Antimicrobial activities of lactoferrin extracted from commercially available milk samples

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT 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 "**Antimicrobial activities of lactoferrin extracted from commercially available milk samples**" submitted by Fahim Bin Najib, has been carried out under the supervision and able guidance of Ms. Zubaida Marufee Islam, Lecturer, Biotechnology Program, Mathematics and Natural Sciences Department, BRAC University and Ms. Kashmery Khan, Lecturer, Biotechnology Program, Mathematics and Natural Sciences Department, BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

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Dedication

To
My Parents

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I am much indebted and would like to express my sincere gratitude and esteem to my respected supervisor Ms. Zubaida Marufee Islam, Lecturer, Department of Mathematics and Natural Sciences, BRAC University, Ms. Kashmery Khan, Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, for her constant supervision, expert guidance, enthusiastic encouragement to follow new ideas and constant support throughout the entire period of my research work. Without their supervision and help this dissertation would not have been possible. They offered invaluable assistance and direction to complete my work on the time.

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Fahim Bin Najib

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Abstract

Lactoferrin, an iron-binding glycoprotein with multifunctional properties is crucial to strengthening the immune system and also useful for commercial applications. The protein's iron-binding capacity makes it undoubtedly advantageous to immune system modulation and different bacterial strains. In the present study, ten locally available commercial milk samples were collected from Dhaka and Chittagong city and the protein lactoferrin was extracted. The milk samples were defatted and casein was separated. The concentration of the isolated protein was measured using the NanoDrop machine. The presence of the specific protein lactoferrin was identified by using SDS-PAGE, where the specific protein was separated through electrical influence. The antibacterial activity of the protein was tested against 18 bacterial strains. The results of the current study indicated that the protein was successful in inhibiting the growth of certain bacteria. The results showed that lactoferrin protein is effective enough to be produced commercially in large scale and can be used as a substitute of medicines to prevent bacteria causing diseases.

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Chapter 1

Introduction

Introduction

1.1 Background

Milk is a nutritious white liquid that is produced in the mammary gland of the mammals. It contains different nutritional compounds such as fats, carbohydrates, proteins, vitamins, growth factors, etc. Among the nutritional compounds the importance of protein is immense. Milk contains different kinds of protein with variant activities without which the human body will not function as a whole. After an infant is born the primary source of protein is colostrum. Colostrum, the first milk contains different kinds of protein for the growth of the infant and also to strengthen the immune system. The major types of protein are the casein proteins and all the other proteins are together called whey proteins. Among the whey proteins some are even effective in the remediation of cancer. Recently researchers are focusing on the use of naturally available proteins through a cost effective process. With the increase in population the demand for a credible source of protein is also in an upsurge.

Lactoferrin is a type of whey protein that meets all the criteria of an ideal immune-boosting protein. Mammalian secretions such as milk, tears, saliva, seminal fluids, vaginal fluids, nasal mucosa, bronchial mucosa as well as some white blood cells and secondary granules of neutrophils have lactoferrin (Rodrigues *et al.*, 2009: Iigo *et al.*, 2009). Lactoferrin is a non-heme iron binding glycoprotein having a molecular weight of 78-80 kDa containing around 690-702 amino acid residues and it belongs to the transferrin family (Legrand *et al.*, 2008). The iron binding property of lactoferrin plays a significant role in retiring microorganism as the iron is removed by the lactoferrin proteins. It even inhibits the microorganism's infectivity and is also used as an antioxidant. Both Gram negative and positive bacteria are also affected by this protein (Moradian *et al.*, 2014). Lactoferrin is currently being used to treat Hepatitis C patients.

In spite of being a crucial element of human body development, over doses of lactoferrin has some consequences also like diarrhea, skin rashes, etc. As the positive impact of this protein is much greater than the negativity and the consequences can be brought under control, the overall process of making a proper use of the protein is being taken under

consideration. The use of naturally available proteins reduces the usage of commercial variants. Commercially available proteins are expensive and can't be utilized in day to day use. The process involves methods that isolate the fat layer making it skimmed. However, the concentration of lactoferrin varies from sample to sample. The more the concentration, the chance of obtaining an ideal source for large scale production increases.

More than 40 years have passed since Grooves (1960) and Johansson (1960) reported the isolation of a multi-functional red protein currently called lactoferrin which is an iron-binding protein belonging to the transferrin family. The X-ray structure of the human lactoferrin protein (Anderson *et al.,* 1987) provided the first knowledge about the iron-binding properties of the transferrin family. Currently there are extensive high resolution data on wide range of lactoferrin in their metal free and metal loaded state. Many studies have been done and are still going on regarding the properties of the lactoferrin some of which are independent of the metal binding properties. It was identified earlier that lactoferrin is responsible to prohibit bacterial growth through iron sequestering method which is now cleared that lactoferrin can even have its impact without the iron sequestering method. The protein can have direct interaction with the target to eliminate its growth. Although there are no general agreement on the properties of the protein but studies are going on to understand the mode of action of this antimicrobial proteins.

1.2 Lactoferrin extraction from different milk sources

Parkar *et al.,* (2016) assessed the extraction and characterization of the lactoferrin protein from commercially available milk samples. Pasteurized milk samples and tetra pack milk samples from locally available vendors were taken. The overall analysis was conducted in triplicate. The extraction procedure was conducted in two stages. In order to isolate the protein, fat layer and casein was separated through centrifugation at different parameters and adjustment of pH using hydrochloric acid and sodium hydroxide. The extraction procedure included the addition of different concentrations of Ammonium Sulphate along with pH adjustment and centrifugation. SDS-PAGE was used in a manner to determine the molecular weight of the protein. On the contrary, the tetra pack samples showed more concentration of the lactoferrin than the pasteurized milk samples. Overall, the study

demonstrates that the quality of both pasteurized and tetra pack milk samples was satisfactory hence it can help in the eradication of many pathogenic bacteria by consuming the milk samples as a nutritional supplement or by consuming the purified lactoferrin powder in order to stimulate the immune system.

Moradian *et al.*, (2014) conducted their study by using colostrum of cows from faculty dairy farms. The isolation and purification procedures were regulated by removing casein through acidic conditions and extra proteins were precipitated by using ammonium sulphate. Lactoferrin was purified by cation exchange chromatography. The antimicrobial activity of lactoferrin was observed after the isolation and purification from cow's colostrum against *Pseudomonas aeroginosa*. Biochemical tests were used to confirm the microbial activity and the bacterial samples were isolated from scald patients. Lactoferrin with different concentration was treated on *Pseudomonas* colonies as well as *E.coli* (DH5α) for two days as positive control. The study came out successful as lactoferrin showed effectiveness on *Pseudomonas* growth as well as *E.coli* and it has more strong effect than other previous studies.

Rachman *et al.*, (2015) investigated the concentration and isolation of lactoferrin from colostrum and milk of various goat samples available in Indonesia. Peranakan Etawah (PE) goats, Jawarandu and Saanen goats crossed with PE goats (SAPE) were obtained from local farms. The aim of their study was to study the influence of the chemical composition of the colostrum and milk of the three breeds and to identify the presence of lactoferrin in order to determine the concentration of lactoferrin content in the samples. The method followed in this experiment was Completely Randomize Design (RAL) factorial pattern (3X8) with three repetitive treatments. The results of the experiment indicated that the chemical composition of colostrum and milk includes different levels of dry matter, non-fat dry matter, fat protein, specific gravity and pH. The identification of lactoferrin was conducted using the spectrophotometric method. The study came out with positive result as the bands were visible in the electrophoresis indicating the molecular weight of colostrum and goat milk lactoferrin. The composition of colostrum has a higher value than the levels of milk and the differences in the breeds affected the concentration of lactoferrin.

Masson *et al.*, (1971) conducted their experiment by using a variety of mammals (guinea-pig, cow, goat, mare and mouse) to identify lactoferrin based on electrophoretic mobility. In this study, they attempted to identify and estimate the concentration of lactoferrin in milk isolated from various other species. The milk samples were collected from local supplies and at different times of lactation period. The milk samples were defatted and casein was separated followed by electrophoresis and radioautography. Radial immunodiffusion was used to carry out the quantitative determination of lactoferrin (Mancini *et al.*, 1965). Pure human lactoferrin and transferrin were used as standards. The results obtained after radioautography showed a pattern of radioactive bands each differed from one sample to another. Transferrin was recognized by its electrophoretic mobility but lactoferrin could not be recognized as they considered the slower bands than transferrin to be lactoferrin. In order to confirm these bands, the samples were dialysed. Furthermore, immunological analysis confirmed that the antisera became active against whey proteins. The concentration of lactoferrin was found progressively decreasing during the first part of the lactation period and increases again towards the end.

