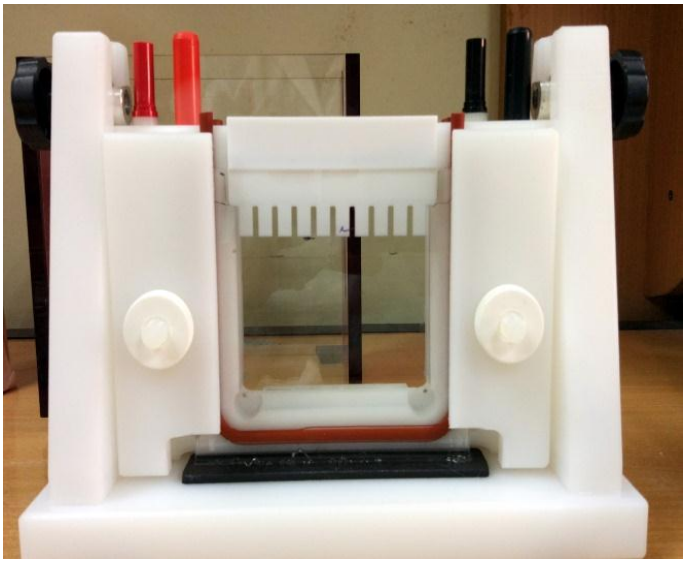


Figure 10: Gel preparation for SDS-PAGE

- 1x TGS running buffer was poured into the upper and lower chambers. Buffer was added very slowly to avoid any kind of bubble formation. The comb was removed gently.
- 15 µL of sample was added to 15 µL of 4x SDS loading dye and then kept in the water-bath for 2min at 95°C.
- Then 20 µL of each samples and ladder were loaded into each well and electricity was supplied at 100V for 2 hours.

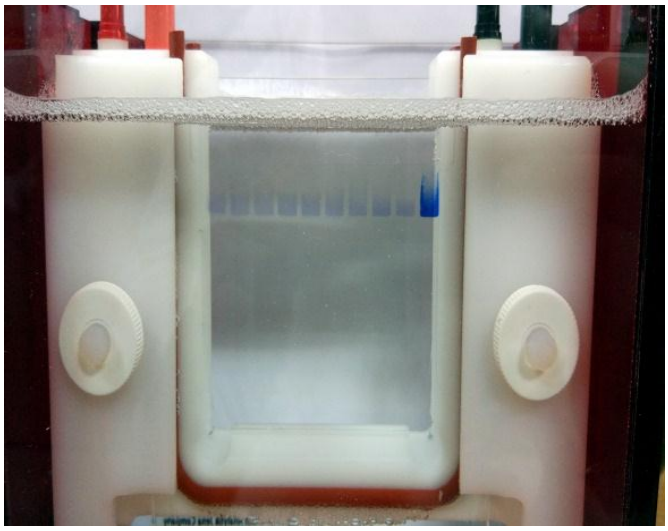


Figure 11: Samples have been loaded

- The gel was then poured into staining solution and kept in water-bath for 20min at 55°C. After that the gel was kept in the distaining solution followed by 20min water-bath treatment at 55°C. The gel was then kept overnight in the distaining solution and observed next day.
- Thus all the samples were run in two batches. The last batch was run for another time to recheck the result.

2.6 Antimicrobial activity test

We have already known about the proposed activities of Lf. One of the most important activities is the antimicrobial activity. It was tested against 18 pathogens collected from university laboratory stock. Initially the pathogens were sub-cultured on NA media plates. After 24 hour incubation at 37°C, desired growth was obtained and stored at 4°C for further analysis. The chart of the pathogens that used is given:

Table 3: Table of pathogens used for antimicrobial activity test

Name of pathogens	Name of pathogens
Staphylococcus aureus	Minor skin disease like pimples, impetigo, boils, scalded skin syndrome etc. and life threatening diseases like pneumonia, toxic shock syndrome, bacteremia, sepsis etc.
Streptococcus pneumoniae	Infection can result in pneumonia, bacteremia or sepsis, otitis media, bacterial meningitis etc.
Klebsiella pneuminiae	Pneumonia, bloodstream infections, wound infections, urinary tract infections and meningitis.
Proteus vulgaris	Urinary tract infections and hospital-acquired infections.
Salmonella typhi	Salmonellosis, typhoid fever, food

	poisoning, gastroenteritis, enteric fever etc.
Shigella dysenteriae	Epidemic dysentery.
Shigella flexneri	Shigellosis a type of diarrhoea.
Vibrio cholera	Cholera which can cause watery diarrhoea and can lead to dehydration & even death if untreated.
Bacillus subtilis	Sometimes cause food poisoning.
Bacillus cereus	Diarrheal illness and emetic illness.
STEC	STEC infections can lead to Hemolytic-uremic syndrome (HUS).
Enterococcus faecalis	Causes nosocomial infections from which urinary tract infections are very common.
EAEC	Acute and persistent diarrhoea.
ETEC	Causes diseases in human and domestic animals by producing heat stable toxin. It is called "Travelers's diarrhoea".
EPEC (typical)	Childhood diarrhoea
EPEC (atypical)	Childhood diarrhoea
Pseudomonas aeruginosa	Urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, systemic infections in patients with burns and cancer etc.
Streptococcus pyogenes	Pharyngitis, scarlet fever, impetigo, Erysipelas. Invasive infections can result in necrotizing fasciitis, myositis and streptococcal toxic shock syndrome.

Well diffusion method was followed to test the antimicrobial activity of Lf samples against these pathogens.

2.6.1 Well diffusion method for antimicrobial activity test

Well diffusion method is one of the most effective methods to test the antimicrobial susceptibility. This method requires a 24 hour culture of the pathogens (was suspended in 0.9% saline), which is used to prepare a lawn of the indicator strain by spreading the cell suspension over the surface of MHA plates with a sterile cotton swab. After the plates dry, wells are made in the MHA using a sterile cork borer of diameter (4 mm). Each well is then filled with the Lf samples. Antimicrobial activity from the samples was observed after overnight incubation at 37°C; ZOI around the wells indicate antimicrobial activity. Results were considered positive if the diameter (mm) of the ZOI was greater than 1mm (Kazemipoor *et al.*, 2012). There are some reasons of using MHA plates for well diffusion method such as it is a non-selective, non-differential medium. This means that almost all organisms plated on here will grow. It also contains starch. Starch is known to absorb toxins released from bacteria, so that they cannot interfere with the antibiotics (Adebayo *et al.*, 2014). It also mediates the rate of diffusion of the antibiotics through the agar. Lastly, it is a loose agar. This allows for better diffusion of the antibiotics than most other plates. A better diffusion leads to a truer zone of inhibition (https://microbiologyinfo.com/mueller-hinton-agar-mha-composition-principle-uses-and-preparation/).

2.7 Differentiation of the amount of Lf between raw milk and commercial milk

The raw milk does not undergo with any kind of treatment whereas the commercial milk undergoes different types of treatment like boiling, pasteurization etc. Various types of nutritional components are sometimes added to the commercial milk by the companies to increase the nutritional value of their product. On the other hand, raw milk used in this research work was collected directly from the farms without any kind of fortification. As chromatography technique had not been used for laboratory limitations, initially the measured concentration of all protein samples by using nanodrop technology were collected and then the SDS-PAGE result of all the samples were also collected. Then both these results were compared to determine the difference of the quantity of Lf present between raw and commercial milk samples. Through this process exact amount of Lf present in the samples cannot be detected.

As a result, the acquired concentration difference between the quantity of Lf in raw and commercial milk is hypothetical.

Chapter Three

Results

Results

Lf was isolated from 11 milk samples from which four were raw milk samples and the others were commercial. Then nanodrop technology and SDS-PAGE were used to identify the isolated Lf. After that, antimicrobial test was done to observe the antimicrobial activity of Lf. A total of 18 pathogens were used to observe the antimicrobial activity.

3.1 Protein quantification by nanodrop technology

Almost all the samples showed protein concentration in the test. Some of the samples contained good amount of protein and some others contained small amount in contrast. Measurements were taken more than one time for each sample for better accuracy.

Table 4: Protein (Lf) concentrations of milk (RAW) samples

Sample No.	Name of the Sample	Concentration of Protein	A280	260/280
Std	BSA	0.161mg/ml	0.161	0.44
Std	BSA	0.229mg/ml	0.229	0.67
Std	BSA	0.43mg/ml	0.43	0.34
7.	Goat Milk	-2.669mg/ml	-2.669	1.2
7.	Goat Milk	-2.445mg/ml	-2.445	1.16
7.	Goat Milk	12.067mg/ml	12.067	1.05
7.	Goat Milk	11.716mg/ml	11.716	1.01
17.	Cow Milk	5.43mg/ml	5.43	0.95
17.	Cow Milk	4.998mg/ml	4.998	0.92
20.	Breast Milk	4.224mg/ml	4.224	0.90
20.	Breast Milk	4.18mg/ml	4.18	0.90
21.	Buffalo milk	2.293mg/ml	2.293	0.82
21.	Buffalo milk	2.725mg/ml	2.725	0.87

- **1 Abs = 1 mg / mL**

The red marked boxes are of the considered results. From all the raw milk samples, goat milk showed the highest amount of protein present which is 12.067mg/ml and the buffalo milk showed the lowest amount of all which is 2.725mg/ml. The same samples showed the highest and lowest OD which is respectively 1.05 and 0.87.

Table 5: Protein (Lf) concentrations of milk (commercial) samples

Sample No.	Name of the Sample	Concentration of Protein	A280	260/280
Std	BSA	0.161mg/ml	0.161	0.44
Std	BSA	0.229mg/ml	0.229	0.67
Std	BSA	0.43mg/ml	0.43	0.34
01.	Fresh	3.379mg/ml	3.379	1.23
01.	Fresh	3.141mg/ml	3.141	1.22
08.	Red cow	1.224mg/ml	1.224	1.11
08.	Red cow	1.426mg/ml	1.426	1.07
09.	King	-0.049mg/ml	-0.049	1.76
09.	King	-0.029mg/ml	-0.029	2.46
09.	King	2.261mg/ml	2.261	1.26
09.	King	3.972mg/ml	3.972	1.24
10.	Dutch Lady	4.226mg/ml	4.226	0.96
10.	Dutch Lady	4.57mg/ml	4.57	0.99
12.	Nido	2.347mg/ml	2.347	1.06
12.	Nido	2.79mg/ml	2.79	1.06
15.	Diploma	-0.01mg/ml	-0.01	-0.73
15.	Diploma	-0.087mg/ml	-0.087	1.56
15.	Diploma	-0.073mg/ml	-0.073	1.54
15.	Diploma	2.828mg/ml	2.828	1.07
15.	Diploma	2.043mg/ml	2.043	0.98
19.	Olympic	7.296mg/ml	7.296	1.28
19.	Olympic	5.983mg/ml	5.983	1.30

- **1 Abs = 1 mg / mL**

The red marked boxes are of the considered results. From all the commercial milk samples, olympic milk showed the highest amount of protein present which is 7.296mg/ml and the redcow milk showed the lowest amount of all which is 1.426mg/ml. The same sample olympic showed the highest OD which is 1.28 but dutch lady milk sample showed the lowest OD which is 0.99.

3.2 Lf identification by using SDS-PAGE

SDS-PAGE was run in total three times for 11 samples. Eleven samples were divided into two batches where one batch was run for two times for better result.

3.2.1 First batch of SDS-PAGE run

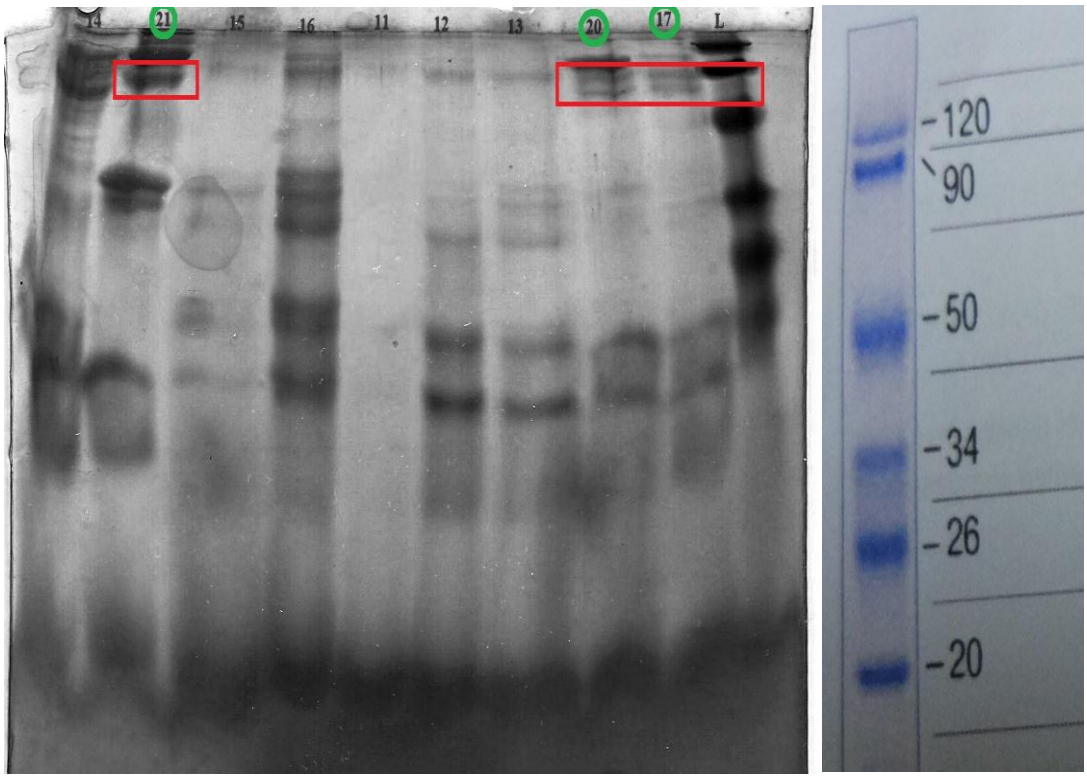


Figure 12: SDS-PAGE result of first batch of samples (the left one) and the size of ladder used (the right one). The number of samples are denoted above of the bands of respective samples