1.3 Roles of Lactoferrin

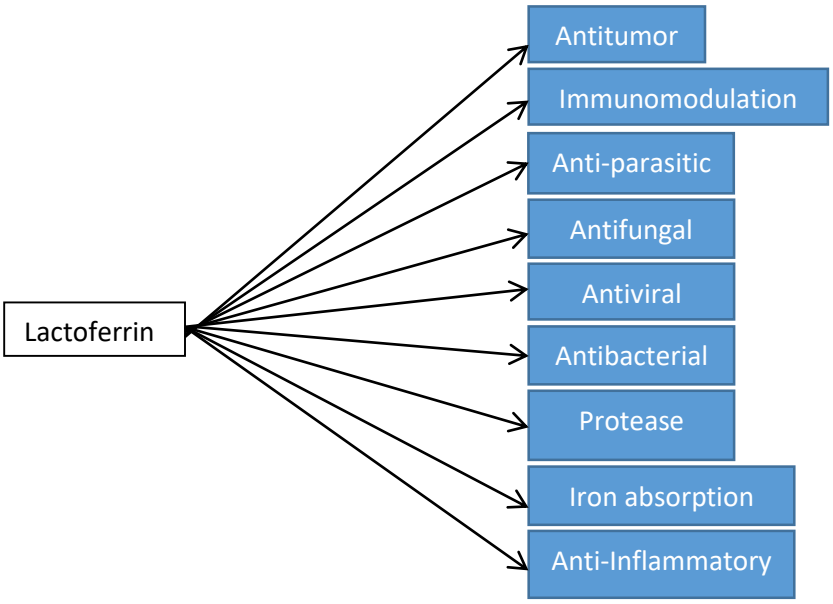


Fig: 1. Roles of Lactoferrin (Brock, J.H., 2002)

1.3.1 Antibacterial Activity

The antimicrobial activity of lactoferrin is widely accepted as there were experiments performed earlier regarding this specific protein and most of the results showed positive outcome. Bacterial strains such as *Streptococcus mutans* and *Vibrio cholerae* are very much affected by the bactericidal protein (Gifford *et al.*, 2005). But the antimicrobial effect does not imply on *Escherichia coli (*Arnold *et al.*, 1977). Researches have shown that the bactericidal effect of lactoferrin is only present in iron free state and the antimicrobial activity is reduced in iron saturated state (Arnold *et al.*, 1980; Kalmar and Arnold, 1988; Yamauchi *et al.*, 1993).

It has now being commercially produced against many infectious diseases. Most of the physicians nowadays prescribe the insertion of lactoferrin as a substitute of antibiotics and other drugs. The protein samples sequestered from natural resources revealed healthy impact on the host. The percentage of its effectiveness made the researchers enthusiastic about sequencing the protein and modifying it for greater good. Apart from the iron binding capacity, Lactoferrin also perceives many other properties against infectious agents which are still under research from the time of its discovery.

1.3.2 Antifungal Activity

The antifungal activity of lactoferrin was first reported by Kirkpatrick *et al.*, (1971). In some research by Kuipers *et al.*, (1999), Fluconazole an antifungal medication in combination with lactoferrin showed positive result in killing the drug-resistant *Candida* species. Some HIV infected patients have fluconazole-resistant species found in them which is believed to be delayed by combining Lactoferrin (Kuipers *et al.*, 1999).

1.3.3 Antiviral Activity

The antiviral activity of Lactoferrin has shown that this protein is capable of inhibiting the viral replication. Lactoferrin directly binds to the viral particle and thereby preventing the infection of the target cell. Ikeda and co-workers in 2000 found that Hepatitis C virus, Polio virus, Rotavirus, Herpes Simplex Virus (HSV) and possibly Human Immunodeficiency Virus (HIV) can be dismantled by using lactoferrin. There are many mechanisms which lactoferrin uses to conduct its antiviral activity. For instance, virus particles firstly attaches

to Heparin Sulphate Proteoglycans (HSPGs), which is like a dock on the host cell (Laquerre *et al.*, 1998). Lactoferrin binds to this dock and prevents the first contact between the virus and the HSPGs preventing the subsequent infection.

1.3.4 Anti-parasitic Activity

The Anti-parasitic activity of lactoferrin is yet under study but Omata *et al.* (2001) observed that if *Toxoplasma gondii* and *Eimeria stiedai* are pre-incubated with lactoferrin peptides isolated from bovine, had reduce the parasitic activity. Lactoferrin also disturbed the iron uptake pathway of *Pneumocystis carnii* thereby reducing its attributes (Cirioni *et al.*, 2000).

1.3.5 Activity against other microbes

Lactoferrin has also shown activity towards a wide range of eukaryotic microbes. For instance, it has shown anti-parasitic activity towards *Pneumocystis carinii* and *Entamoeba histolytica* through iron sequestration method (Weinberg *et al.*, 1994). But now some parasites have evolved in such a manner that they are benefitted by the sequestration method. Studies showed that *Tritrichomonas foetus* if grown under iron deficiency, lactoferrin tends to increase the growth of the parasite by working as iron source. The parasite also releases and takes up Lactoferrin in an energy-dependent mechanism (Tachezy *et al.*, 1996). In some cases, it appeared that Lactoferrin triggers anti-parasitic mechanism in epithelial cells (Dzitko *et al.*, 2007).

1.4 Necessity of pH adjustment for Lactoferrin precipitation

The term “pH” is used to measure the acidity and alkalinity of a solution. Nowadays pH measurement has become compulsory in a variety of fields such as agriculture, wastewater treatment, industrial processes, environmental monitoring, and in research and development programs. The measurement is based upon the relative quantity of Hydrogen ions contained in the solution. pH is defined as the negative logarithm of hydrogen ion. With the increase in hydrogen ion concentration pH goes down by making the solution acidic in nature. A standard pH measurement is based upon the following three constituents, such as, a pH electrode, temperature compensation element and a pH meter or controller. The pH electrode should be handled carefully as it is very sophisticated and easily broken. Milk consists of different bioactive components and each has a definitive role to play in the immune system of an infant as well as an adult.

In order to isolate lactoferrin from the other bioactive molecules of milk, pH adjustment is required to precipitate lactoferrin from fat layer, casein, etc.

1.5 Determination of protein concentration using NanoDrop

A NanoDrop is a type of spectrophotometer that determines the concentration of a DNA, RNA or protein indulged sample. The machine can read as much as 2µl on a pedestal. The NanoDrop process require less time and cleanup procedures which makes it an ideal concentration measurement instrument. It is more accurate and gives more specific result than traditional spectrophotometers. Nowadays most of the laboratories around the world are using NanoDrop for lab experiments for its persistence. The machine can actually determine what is on the pedestal by using the A260/280 value. Additionally, the concentration of your sample will be reported in ng/µl. If there are any error while reading the samples one can always reread the samples as it just a click away. The concentration of lactoferrin in milk samples differ from each other. The NanoDrop machine is required in this experiment to determine which sample has the highest lactoferrin concentration than other samples.

1.6 Protein separation using SDS PAGE

The electrophoresis is a method for separating macromolecules by using an electric field. The Sodium Dodecyl Sulphate (SDS) in addition with a discontinuous polyacrylamide gel is used for protein separation. This macromolecule separation method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is generally used to dissect the proteins in complex concentrates. The most ordinarily utilized strategies are obtained from the broken SDS-PAGE framework initially depicted by Laemmli (1970). The framework really comprises of two gels - a settling (otherwise known as running) gel in which proteins are settled on the basis of their molecular weights (MWs) and a stacking gel in which proteins are concentrated before entering the settling gel. Contrasts in the compositions of the stacking gel, settling gel and electrophoresis buffer create a framework that is able to separate proteins based on their MWs. Milk contains different whey proteins and the molecular weight of each protein differs from each other. SDS PAGE is used in this experiment to separate proteins based

on their molecular weights. The protein with higher molecular weight migrates through the sieving gel under the influence of an applied electric field and the proteins with less molecular weight stays above.

1.7 Rationale of the experiment

The usage of antibiotics and other drugs created adverse effects on the human body as most of the bacteria and viruses are becoming resistant. The need of an alternative source for preventing bactericidal activity is increasing. Some milk proteins fulfill these criteria. Researchers have found that milk contains certain proteins that help build the immune system of an infant as well as an adult. The significance of these proteins has created an urge for developing a standard protocol for the isolation of proteins.

1.8 Objectives

The objective of the study is to assess the quality of the commercially available milk samples and the microbial activity in details.

- Establishment of a standard protocol for the isolation of Lactoferrin protein from commercial samples.
- Determination of the concentration of the protein in different samples.
- Comparative analysis of the proteins.
- Determination of the molecular weight of the protein.
- Isolation of a purified form of the protein.
- Observation of antimicrobial activities against different bacterial strains.

Chapter 2

Materials and Methods

Materials and methods

2.1 Study place

The laboratory works of this research was done in the biotechnology research laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

2.2 Study period

This research work was carried out from January, 2017 to July, 2017.

2.3 Materials

2.3.1 Equipment

- Laminar airflow cabinet (Model-SLF-V, vertical, SAARC group Bangladesh)
- Incubator (Model-0SI-500D, Digisystem Laboratory Instruments Inc. Taiwan)
- Vortex machine (Digisystem Taiwan, VM-2000)
- Autoclave machine (Model: WIS 20R Daihan Scientific Co. ltd, Korea)
- Centrifuge Machine (Digisystem laboratory Instruments Inc.)
- NanoDrop Machine (Thermo Scientific)
- Magnetic Stirrer (JSR)
- Glass wares, pH meter petri-dishes, micro-pipettes, Bunsen burner, hot plate, electric balance, falcon tubes, McCartney bottle, PCR tubes, Cork borer, etc.