There were in total 5 samples in the gel. As we already know that the size of Lf is about (77-80) kDa, the bands found were compared with the ladder to see whether Lf

is present there or not. The bands those matched with the ladder size of Lf are marked with red coloured box in the picture and their sample numbers are marked with green coloured round box. The sample's numbers are 17, 20, 21 which stand for respectively **cow milk, breast milk and buffalo milk**. The other samples did not show the bands that match with Lf size.

3.2.2 Second batch of SDS-PAGE run

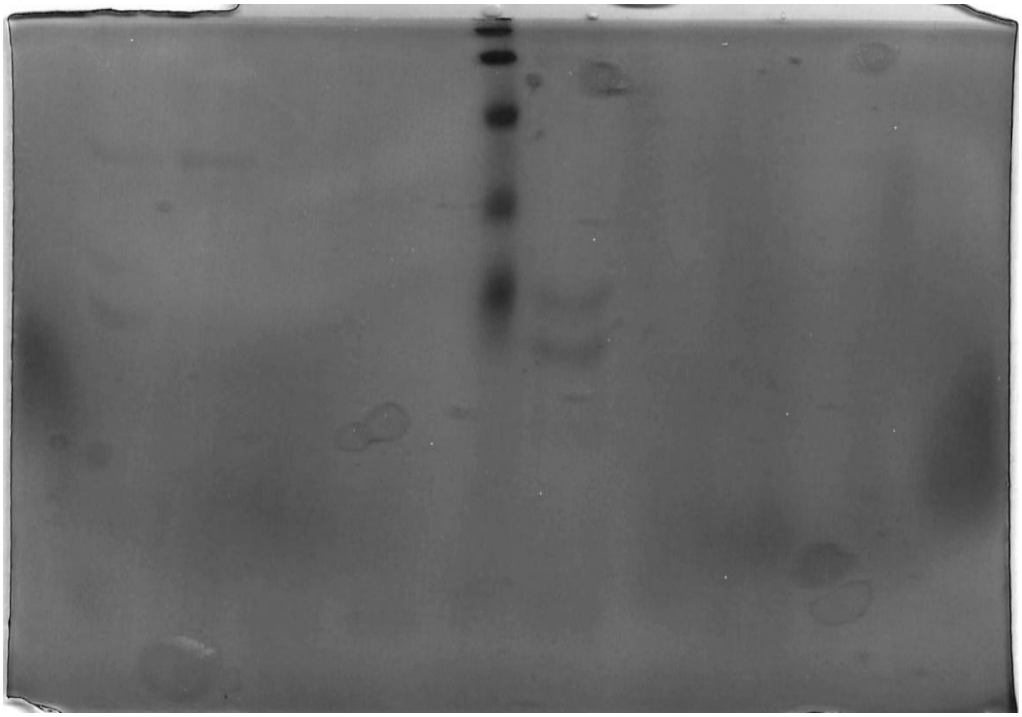


Figure 13: SDS-PAGE result of second batch of milk samples

There were total 6 samples in the gel. In the second batch of gel run, no significant band was observed as shown in the figure 13.Thats why this batch was run for another time.

3.2.3 Third time SDS-PAGE run

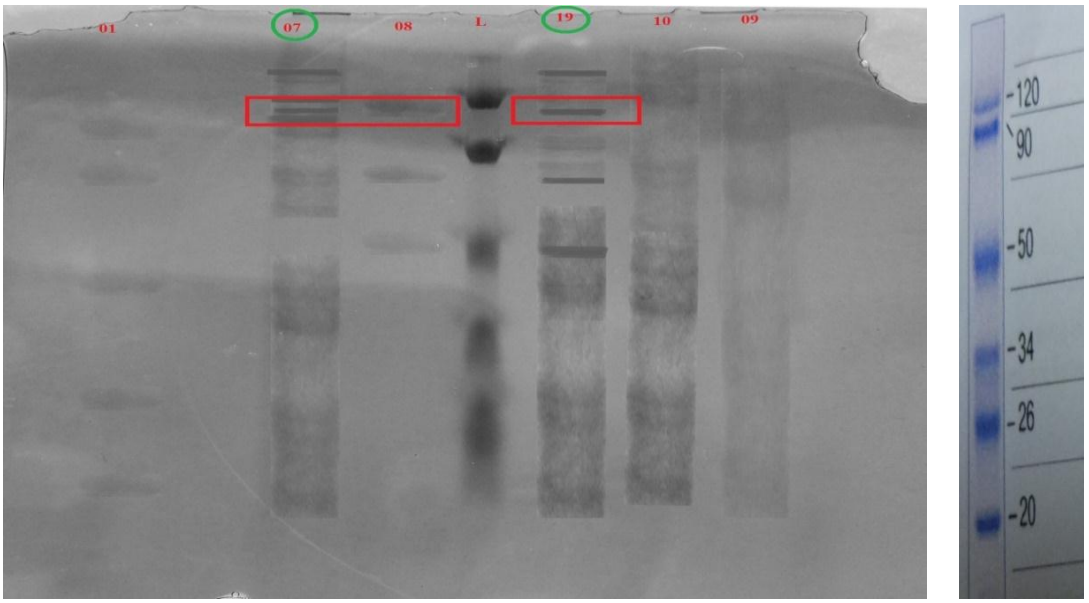


Figure 14: SDS-PAGE result of second batch of samples for second time (the left one) and the size of ladder used (the right one). The number of samples are denoted above of the bands of respective samples

There were total 6 samples in the gel. As we already know that the size of Lf is about (77-80) kDa, the bands found were compared with the ladder to see whether Lf is present there or not. The bands those matched with the ladder size of Lf are marked with red coloured box in the picture and their sample numbers are marked with green coloured round box. The sample's numbers are 7, 19 which stand for respectively **goat milk and Olympic milk sample**. The other samples did not show the bands that match with Lf size.

3.3 Antimicrobial activity test

All the eleven milk samples were tested against 18 pathogens to observe the antimicrobial activity of them. Well diffusion method was followed for this purpose.

Table 6: The result of antimicrobial test of milk samples against 18 pathogens collected from laboratory stocks.

Sample / Pathogen	01	07	08	09	10	12	15	17	19	20	21
Staphylococcus aureus	X	✓	X	X	X	X	X	X	X	X	X
Streptococcus pneumonia	X	X	X	X	X	X	X	X	X	X	X
Klebsiella pneuminiae	X	X	X	X	X	X	X	X	X	X	✓
Proteus vulgaris	X	X	X	X	X	X	X	X	X	X	X
Salmonella typhi	X	X	X	X	X	X	X	X	X	X	X
Shigella dysenteriae	X	X	X	X	X	X	X	X	X	X	X
Shigella flexneri	X	X	X	X	X	X	X	X	X	X	X
Vibrio cholera	X	✓	X	X	✓	X	✓	X	X	X	X
Bacillus subtilis	X	X	X	X	X	X	X	X	X	X	X
Bacillus cereus	✓	✓	X	X	✓	X	✓	X	✓	X	X
STEC	X	X	X	X	X	X	X	X	X	X	X
Enterococcus faecalis	X	X	X	X	X	X	X	X	X	X	X
EAEC	X	X	X	X	X	X	X	X	X	X	X
ETEC	✓	X	X	✓	✓	X	X	X	✓	X	X
EPEC (typical)	X	X	X	X	X	X	X	X	X	X	✓
EPEC (atypical)	X	X	X	X	X	X	✓	X	X	X	X

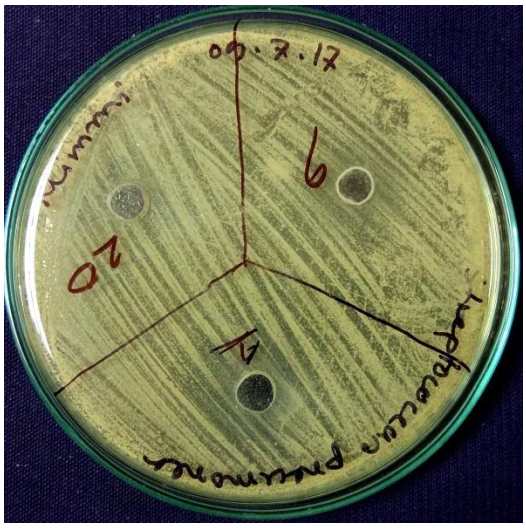
Pseudomonas aeruginosa	✓	✓		✓	X	X	✓	X	✓	X	X
Streptococcus pyogenes	✓	X	X	X	X	X	X	✓	X	X	X

Key:

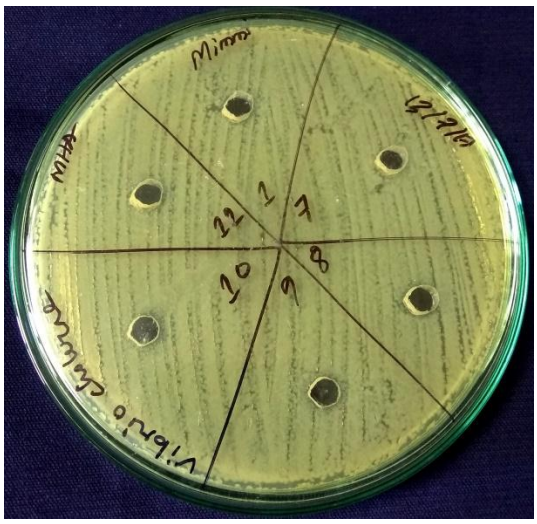
X means milk sample did not kill pathogens and

✓ Means milk sample killed pathogens & gave ZOI.

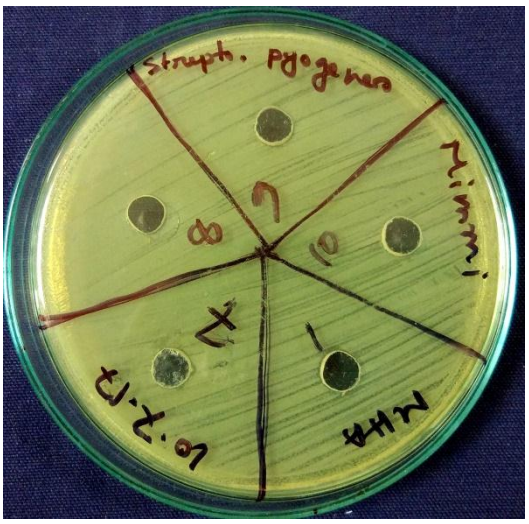
In the table of results of antimicrobial test against pathogens, very few samples showed positive results whereas most of the samples showed negative results. That means most of the milk samples were not able to kill pathogens under specified conditions. On the other hand some of the samples showed very promising results against some pathogens. The samples which showed positive results are coloured green.

(a)	**(b)**
	

(c)



(d)



Key:

a= *Streptococcus pneumoniae* **b = *Staphylococcus aureus***

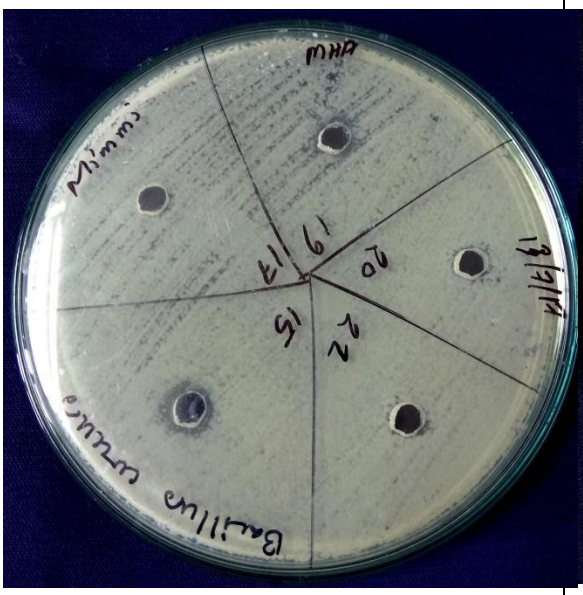
c= *Vibrio cholerae* **d=*Streptococcus pyogenes***

Figure 15: Some pictures of negative result of ZOI by the samples against some pathogen. The names of the pathogens are mentioned there. 4-6 samples were diffused in one plate of pathogen culture. Total numbers of 18 pathogens were used to observe antimicrobial test but only pictures 4 pathogens are given here. The negative results for ZOI for samples against other pathogens are same. That's why those pictures are not mentioned.