2.3.2 Media

Different types of media were used for selective growth, enrichment culture, and indication of specific properties. Media preparation and sterilization were done according to the protocol and standard recipe.

Fig: Nutrient Agar Plate

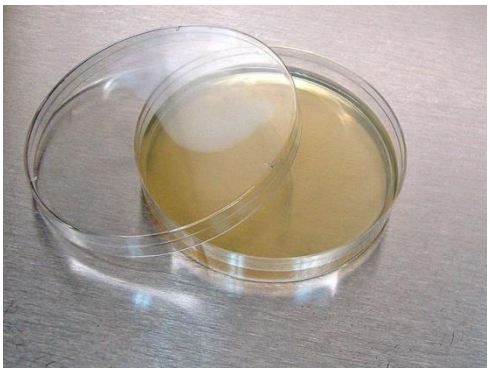


Fig: Mueller Hinton Agar plate

2.4 Flowchart of the study design

Sample Collection

↓

Fat separation

↓

Casein Separation

↓

Lactoferrin extraction

↓

Protein concentration determination

↓

Protein Separation

↓

Antibacterial Assessment

2.5 Methods

2.5.1 Sample Collection

Milk samples were collected from different locations of Dhaka and Chittagong city. Total ten milk samples were collected, where seven samples were powdered milk and three samples were liquid milk obtained from local markets.

Table: 2.1 Name of the commercially packed milk samples.

Milk Samples	Sample No.	Form	Collection Area
Milk Vita	14	Liquid	Dhaka
PRAN	11	Liquid	Dhaka
Aarong	03	Liquid	Dhaka
DANO	02	Powder	Dhaka
Farm Fresh	06	Powder	Dhaka
Marks	16	Powder	Dhaka
AMA	05	Powder	Dhaka
Farmland Gold	04	Powder	Chittagong
No.1	18	Powder	Chittagong
Super Pure	13	Powder	Chittagong

2.5.2 Sample Processing

After collecting the samples, all the ten samples were kept in refrigerator at 4ºC. As some of the samples were liquid, so they need to freeze for further usage. In the beginning of the experiment the samples were taken out of the fridge and normalized. Falcon tubes were

used initially and 5 ml of each sample were taken making a total volume of 20ml per sample.

2.5.3 Casein Separation

2.5.3.1 Fat Layer Separation

After the pouring the samples in the respective falcon tubes, the samples were defatted. The falcon tubes were inserted into the chambers of the centrifuge machine. The rotation was set to 4000 rpm and it was set for 10 minutes. As some of the milk samples contained little amount of fat the samples were centrifuged twice. The centrifugation process being completed the fat layer on the top of the sample were removed by using a spatula and discarded.

2.5.3.2 pH Measurement

The volume of the defatted milk samples was noted and an equal volume of distilled water was added. Three beakers were rinsed with water and ethanol, dried and labeled (sample, control and distilled water). After rinsing the pH meter with distilled water it was dipped into the beaker poured with milk sample. After 30 sec the reading of the pH meter was noted down and the pH meter was rinsed with distilled water and ethanol, dried and switched off. The process was followed for next all samples. As all the samples were added with distilled water so the initial pH was lower than 7 in all the samples.

Table: 2.2 Initial pH of the samples.

Sample Name	Sample No.	Initial pH
Milk Vita	14	6.54
PRAN	11	6.59
Aarong	03	6.62
DANO	02	6.16

Farm Fresh	06	6.54
Marks	16	6.29
AMA	05	6.45
Farmland Gold	04	6.50
No.1	18	6.27
Super Pure	13	6.23

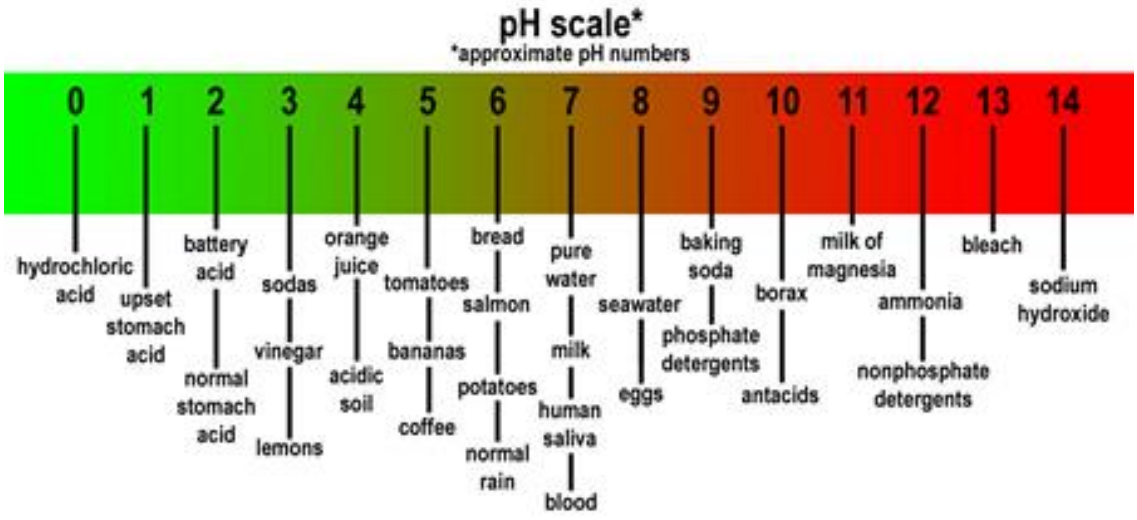


Figure: pH scale

2.5.3.3 pH Adjustment

After measuring the initial pH, all samples were kept in a stand. 1N HCl was added slowly to each of the milk samples drop wise and with constant stirring until the pH was adjusted to 4.6 in order to precipitate casein. When the pH goes down below 4.6 then a drop of 1N NaOH was added in order to increase the pH to 4.6.

2.5.3.4 Centrifugation

All the samples were kept in refrigerator at -20ºC for 5 minutes. The samples in the falcon tube were then centrifuged at 2000rpm for 10 minutes. The supernatants were taken as much as possible from each sample and were transferred in McCartney bottles.

Fig: Centrifuge Machine

2.5.3.5 Storage

All the supernatants of the respective samples were stored in refrigerator at 4ºC for further usage.

Fig: McCartney bottle containing supernatant

2.5.4 Lactoferrin extraction

2.5.4.1 pH Adjustment

3ml from each sample supernatant were taken in Falcon tubes and 1N NaOH was added with continuous stirring until the pH reached 6.0.

Fig: pH meter

2.5.4.2 Magnetic Stirring

An equal volume of 45% Ammonium Sulphate was added in each of the falcon tubes making a total volume of 6ml. The samples were then poured in a beaker and a magnetic bead was transferred inside. The beaker was kept on the flat metallic platform the magnetic stirrer machine. The stirring was fixed to 420 rpm and kept for 1 hour at room temperature.

Fig: Magnetic Stirrer

2.5.4.3 pH Adjustment

The samples were then subjected to addition of 1n HCl slowly with constant stirring until the pH reaches 4.0 and then the samples were again subjected to 1N NaOH with constant stirring until the pH reached 8.0 At pH 8.0 an equal volume of 80% Ammonium Sulphate was added.

2.5.4.4 Magnetic Stirring

The mixture of the samples and reagents were again transferred to beaker with magnetic bead inside. The samples were stirred for approximately 1hr at 420rpm after the addition of the whole Ammonium Sulphate solution.

2.5.4.5 Incubation period

All the samples were then transferred to falcon tubes incubated at 4ºC overnight for the lactoferrin to precipitate.

2.5.4.6 Centrifugation

After the overnight incubation, the samples were centrifuged at 4000rpm for 10 minutes.

2.5.4.7 Precipitation

The lactoferrin precipitates were obtained and dissolved and resuspended in 500µl 1X PBS buffer. The pH of the PBS buffer was previously adjusted to 7.4.

2.5.4.8 Storage

The lactoferrin stock obtained was stored in refrigerator at 4ºC in the respective tubes for further analysis.

2.6 Protein concentration determination

The protein concentration determining process was conducted in NanoDrop machine in the laboratory of Invent Technologies Ltd. The samples were froze at -20ºC the previous night. 20µl of each samples were taken in PCR tubes. TE buffer was added with the samples. The samples were then poured in the small well of the NanoDrop machine and measured. A

computer was also used in the process that was connected to NanoDrop to visualize the results.

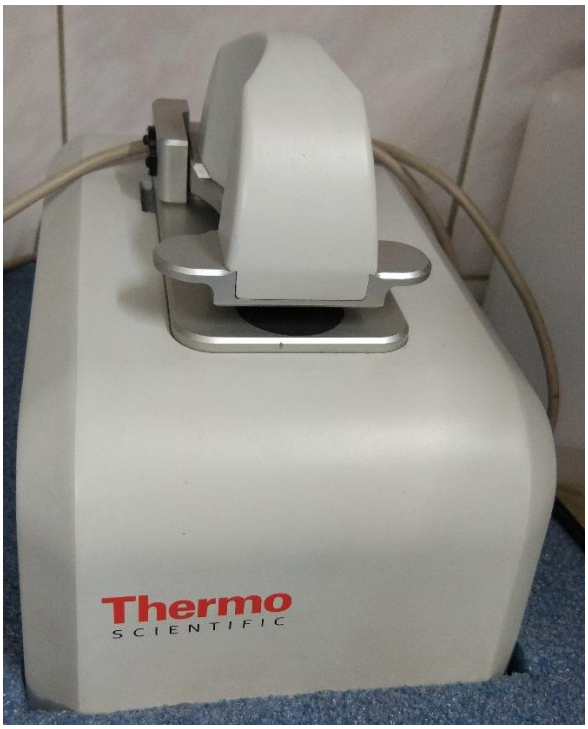


Fig: NanoDrop Machine

2.7 Protein separation

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) method was used in this perspective.