There are some pathogens against which 4/5 of 11 samples showed positive result which means these pathogens were killed by 4/5 milk samples whereas maximum pathogens could not be killed .The name of such positively killed pathogens are *Vibrio cholerae***,** *Bacillus cereus, Pseudomonas aeruginosa and* ETEC. The samples that showed most promising result in giving ZOI are 7 (against 4 pathogen), 1 (against 4 pathogens), 19 (against 3 pathogen), 10 (against 3 pathogens) and 15 (against 4 pathogens). Among these samples only number 7 is raw milk sample which stands for goat milk and the others are commercial milk. Some other samples showed ZOI for one or two pathogens such as 9 (against 2 pathogens), 17 (against one pathogen) and 21 (against 2 pathogens).

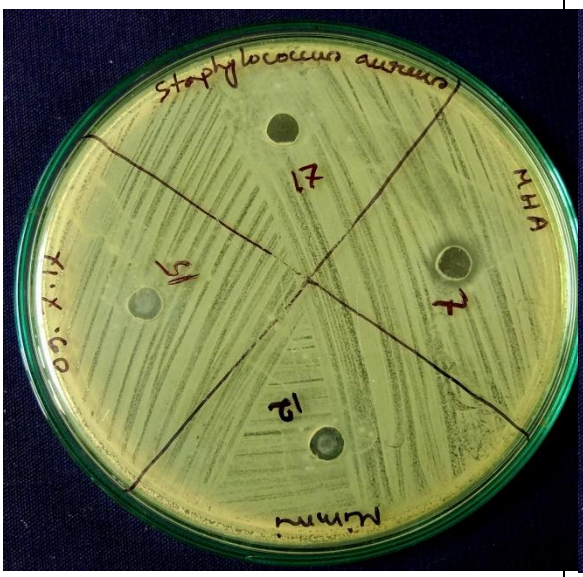
- **1mm of ZOI was considered as positive result** (Kazemipoor *et al.*, 2012).

(a)

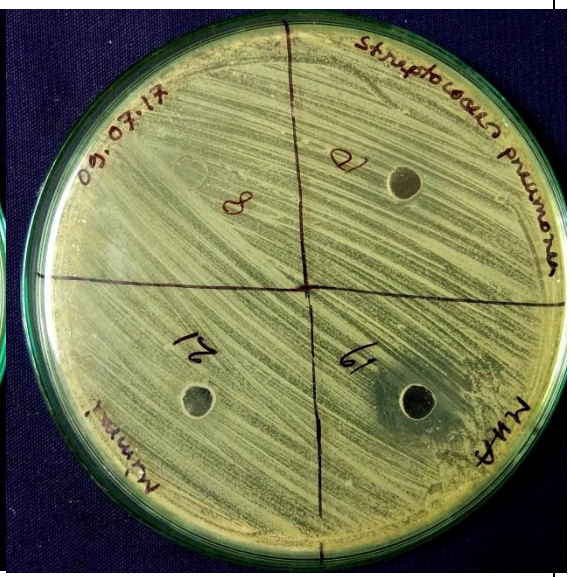


(b)

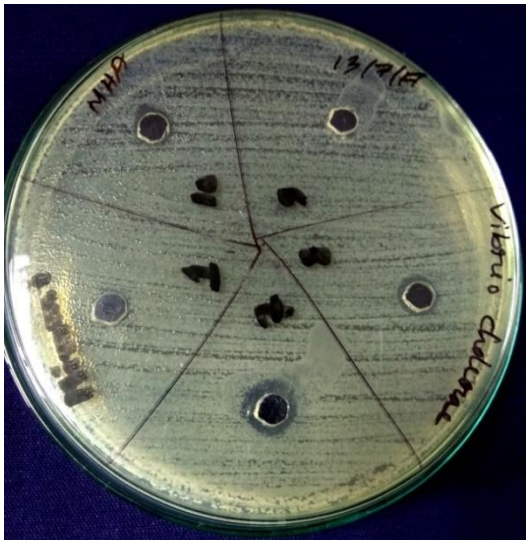
(c)



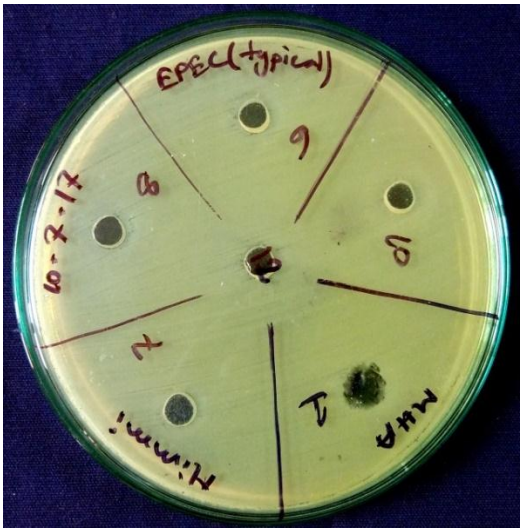
(d)



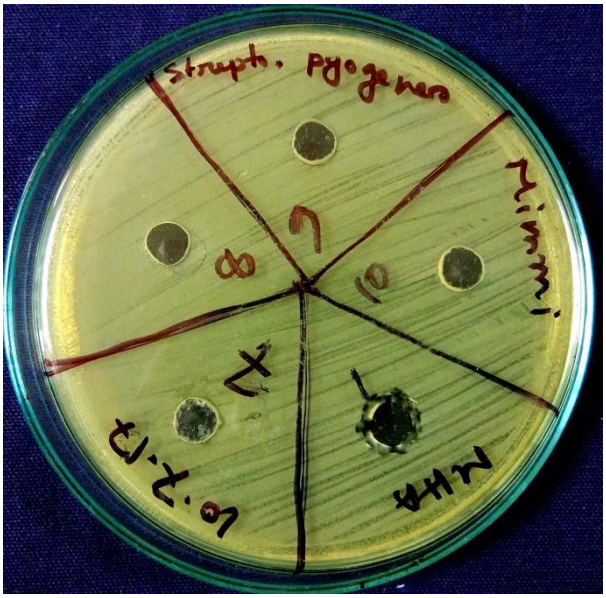
(e)



(f)



(g)



Key:

a=*Bacillus cereus* **b=*Klebsiella pneuminiae***

c=*Staphylococcus aureus* **d=*Streptococcus pneumonia***

e=*Vibrio cholerae* **f= EPEC (typical)**

g= *Streptococcus pyogenes*

Figure 16: Some pictures of some positive results of ZOI by some of the samples against pathogens. The names of the pathogens are mentioned there. 4-6 samples were diffused in one plate of pathogen culture. Total numbers of 18 pathogens were used to observe antimicrobial test but only pictures 5 pathogens are given here. The given ZOI for samples against other pathogens are almost same. That's why those pictures are not mentioned.