2.7.1 Sample preparation

15µl of the sample is added with 15µl of 4X loading dye in a PCR tube.

2.7.2 Preparing SDS Page gel

2.7.2.1 Cleaning the plates and combs

For each gel, one short plate, one spacer plate and one comb were used. A little bit of 70% ethanol was sprayed on the plates, and wipe dry using tissue paper. The combs were washed thoroughly with tap water and then with distilled water.

2.7.2.2 Setting the plates on the rack

The short plate was layered on the spacer plate, with the spacers in between and slides the two plates into the holder. The bottom edges of the two plates were flushed to avoid leakage. The plates were locked in and was placed the holder on the rack, with the bottom edges of the plates pushed into the rubber pad to make a water-tight seal. The seal was ensured by pipetting a small volume of water between the plates and making sure there is no leakage. Blotted dry with filter paper

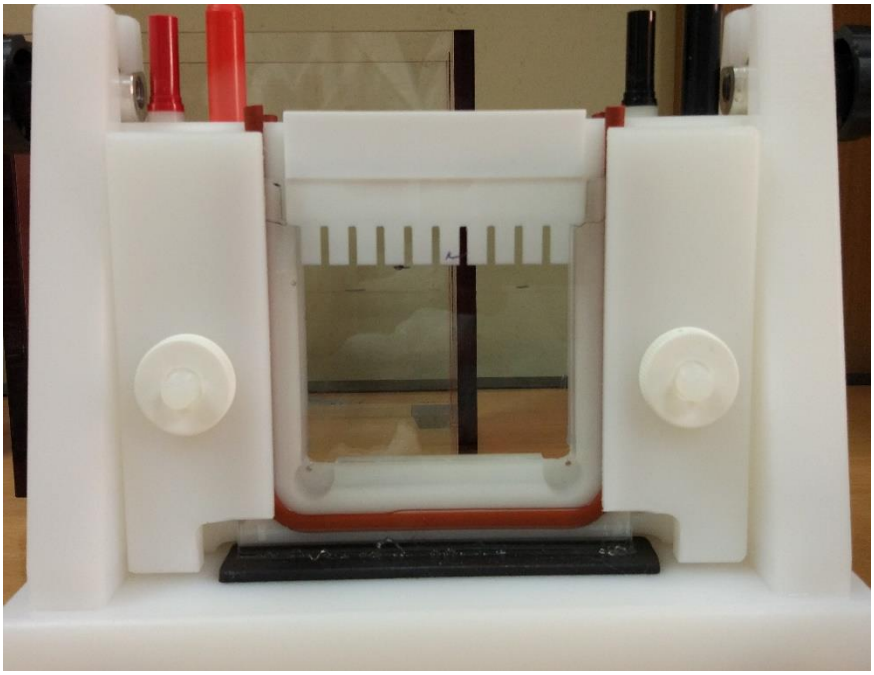


Fig: Setting plates on the rack

2.7.2.3 Pouring the separating gel

The solutions were pipetted in order. Bubbles were avoided during pipetting, which will inhibit polymerization. The solution was swirled gently to mix thoroughly after addition of each component. Once TEMED was added, the gel began to polymerize. The gel mix was pipetted between the plates, making sure of leaving enough space at the top for the stacking gel and comb. The gel has polymerized after about 10-15 minutes. The top level of the separating gel was marked on the plate to differentiate between separating gel and the stacking gel.

2.7.2.4 Pour the stacking gel

For each gel, the solutions were pipetted carefully and swirled to mix after addition of each component. The gel mix was pipetted between the plates up to just below the edge of the short plate. The comb was placed carefully. Once the gel has polymerized, the comb was removed

2.7.2.5 Gel storage

The gel prepared was then stored inside a box containing 1X TGS buffer and kept in the refrigerator at 4ºC for further use.

2.7.3 Gel run

The plates containing the gel were placed on the rack and along with it the whole complex was inserted into the glass chamber. The 1X TGS buffer made earlier was poured inside the glass chamber up to the maximum limit pointed in the glass chamber. The sample mixture was then poured into the wells of the gel sequentially. A ladder sample was also placed in the well on the right side of the gel. The lid of the glass chamber was fixed on the top and constant current of 100V was provided.

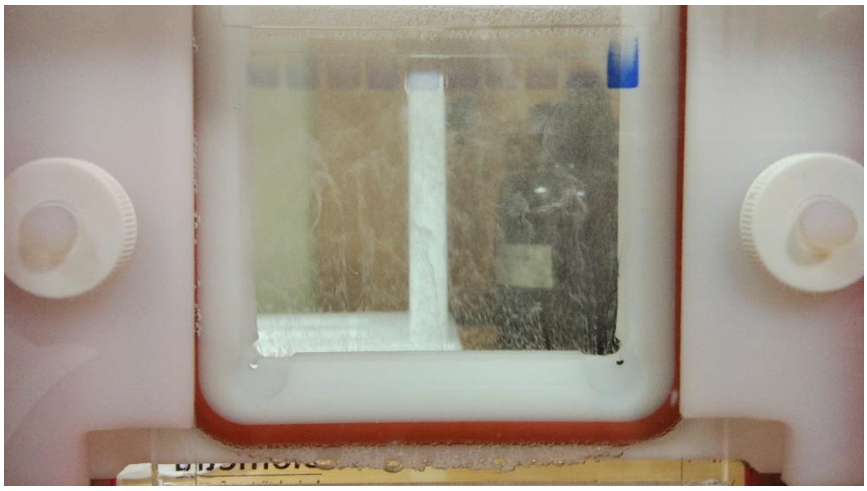


Fig: SDS PAGE gel run

2.8 Antibacterial Assessment

2.8.1 Bacterial strain collection

A total of 18 bacterial strains were used in this experiment to identify the antibacterial activity of the protein.

Table: 2.3 Name of the bacterial strains

Name	Source	Type
Vibrio cholera	ICDDR,B	Gram-negative
Shigella flexneri	ICDDR,B	Gram-negative
Shigella dysenteriae	ICDDR,B	Gram-negative
Salmonella typhi	ICDDR,B	Gram-negative
Bacillus subtilis	ICDDR,B	Gram-positive
Bacillus cereus	ICDDR,B	Gram-positive
Enterococcus faecalis	ICDDR,B	Gram-positive
STEC	ICDDR,B	Gram-negative
Enteroaggregative Escherichia coli (EAEC)	ICDDR,B	Gram-negative
Enterotoxigenic Escherichia coli (ETEC)	ICDDR,B	Gram-negative
Enteropathogenic Escherichia coli (typical) (EPEC)	ICDDR,B	Gram-negative
Enteropathogenic Escherichia coli (atypical) (EPEC)	ICDDR,B	Gram-negative
Pseudomonas aeruginosa	SHISHU	Gram-negative
Staphylococcus aureus	SHISHU	Gram-positive

Klebsiella pneumonia	ICDDR,B	Gram-negative
Proteus vulgaris	ICDDR,B	Gram-negative
Streptococcus pyogenes	ICDDR,B	Gram-positive
Streptococcus pneumonia	SHISHU	Gram-positive

2.8.2 Media Preparation

Two types of media were used in this experiment. Nutrient Agar (NA) media was prepared for bacterial sub-culture and Mueller Hinton Agar (MHA) media was prepared for well diffusion**.**

2.8.3 Preparing bacterial sub-culture

Streaking method was used in this section. A loop was used to streak the bacterial strains on the NA media. The loop was sterilized in the Bunsen burner before streaking. A small amount of bacterial stock is touched by the tip of the loop and streaked on the media evenly. The whole process was conducted in laminar air flow cabinet.

2.8.4 Incubation

The bacterial subcultures were then kept in the incubator overnight at 37ºC for the bacterial growth.

2.8.5 Lawn Culture

Autoclaved cotton swab and previously prepared saline was used. A loop is sterilized by Bunsen burner and touched on the top of the sub-culture plate. Then the loop is inserted into the saline solution. The saline solution is then vortexed until the solution turns a little turbid. The saline solutions were then compared to Mcfarland standard solution as a reference. Autoclaved cotton swab is dipped in the saline solution and spread on the surface of MHA plate avoiding gaps.

2.8.6 Well diffusion

A Cork borer is heated on the Bunsen burner and dipped on the MHA plate creating a well. The milk extracts were poured in the well until the well is filled. All the other samples were inserted in the well sequentially. After the samples were poured, the MHA plates were kept in the incubator for overnight incubation.

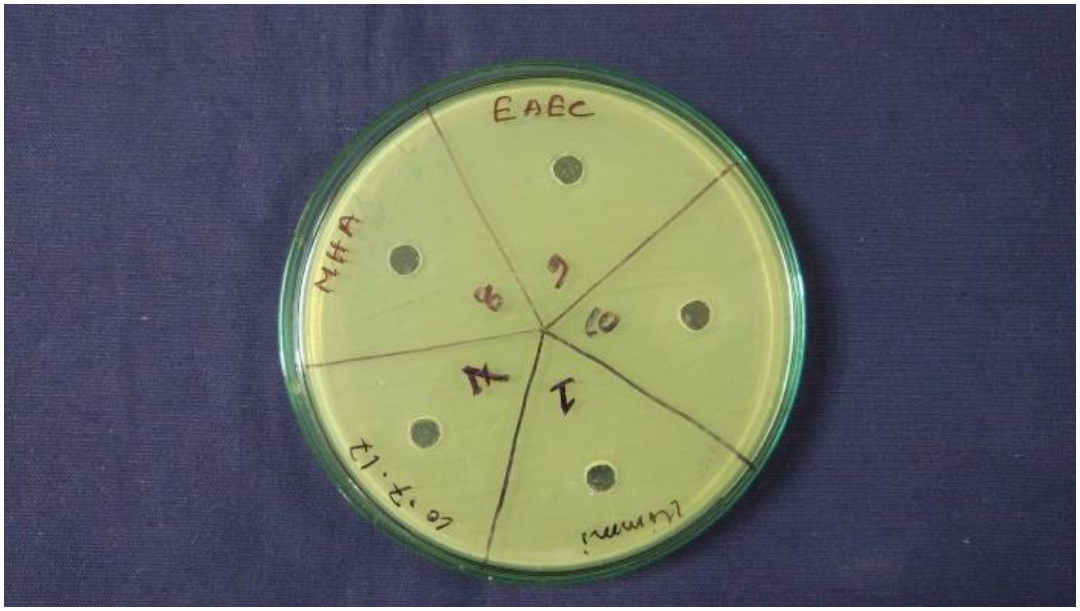


Fig: Well diffusion

Chapter 3

Results

Results

Milk is a very common and potential substitute for human health but many concerns have risen regarding their nutritional components, quality, etc. Many diary industries have already started producing different milk products and many of them are going to launch in the market, but most of the companies are not concerned about the quality of the milk products. On the other hand, most of the families prefer raw milk as they only think about the nutritional benefits other than the quality of the milk. In the present study, ten commercial milk samples (three commercially packed liquid milk samples and seven commercially packed powder milk samples) were examined for protein concentration determination and antibacterial activity.

3.1 Protein concentration of different milk samples

The more the protein concentration, the better the milk sample. Commercially packed milk samples are believed to have lower protein concentration than raw milk. The three pasteurized liquid milk samples that were used showed higher protein concentration than the rest of the powdered milk samples except for the PRAN liquid milk sample. The PRAN milk sample showed lower protein concentration than its other two variants Milk Vita and Aarong. Among the powdered milk samples AMA milk and Farm Fresh milk samples showed higher concentration than the other powdered samples.

Overall protein concentration ranges from 0.184 to 10.335 mg/ml. In this study, the highest protein concentration was found in the Aarong liquid milk sample and the lowest concentration was found in the Farmland Gold powder milk sample. A standard sample was used having a concentration of 0.43 mg/ml.

Table: 3.1 Protein concentrations of the liquid milk samples

Sample ID	Sample Name	Protein Concentration	Unit	260/280
03	**Aarong**	10.335	mg/ml	0.99
11	**PRAN**	1.123	mg/ml	1.03
14	**Milk Vita**	8.061	mg/ml	1.23

Table: 3.2 Protein concentrations of the powdered milk samples

Sample ID	Sample Name	Protein Concentration	Unit	260/280
02	**DANO**	3.356	mg/ml	1.37
04	**Farmland Gold**	0.184	mg/ml	1.11
05	**AMA**	7.722	mg/ml	1.12
06	**Farm Fresh**	5.293	mg/ml	1.24
13	**Super Pure**	0.576	mg/ml	0.91
16	**Marks**	3.134	mg/ml	0.95
18	**No. 1**	3.643	mg/ml	1.09

The protein concentration of all the commercial milk samples are shown in the pie chart below-

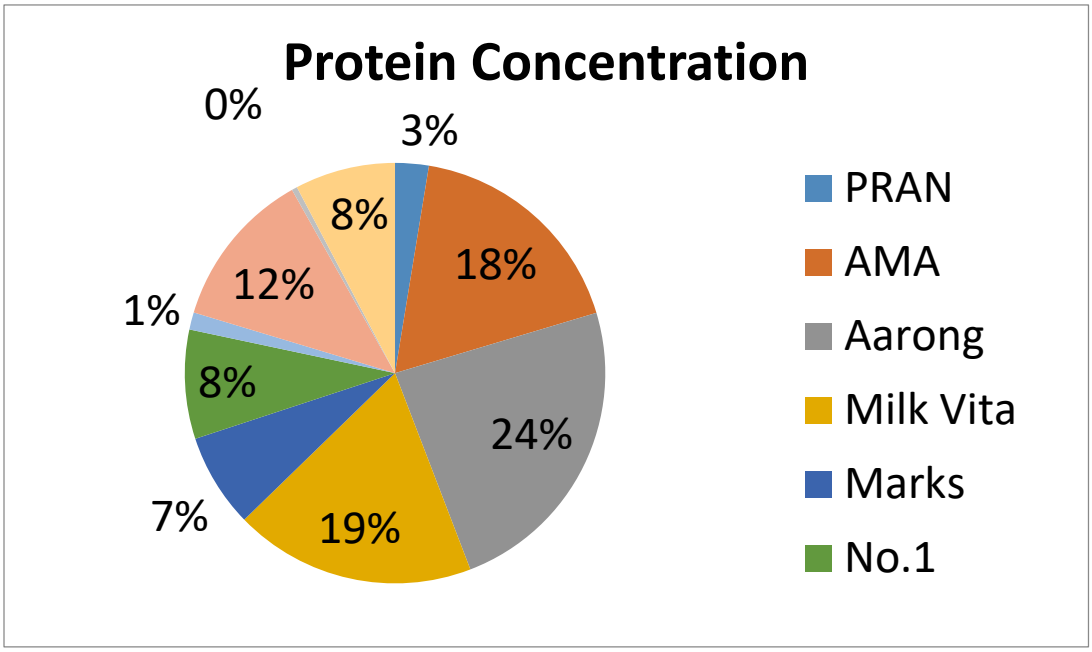


Fig: Pie chart of protein concentration

3.2 Protein Separation

The SDS-PAGE ran twice with 100V-120V electric influence. The ladder (10-120 kDa) on the right showed bands as expected but it was a little hazy. As the molecular weight of lactoferrin is 77-80 kDa only a few samples showed bands in that range. In the first gel run only four samples were used. Milk Vita sample showed band in the standard range of lactoferrin whether Super Pure, PRAN and Marks did not showed any bands.

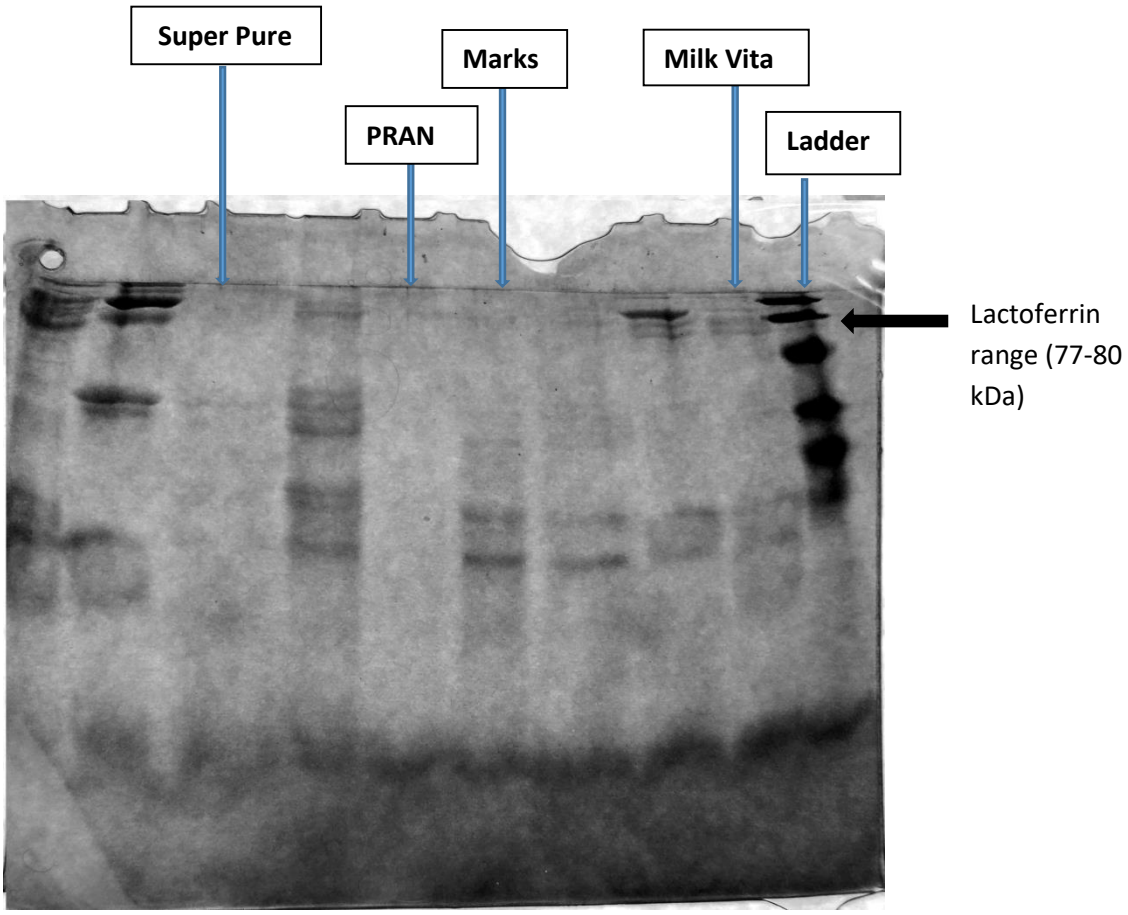


Fig: The test results of the 1st SDS PAGE run.