Chapter Four

Discussion

4. Discussion

Many new and promising treatments for reducing or diminishing the adverse effects of microorganisms are being arisen day by day. Lf is also thought to be effective in this purpose for its structure and components. The research study on Lf was started from this idea of finding antimicrobial activity nature.

In this research study eleven milk samples were collected from different farms and stores of Dhaka, Chittagong and Cox's Bazar. Then Lf was isolated from samples by following the procedure made by studying several research papers. After confirming whether Lf is present in the samples or not, they were tested for antimicrobial activity.

In the first place, Lf was extracted from eleven milk samples and stored at 4°C. Then for identification of Lf protein quantification by nanodrop technology was done. By this experiment the protein concentration present in the sample can be detected but the name of the protein cannot be identified. From the result (table 4 & 5) of this test we can see that the highest amount of protein was present in goat milk sample which is 12.067mg/ml and it's a raw milk sample whereas 7.296mg/ml was the highest amount among the commercial milk samples which is of Olympic milk. On the other hand, 2.725mg/ml and 1.426mg/ml were the lowest amount of protein of respectively buffalo milk and redcow milk samples. So, we can summarise the experiment result as raw milk has better amount of protein than the commercial ones. If we look at the table 4 & 5 and compare the concentrations of protein we can see that other raw milk samples have better quantities of protein than the commercial ones. Only a few numbers of commercial milk samples like Olympic and dutchlady have good amount of protein like raw milk samples. As a moderate quantity of protein is present in almost all the eleven samples, it can be assumed that Lf extraction method was might be correct and there is a high possibility of the presence of Lf in the samples.

In the second place, SDS-PAGE was performed to identify Lf in the milk samples. In the introduction part it was mentioned that Lf is an iron binding glycoprotein of the transferring family which is of 80kDa in size (Sharbafi and Rafiei *et al.*, 2014). The size of Lf was considered from 77kDa to 80kDa (Adam *et al.*, 2008). In the figure 12, 3 bands of (77-80) kDa size were found. The bands were of cow milk, breast milk and buffalo milk. In the figure 13, no bands were seen there. For rechecking, this batch was run for one more time. In the figure 14 which is of second time run of second

batch, 2 bands were observed which were of goat milk and Olympic commercial milk. Lf was undoubtedly found in cow milk, goat milk, buffalo milk, breast milk and Olympic commercial milk sample. It is observed that the samples that contain Lf are raw milk sample except Olympic which is commercial milk sample. Theoretically there was 100% chance of getting Lf in raw milk if the extraction method is proper and we found the same result. As the other 6 samples did not show any band that matches with Lf, so it is obvious that those do not contain Lf. But Olympic milk sample contains Lf. The industry ensures the presence of Lf may be by following the method that does not harm Lf or they add Lf from outside as additives. There is also a possibility that the other samples those do not contain Lf were stored for almost 3 months. As a result, the isolated Lf might be destroyed due to long time storage or might be there was any error while performing the extraction method for these samples.

After that, the concentration of Lf was differentiated between raw and commercial milk samples. Now it is known that all the 4 raw milk samples contain Lf whereas only 1 sample from commercial milk contains Lf. So, it can be said that raw milk contains Lf for sure but the commercial may have Lf and sometimes may not. The exact concentration of Lf is unknown because of the limitation of lab apparatus but the concentration of Lf can be assumed. From the table 4 & 5 the protein concentration for these raw samples were compared with the commercial one. The concentration of cow milk, breast milk, goat milk, buffalo milk and Olympic are respectively 5.43 mg/ml, 4.224 mg/ml, 12.067 mg/ml, 2.725 mg/ml and 7.296 mg/ml. It is observed that the raw milks contain a moderate amount of protein so they might contain a good amount of Lf also. On the other hand, Olympic also contains very good amount of protein in comparison with raw milk but almost all the commercial milks did not have Lf. So it can be said that it also may contain good quantity of Lf which is may be added as commercial milk gave fully negative result for Lf except this one.

By observing the biochemical tests, it can be concluded that some samples contain Lf and some don not. So the method of Lf isolation can be considered as the right one and can be used for further Lf related studies. Finally, it can be used as optimal protocol for Lf isolation.

Then, all the Lf samples were tested for antimicrobial activity. 18 pathogens were used against which the antimicrobial tests were done. 1mm of ZOI was considered as positive result (Kazemipoor *et al.*, 2012). From table 6 it can be observed that most of the pathogens could not be killed by the Lf samples and some samples that gave ZOI effectively. Theoretically all the raw milk should give ZOI against pathogens but surprisingly, breast milk Lf did not give any ZOI against any of the 18 pathogens. The reasons behind this could be breast milk could not be collected in large amount and it was also stored for a long period of time. So might be it has loses its antimicrobial activity. Besides this, cow milk gave ZOI only against *Streptococcus pyogenes* whereas buffalo milk Lf gave ZOI against *Klebsiella pneuminiae* and EPEC (typical). Goat milk Lf showed very promising result by giving ZOI against 4 pathogens which are *Staphylococcus aureus, Vibrio cholerae, Bacillus cereus, Pseudomonas aeruginosa.* On the other hand, only Olympic among commercial milk samples contain Lf but some more commercial milk samples also gave ZOI. Olympic gave ZOI against *Pseudomonas aeruginosa, Bacillus cereus* and ETEC. Surprisingly some other commercial milk samples such as fresh, dutchlady, diploma and king. Fresh and diploma milk samples gave ZOI against 4 different pathogens though they do not contain Lf. So, may be they do not contain Lf but they contain some other protein that are also effective against pathogens. There are some pathogens which were not affected by any of these raw and commercial samples such as *Streptococcus pneumonia, Proteus vulgaris, Salmonella typhi, Shigella dysenteriae, Shigella flexneri.* So, the Lf and the other proteins were not effective for these pathogens.

If more samples could be collected then the result would have been more effective and helpful. But it was very difficult to collect the raw milks from different farms. As a result the sample number was not upto the expectations.

Purifications of Lf could not be done due to the unavailability of chromatography column. If the purification could be done, then the exact concentration of Lf could have been known as well as all the results would have been more accurate for Lf. The difference between the concentration of Lf for raw and commercial milk could be determined properly with exact concentration.

While measuring absorbance by nanodrop it is good to use the buffer that is used to store the sample as blank. But the INVENT technologies Ltd. Used TE buffer instead of using PBS buffer as they did not have PBS buffer in stock.

There was mentioned to use centrifugation at 4°C in the protocol of Lf extraction but large centrifugation machine with temperature adjustment system was not available. As a result alternative way like keeping the sample at 4°C and then putting it into centrifugation machine was followed.

The antimicrobial activity of Lf is very promising and can be applied in various purposes to reduce or diminish microbial growth. So the research regarding Lf should be continued. There are some drawbacks in this research which have been mentioned before. The first aim is to reduce the drawbacks.

More samples of milk would have enriched this research work. There are cows, buffaloes, goats of different species. So milk from different species of same animal can be used because the Lf can differ in their structures and confirmations according to their genetic makeup. On the other hand, breast milk was collected from only one mother but this sample can be collected from more mothers. Lf can also differ according to their genetic makeup of different mother. As the possibility of getting Lf in raw milk is 100%, so these samples should be collected more.

For best purification of Lf, chromatography must be used. Otherwise it is very difficult to know whether the activity is because of Lf or any other protein. If purification is not ensured then it would be very difficult to come to a proper result.

Large amount of sample should be used during isolating Lf for getting higher concentration.

Animal model can be used to observe the antimicrobial activity of Lf which can be further used to determine whether Lf will work in human body or not.

Then Lf can be developed as an edible product which can be used as the alternative of antibiotic. Nowadays many antibiotic resistant strains have developed. There is a higher possibility that these strains can be diminished by using Lf. On the other hand, by taking antibiotics frequently microbial strains are becoming resistant. As a result

the antibiotics are not working against pathogen properly. If the use of Lf as an alternative can be started then developing of resistant strains also can be reduced.

Lf can also be added to the dairy foods to preserve them to reduce microbial deterioration. So, further research can be conducted how to add Lf to different products that can be damaged by microorganism. Lf is a protein itself. As a result, the use of chemicals can be reduced in food industry by using Lf as an alternative. Besides this, Lf can be added to foods as nutritional substitution.

In this research, only antibacterial test was done for Lf. The other aspects of antimicrobial property can also be tested such as antiviral, antifungal etc.

Some researches about Lf also showed anticancer activity of Lf. So, this property can be researched more to identify new treatment for cancer.

Recommendations for future work:

1. The use of chromatography can lead to better purification of Lf.
2. More samples for Lf isolation will increase the research aspects. The main focus should be to the raw milk samples.
3. Animal modelling will be very helpful in understanding the antimicrobial activity of Lf.
4. Addition of Lf to food industry will create a new era.
5. Centrifuge machine with temperature adjustment should be used to maintain 4°C temperature.

Conclusion

Lf has antibacterial movement towards a range of diverse bacterial pathogens. It is a very promising field to study with. There are some errors in this research study as it was the very first work with Lf in BRAC university laboratory. Further research with Lf can open a new era for treatment of many diseases and food industry most importantly. This study showed that Lf can be effective against bacteria sothat it can be used to diminish those bacteria. It also showed that Lf is very much available in raw milk in comparison with the commercial milk. So, in order to enrich this research work, further study is very important. Both nutritional and functional use as natural antioxidants are also foreseen due to Lf's ability to tightly scavenge iron thereby

removing an important catalyst for radical formation (Steijns *et al.*, 2000). So after finishing proper research Lf can be used in food and feed industry as well as in medicine industry.

REFERENCES

Actor, J. K., Hwang, S. A., and Krunzel, M. L. (2009). Lactoferrin as a Natural Immune Modulator. *Curr Pharm Das*, 15(17), 1956-1973.

Adam, V., Zitka, O., Dolezal, P., Zeman, L., Horna, A., Hubalek, J., Siley, J.,Krizkova, S., Trnkova, L., and Kizek, R. (2008). Lactoferrin Isolation Using Monolithic Column Coupled with Spectrometric or Micro-Amperometric Detector. *Sensors*, 8, 464-487.

Appelmelk, B.J., An, Y.-Q., Geerts, M., Thijs, B.G., de Boer, H.A., MacLaren, D.M., de Graaff, J., and Nuijens, J.H., (1994). Lactoferrin is a lipid A-binding protein. Infect. *Immun.*, 62, 2628–2632.

Arnold, R.R., Brewer, M., Gauthier, J.J., (1980). Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. *Infect. Immun.*, 28, 893–898.

Baker, E.N., Baker, H.M., and Kidd, R.D. (2002). Lactoferrin and transferrin: Functional variations on a common structural framework. *Biochem. Cell Biol.*, 80, 27-34.

Baker, E.N., Rumball, S.V., and Anderson, B.F. (1987). Transferrins: Insights into structure and function from studies on lactoferrin. *Trends Biochem*. *Sci.*, 12, 350-353.

Berkel, P.H.C.V., Geerts, M.E.J., Veen, H.A.V., Mericskay, M., Boer, H.A., and Nuijens, J.H. (1997). The N-terminal stretch Arg2 Arg3, Arg4, and Arg5 of human lactoferrin is essential for binding to heparin, bacterial lipopolysaccharide, human lysozyme and DNA. *Biochem. J.*, 328, 145-151.

Berlutti, F., Pantanella, F., Natalizi, T., Frioni, A., Paesano, R., Polimeni, A., and Valenti, P. (2011). Antiviral Properties of Lactoferrin-A Natural Immunity Molecule. *Molecules,* 16, 6992-7018.

Devesh, R., Parkar, Rahul, N. J., and Mukesh, R. P. (2016). Extraction and Characterization of Lactoferrin from Commercial Milk. *International Journal of Pharmacy & Pharmaceutical research*, 6(2), 312-320.

Farnaud, S. J. C., and Evans, R. W. (2003). Lactoferrin- A Multifunctional Protein with Antimicrobial Properties. *Molecular Immunology*, 40, 395-405.

Grossmann, J.G., Neu, M., Pantos, E., Schwab, F.J., Evans, R.W.,Townes-Andrews, E., Appel, H., Thies, W.G., Hasnain, S.S., (1992). X-ray solution scattering reveals conformational changes upon iron uptake in lactoferrin, serum, and ovo-transferrins. *J. Mol. Biol.*, 225, 811-819.

Hancock, R.E.W., and Diamond, G., 2000. Cationic antimicrobial peptides: a major element in mammalian innate immune defences against infection. *Trends Microbiol*, 8, 402–410.

Hara, K., Ikeda, M., Saito, S., Matsumoto, S., Numata, K., Kato, N., Tanaka, K., and Sekihara, H. (2002). Lactoferrin inhibits hepatitis B virus infection in cultured human hepatocytes. *Res. Hepatol., 24*, 228-235.

Jenssen, H., and Hancock, R. E. W. (2008). Antimicrobial Properties of Lactoferrin. *Biochimie*, 91, 19-29.

Julie, A. (n.d.). Bacillus cereus Retrieved from http://food.unl.edu/bacillus-cereus**.**

Karav, S., German, J.B., Rouquie, C., Parc, A. L., and Barile, D. (2017). Studying Lactoferrin N-Glycosylation. *International Journal of Molecular Sciences*, 11, 110-124.

Legrand, D., Pierce, A., Elass, E., Carpentier, M., Mariller,C. and Muzurier, J. (2008). Lactoferrin structure and functions. *Adv. Exp. Med. Biol.*, 606, 163-194.