In the second run, six samples were used. Among them Aarong, AMA and farm fresh showed visible bands in the standard range whereas the other samples such as DANO, Farmland Gold and No.1 didn't showed any visible bands. The bands were hazy.

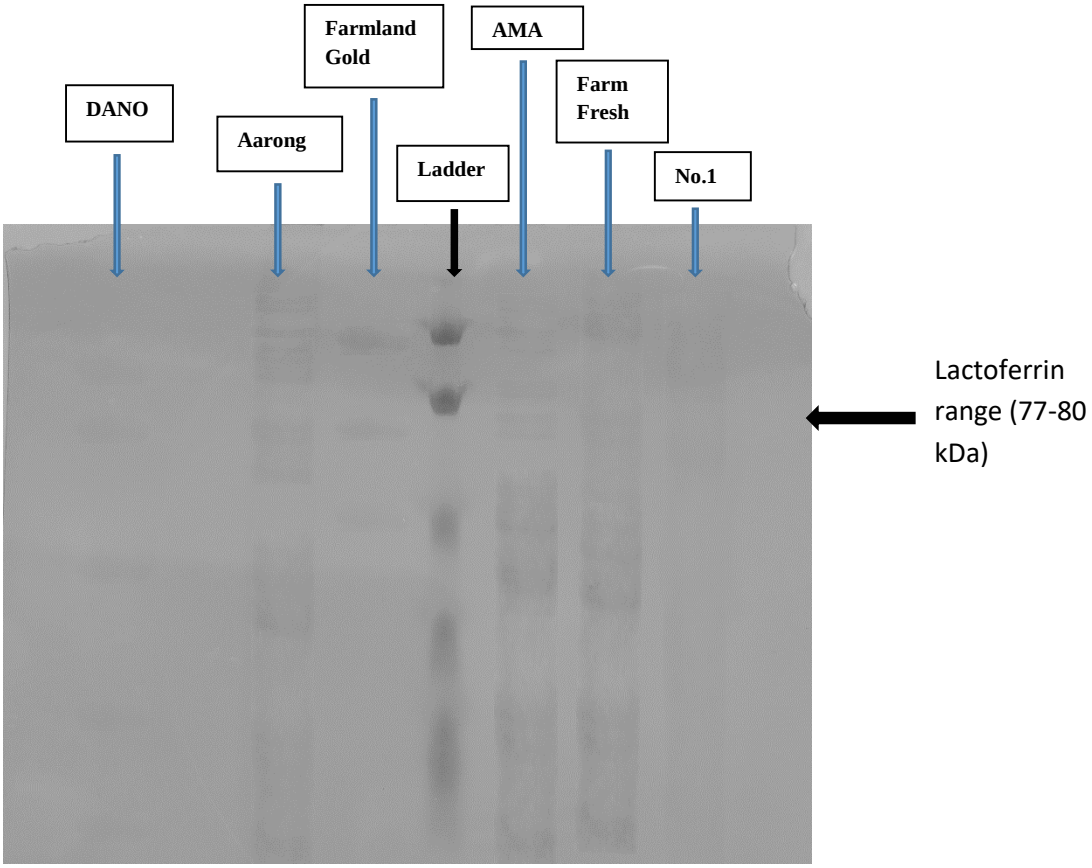


Fig: The test results of the 2nd SDS PAGE run.

3.3 Antibacterial activity

Antibacterial activity test for all the ten commercial milk samples were conducted using the well diffusion method. The zone of inhibition was the main priority in this experiment. The samples poured in the wells were dried off forming zones around the well.

3.3.1 Antibacterial activity of liquid milk samples

Among the three liquid milk samples, PRAN milk sample inhibited the growth of *Vibrio cholera, Shigella dysenteriae, Bacillus subtilis, Bacillus cereus* and *Klebsiella pneumonia* strains. The Aarong milk sample showed inhibition against of *Vibrio cholera, Shigella dysenteriae, Bacillus subtilis* and *Enterococcus faecalis*. Milk Vita showed inhibition only on *Enterococcus faecalis* strain.

Table: 3.3 Antibacterial inhibitions of liquid milk samples.

Bacterial Strains	Milk Vita (14)	PRAN (11)	Aarong (03)
Vibrio cholera	No	Yes, small	Yes, small
Shigella flexneri	No	No	No
Shigella dysenteriae	No	Yes, small	Yes, small
Salmonella typhi	No	No	No
Bacillus subtilis	No	Yes, small	Yes, small
Bacillus cereus	No	Yes, small	No
Enterococcus faecalis	Yes	No	Yes
STEC	No	No	No
Enteroaggregative Escherichia coli (EAEC)	No	No	No
Enterotoxigenic Escherichia coli (ETEC)	No	No	No
Enteropathogenic Escherichia coli (typical) (EPEC)	No	No	No
Enteropathogenic Escherichia coli (atypical) (EPEC)	No	No	No
Pseudomonas aeruginosa	No	No	No
Staphylococcus aureus	No	No	No
Klebsiella pneumonia	No	Yes	No

Proteus vulgaris	No	No	No
Streptococcus pyogenes	No	No	No
Streptococcus pneumonia	No	No	No

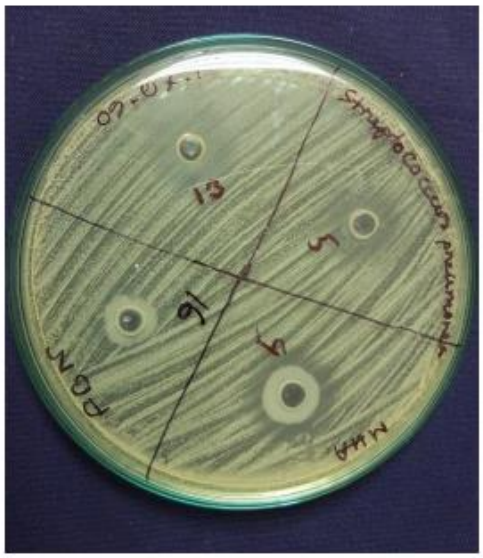
3.3.2 Antibacterial activity of powdered milk samples

The samples Farm fresh showed antibacterial activity against seven strains, towards *Vibrio cholera, Shigella flexneri, Bacillus subtilis, Bacillus cereus, Enterococcus faecalis, Enterotoxigenic Escherichia coli (ETEC)* and *Streptococcus pneumonia.* The Farmland Gold samples showed zone against six bacterial strains**.** The DANO milk sample showed inhibition against five bacterial strains and the No.1 sample showed against four strains. The samples named Marks, AMA and Super pure showed antibacterial activity against three strains.

Table: 3.4 Antibacterial inhibitions of powdered milk samples

Bacterial Strains	**DANO (02)**	**Farm fresh (06)**	**Marks (16)**	**AMA (05)**	**Farmland Gold (04)**	**No.1 (18)**	**Super pure (13)**
Vibrio cholera	**No**	**Yes, small**	**No**	**No**	**Yes**	**Yes, small**	**No**
Shigella flexneri	**Yes**	**Yes, small**	**No**	**No**	**No**	**No**	**No**
Shigella dysenteriae	**No**	**No**	**Yes, small**	**No**	**No**	**Yes, small**	**No**
Salmonella typhi	**No**	**No**	**No**	**No**	**No**	**No**	**No**
Bacillus subtilis	**Yes, small**	**Yes, small**	**Yes, small**	**No**	**Yes, small**	**Yes, small**	**No**
Bacillus cereus	**No**	**Yes, small**	**No**	**No**	**Yes**	**No**	**Yes**
Enterococcus faecalis	**No**	**Yes, small**	**No**	**Yes**	**Yes**	**No**	**Yes**

STEC	**No**	**No**	**No**	**No**	**No**	**Yes, small**	**No**
Enteroaggregative Escherichia coli (EAEC)	**No**	**No**	**No**	**No**	**No**	**No**	**No**
Enterotoxigenic Escherichia coli (ETEC)	**No**	**Yes, small**	**No**	**No**	**No**	**No**	**No**
Enteropathogenic Escherichia coli (typical) (EPEC)	**No**	**No**	**No**	**No**	**No**	**No**	**No**
Enteropathogenic Escherichia coli (atypical) (EPEC)	**No**	**No**	**No**	**No**	**No**	**No**	**No**
Pseudomonas aeruginosa	**No**	**No**	**No**	**No**	**No**	**No**	**No**
Staphylococcus aureus	**No**	**No**	**No**	**No**	**No**	**No**	**No**
Klebsiella pneumonia	**Yes**	**No**	**No**	**No**	**No**	**No**	**No**
Proteus vulgaris	**No**	**No**	**No**	**Yes, small**	**No**	**No**	**No**
Streptococcus pyogenes	**Yes, small**	**No**	**No**	**No**	**Yes, small**	**No**	**No**
Streptococcus pneumonia	**Yes, small**	**Yes**	**Yes**	**Yes**	**Yes**	**No**	**Yes**

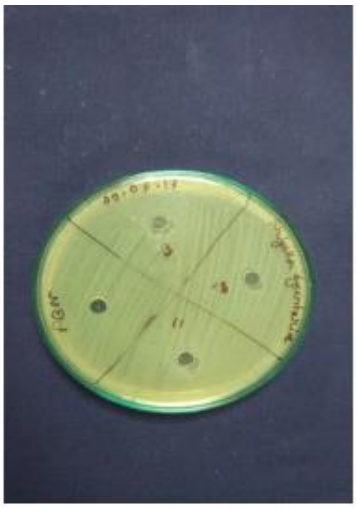


Streptococcus pneumonia

Bacillus subtilis

Bacillus subtilis

Vibrio cholera

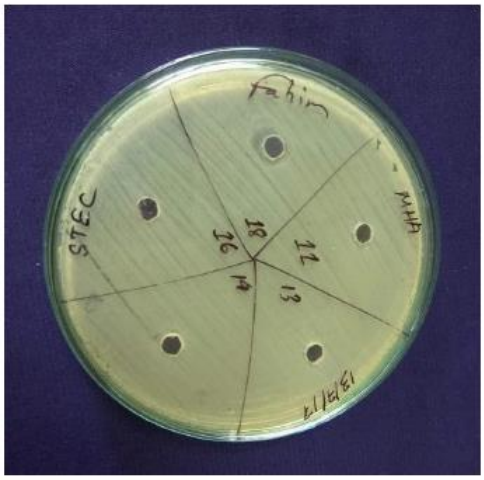


Shigella dysenteriae

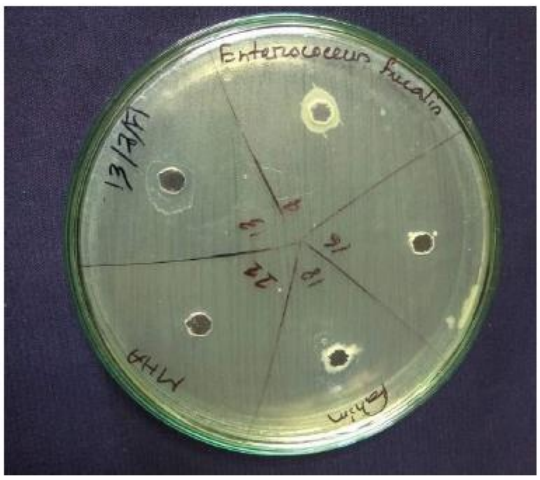
Bacillus cereus

Shigella flexneri

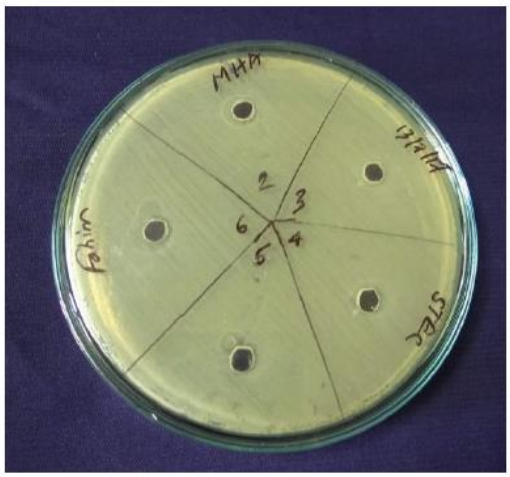
Shigella flexneri



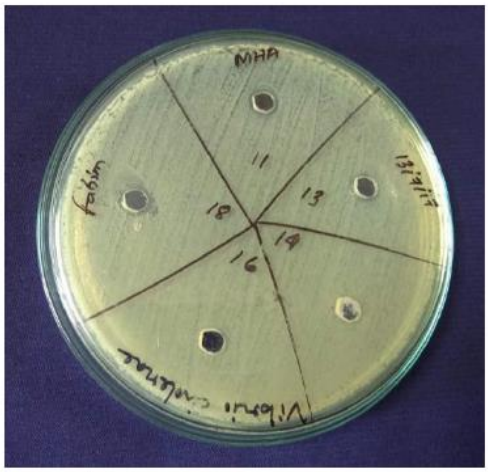
STEC



Enterococcus faecalis



STEC



Vibrio cholera

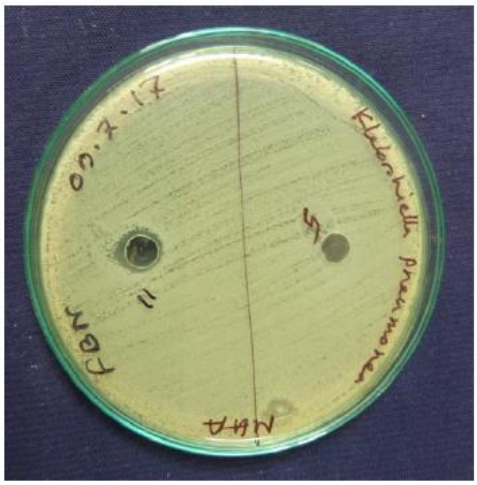
Enterococcus faecalis

Streptococcus pneumonia

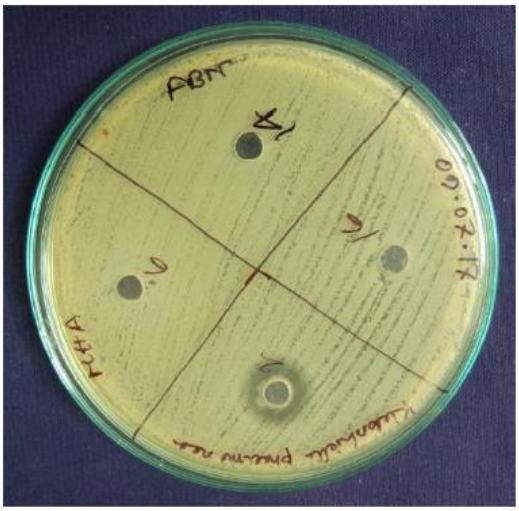
Streptococcus pneumonia

Shigella dysenteriae

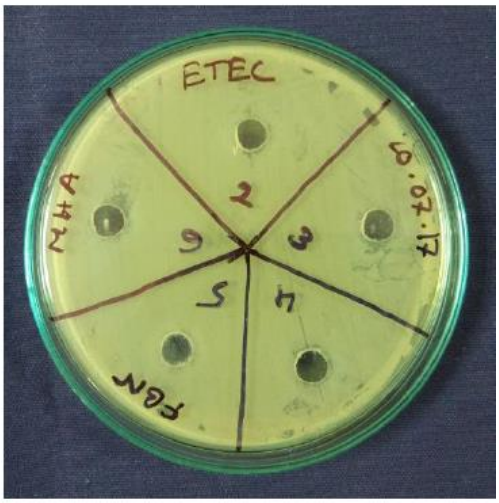
Proteus vulgaris



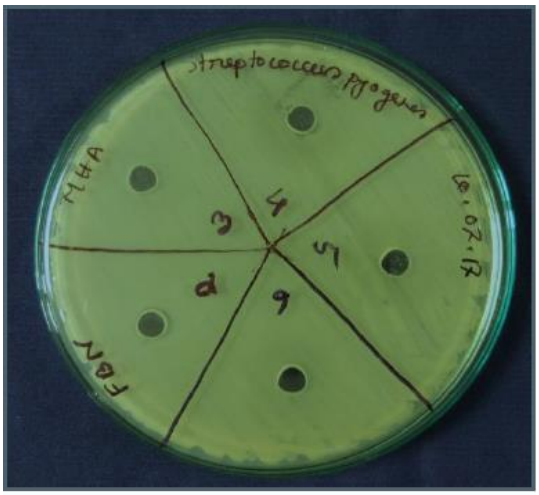
Klebsiella pneumonia



Klebsiella pneumonia



ETEC



Streptococcus pyogenes

Fig: Antibacterial activity against different bacterial strains

Chapter 4

Discussion

Discussion

Developing countries such as Bangladesh, where millions of peoples are consuming commercially packed milk variants throughout the whole year as it is a source of their protein source and nutrition. The commercially packed milk variants being good in nutrition and is available at affordable price in Bangladesh. The commercial milk samples available in the local market are admirable by the consumers. Many diary industries are producing and marketing different milk products, but most of the companies have no concern regarding the quality of the milk. Moreover, the dairy sector in Bangladesh has not received adequate attention like the other sectors with current policies and issues. The national milk production can only meet 13% of the present consumption (Shamsuddhoha *et al.*, 2000). The demand for milk and milk products is increasing with the rapid population growth creating an imbalance between the demand and supply. Milk contains certain bioactive components that strengthen the immune system of infants. The absence of such compounds can cause catastrophic effects. Several authors have conducted surveys on foodborne pathogens in US and they found that apart from conventional diaries most of the commercial diaries used pasture-based management systems promoted by the Weston A. Price Foundation (Hancock *et al.*, 1998; Jayarao *et al.*, 2006; LeJeune *et al.*, 2009; Oliver *et al.*, 2005; Oliver *et al.*, 2009; Shere *et al.*, 1998). But in case of Bangladeshi diaries, some steps may be unavailable regarding the quality improvement of the milk variants. In this study the specific protein lactoferrin was isolated from milk samples to identify its concentration and its impact against various pathogens.