Mazurier, J., and Spik, G. (1980). Comparative study of the iron-binding properties of human transferrins. I. Complete and sequential iron saturation and desaturation of the lactotransferrin. *Biochim.Biophys. Acta*, 629, 399-408.

Moguilevsky, N., Retegui, L.A., Masson, P.L., (1985). Comparison of human lactoferrins from milk and neutrophilic leucocytes. Relative molecular mass, isoelectric point, iron-binding properties and uptake by the liver. *Biochem. J.*, 229, 353–359.

Moradian, F., Sharbafi, R., and Rafiei, A. (2014). Lactoferrin, Isolation, Purification and Antimicrobial Effects. *Journal of Medical and Bioengineering,* 3(3), 407-410.

Naidu, A.S., & Arnold, R.R. (1997). Influence of lactoferrin on host microbe interactions. Kang, J.H., Lee, M.K., Kim, K.L., & Hahm K.S. (1996). Structure biological activity relationships of 11-residue highly basic peptide segments of bovine lactoferrin. *International Journal of Peptide and Protein Research,* 48, 357-363.

Ninfa, A. J., Ballou, D. P., and Benore, M. (2009). Fundamental Laboratory Apparatus for Biochemistry and Biotechnology. *Fitzgerald Science Press,* 72, 113.

Redwan, E.M., and Tabll, A. (2007). Camel lactoferrin markedly inhibits hepatitis C virus genotype 4 infection of human peripheral blood leukocytes. *J. Immunoass. Immunoch.*, 28, 267-277.

Rossi, P., Giansanti, F., Boffi, A., Ajello, M., Valenti, P., Chiancone, E., and Antonini, G. (2002). Ca2+ binding to bovine lactoferrin enhances protein stability and infl uences the release of bacterial lipopolysaccharide. *Biochem. Cell Biol.*, 80, 41-48.

Siciliano, R., Rega, B., Marchetti, M., Seganti, L., Antonini, G., and Valenti, P. (1999). Bovine lactoferrin peptidic fragments involved in inhibition of herpes simplex virus type-1 infection. *Biochem. Biophys. Res. Commun.*, 264, 19-23.

Singh, P.K.V (2002). A component of innate immunity prevents bacterial biofilm development. *Nature*, 417, 552-555.

Steijins, J. M., and Hooijdonk, A.C.M.V. (2000). Occurrence, Structure, Biochemical Properties and Technological Characteristics of Lactoferrin. *British Journal of Nutrition*, 84(1), 511-517.

Thorpe, C. M. (2004). Shiga Toxin—Producing *Escherichia coli* Infection. *Clinical Infectious Diseases*, 38, 1298–1303.

Valenti, P., Antonini, G. (2005). Lactoferrin: An important host defence againstmicrobial and viral attack. *Cell. Mol. Life Sci.*, *62*, 2576-2587.

Wakabayashi, H., Kurokawa, M., Shin, K., Teraguchi, S., Tamura, Y., and Shiraki, K. (2004). Oral lactoferrin prevents body weight loss and increases cytokine responses during herpes simplex virus type 1 infection of mice. *Biosci. Biotechnol. Biochem.*, 68, 537-544.

Wong, C. (n. d.). The Benefits of Lactoferrin. Retrieved from https://www.verywell.com/the-benefits-of-lactoferrin-89471 .

Yamauchi, K., Tomita, M., Giehl, T.J., Ellison III, R.T., (1993). Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect. Immun.,* 61, 719–728.

APPENDIX-1

Reagents

1. 1X PBS Buffer:

Components	Amount (g/L)
NaCl	8
KCl	.2
Na_2HPO_4	1.44
KH_2PO_4	.24
Final pH	7.4 and store at room temperature

2. 1N NaOH:

To 40ml of dH_2O, 4g of NaOH was mixed and then 60ml of dH_2O was added to make the final volume of 100ml and stored at room temperature.

3. 1N HCl:

8.212ml of HCl from stock solution was mixed to 30ml of dH_2O and then 70ml of dH_2O was added to make final volume of 100ml qnd stored at room temperature.

4. 45% $(NH_4)_2SO_4$:

45g of **(**$NH_4)_2SO_4$ was added and mixed to 100ml of dH_2O and stored at room temperature.

5. 80% $(NH_4)_2SO_4$:

To 100ml of dH_2O, 80g of **(**$NH_4)_2SO_4$ was added and mixed by putting heat and then stored at room temperature.

6. 1X TGS Running Buffer:

Components	**Amount**
Trisma Base	7.5g
Glycine	36g
10% SDS	25ml

dH_2O	Upto 2.5L
Final pH	8.3 And store at 4°C

7. 30% Acrylamide:

Components	Amount
Acrylamide	29g
Bis-Acrylamide	1.05g
dH_2O	100ml

- Acrylamide is neurotoxic.So it is needed to use gloves and mask during working with Acrylamide.
- It is needed to wrap the bottle up with aluminium foil.

8. 1.88M Tris:

Components	Amount
Trisma Base	11.388g
SDS	.2g
dH_2O	50ml
Final pH	8.8 and stored at 4°C

9. 0.62M Tris:

Components	Amount
Trisma Base	3.755g
SDS	.2g
dH_2O	50ml
Final pH	6.8 and stored at 4°C

10. 10% SDS Solution:

10g of SDS was gently mixed to 100ml of dH_2O and stored at room temperature. Shaking was avoided as it will form bubbles due to shaking.

11.10% APS:

.5g of APS was mixed to 5ml of dH_2O and was distributed of 1ml among 5 micro centrifuge tubes and stored at -20°C.

12. Staining:

Components	Amount
Acetic Acid	10ml
Methanol	40ml
dH_2O	70ml
Comassie Blue-R250	0.13g

- Stored at room temperature.

13. Destaining:

Components	Amount
Acetic Acid	20ml
Methanol	20ml
dH_2O	160ml

- Stored at room temperature.

APPENDIX-2

Media Composition

The compositions of all the media used in this study are given below:

Nutrient Agar

Component	Amount (g/L)
Nutrient Agar	28

- The media was autoclaved after mixing by heat.

MHA

Component	Amount (g/L)
MHA	38

- The media was autoclaved after mixing by heat.

APPENDIX – 3

Instruments

The important equipments used through the study are listed below:

Autoclave	SAARC
Freeze (-20ºC)	Siemens
Incubator	SAARC
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Oven, Model :MH6548SR	LG, China
Refrigerator (4ºC) Model: 0636	Samsung
Safety Cabinet Class II Microbiological	SAARC
Vortex Mixture	VWR International
Water Bath	Korea
Weighing Balance	ADAM EQUIPMENTTM, United Kingdom
Conical Flask	Amber
Petri Plate	Amber
Nanodrop 2000 Spectrophotometer	Thermo Scientific, USA