Lactoferrin is considered to be present in bovine (Groves, 1960), human (Johansson, 1960; Montreuil *et al.*, 1960; Grüttner *et al,*. 1960), guinea-pig (Masson, 1970) and goat (Oram and Reiter, 1968) milk. The data presented indicates that the milk concentration in commercial milk samples vary from each other. In case of pasteurized liquid milk samples the amount of protein concentration is higher in contrast to powdered milk samples. Among the pasteurized liquid milk samples protein concentration of Aarong and Milk Vita samples were higher than PRAN sample whereas in case of powdered samples AMA and Farm Fresh samples had the higher concentration of protein. Masson and Heremans (1971) stated that neither the concentration of lactoferrin nor that of transferrin in the milk of different

species bears any relationship to the concentration of the milk iron which signifies that no matter how the concentration increases it has no relationship with the concentration of the iron binding protein lactoferrin. It can be assumed from the results of the NanoDrop protein quantification method that in spite of having a high amount protein concentration the samples can lack lactoferrin and might possess other protein rather than lactoferrin. Rat milk, for instance, is practically does not contain lactoferrin but large amounts of iron. In contrast, human milk is particularly rich in lactoferrin but poor in iron. In some species, such as the rat and rabbit, transferrin seems to compensate for the absence of lactoferrin. However, the milk of the dog, which is very rich in iron, contains only traces of transferrin and apparently no lactoferrin. The estimation of the amounts of lactoferrin by NanoDrop would give false results if these proteins were already saturated with cold iron prior to the addition of Fe (Masson and Heremans, 1971).

The presence of lactoferrin was confirmed by performing SDS-PAGE. The purity of the extracted lactoferrin was confirmed when some of the milk samples showed bands in the region 77-80kda confirming that the extraction procedure gives pure lactoferrin although the bands were slightly visible. The results of the present study is similar to other studies conducted by Younghoon *et al.,* (2009) where SDS-PAGE was used to confirm the purity of the protein. Yafei *et al.*, (2011) also conducted a study based on a single band in the gel of SDS-PAGE in order to confirm the purity of the lactoferrin from defatted bovine colostrum. In this experiment, the liquid samples with higher concentrations of protein showed bands from the sample with lower concentration of protein. In the first gel run only Milk Vita sample showed bands among the other samples such as Super Pure, PRAN and Marks And in the second gel run only Aarong, AMA and Farm Fresh showed bands other than DANO, Farmland Gold and No.1. Some of the samples in spite of having a higher number of protein concentrations didn’t show any bands. The main reason behind this result might be that the samples contained other proteins apart from lactoferrin. Another reason might be the handling issues as the precipitate found in some samples after the extraction procedure was minimal compared to others. Some of the proteins might also been lost during the casein separation method as this step includes the separation of casein precipitates from lactoferrin supernatant.

The iron binding is considered the major mechanism responsible for the bacteriostatic activity of lactoferrin (Roseanu *et al.*, 2010). Lactoferrin has high affinity for iron and together with its presence in an iron-free form in body secretions allows lactoferrin to produce iron-deficient environment that limits bacterial growth (Arnold *et al,*. 1980; Kalmar *et al.*, 1988; Yamauchi *et al,*. 1993). In the present study, the effect of lactoferrin was examined 18 bacterial strains. The results indicated that in case of pasteurized liquid milk samples, PRAN and Aarong showed better bacterial inhibition that the other sample confirming the presence of lactoferrin. The PRAN milk sample however showed lower concentration of protein and no bands in the SDS-PAGE but showed inhibition during the antibacterial test. The reason behind this might be the usage of certain preservatives and other chemicals for the preservation of milk that inhibits the growth of certain bacterial strains. In case of the powdered milk samples Farm Fresh, Farmland Gold and DANO showed better inhibition than the other samples. All of these samples that showed bacterial growth inhibition can be assumed to have lactoferrin presence. For instance, if we consider the antibacterial activity of lactoferrin against *Pseudomonas aeruginosa* a few mechanisms might come in consideration. As lactoferrin is an iron-binding protein which scavenges free iron from its surrounding, the deficiency of iron prevents the biofilm formation by *Pseudomonas* (Caraher *et al.*, 2007). In the present study all the samples could not inhibit the formation of biofilm in order to prevent *Pseudomonas* growth which might be the case that the isolated protein couldn't bind to the iron or the number iron was immense than the number of the proteins. Another reason might be the long preservation period of the samples with PBS buffer which might have affected the protein.

Chapter 5

Conclusion

Conclusion

The data presented in the current study concludes that not all the commercially packed milk variants have the protein lactoferrin and consumption of some commercial milk can also eradicate bacterial growth. However all the samples were collected from different areas of Dhaka and Chittagong city and the samples contained less protein concentration compared to raw milk. The antibacterial activity of lactoferrin was not satisfactory against Enteroaggregative *Escherichia coli* (EAEC), Enteropathogenic *Escherichia coli* (typical) (EPEC), Enteropathogenic *Escherichia coli* (atypical) (EPEC), *Pseudomonas aeruginosa* and *Staphylococcus aureus*. There is a generalized belief among the consumers that, automated machines and some preservatives are used during the processing of commercial milk. Despite all these issues, most of the samples showed better protein concentration and antibacterial activity against *Bacillus subtilis, Enterococcus faecalis, Vibrio cholera* and *Streptococcus pneumonia* which indicate that all most of the samples are locally produced and marketed them are actually beneficial to health. In order to improve the quality of the milk and milk products government authorized institutions like BCSIR and BSTI and also administrative organization like mobile courts should be given authorization to undertake precautionary investigations to check the quality of the commercial milk. Besides, government and non-government institutions should create public awareness regarding the health benefits of consuming milk on daily basis.

Chapter 6

Reference

Reference

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Appendices

Appendix- I

Media compositions

The composition of all media used in the study is given below.

Nutrient Agar

Components	**Amount (g/L)**
Peptone	5.00
Sodium Chloride	5.00
Beef Extract	3.00
Agar	15.00

*****Final pH adjusted to 7.0

Mueller Hinton Agar

Components	**Amount (g/L)**
Beef Extract	2.00 gm.
Acid Hydrolysate of Casein	17.50 gm.
Starch	1.50 gm.
Agar	17.00 gm.

*****Final pH adjusted to 7.3±0.1 (at 25°C)

Appendix- II

Reagents

The composition of all reagents used in the study is given below.

PBS Buffer (1 L)

At first, in 800 ml of distilled water 8 g of NaCl, 0.2 g of KCl, 1.44 g of disodium phosphate and 0.24 g of monopotassium phosphate were added sequentially. Stirred for a while and the rest 200 ml was added making the total volume of 1 liter. pH was adjusted to 7.4 using HCl.

1 N NaOH (100 ml)

4 g of NaOH was added with 40 ml distilled water and stirred. Then, the other 60 ml was added and stirred again making a total volume of 100 ml. Aluminum foil was used to cover the conical flask and stored for usage.

1 N HCl (100 ml)

8.212 ml of HCl was added to 30 ml distilled water and stirred and the rest 70 ml distilled water was added and stirred gently making the total volume 100 ml. Aluminum foil was used to cover the conical flask and stored for usage.

45% Ammonium Sulphate (100 ml)

45 g of Ammonium Sulphate was added to 60 ml of distilled water and stirred. The rest 40 ml distilled water was added gently and stirred making a total volume of 100 ml.

80% Ammonium Sulphate (100 ml)

80 g Ammonium Sulphate was added to 50 ml of distilled water and stirred. The rest 50 ml distilled water was added gently and stirred making a total volume of 100 ml.

30% Acrylamide (100 ml)

29.0 g of Acrylamide and 1.05 g of Bis-Acrylamide was added to 100 ml distilled water. Aluminum foil was used to wrap up and stored at 4ºC.

1.5 M Tris Hydrochloride (pH 8.8) (50 ml)

14.81 g of 1.5 M Trisma base and 0.2 g of SDS was added to 50 ml of distilled water and stored at 4ºC.

0.62 M Tris (pH 6.8) (50 ml)

3.755 g of Trisma Base and 0.2 g of SDS was added to 50 ml of distilled water. The reagent needed rotation and was stored at 4ºC.

10% SDS solution (50 ml)

5 g of SDS was added to 50 ml of distilled water. Shaking was avoided and stored at room temperature.

10% APS (Ammonium persulphate) (5 ml)

0.5 g of APS was added to 5 ml distilled water. Freshly prepared and stored at -20ºC.

2% Agarose Gel

2 g of Agarose powder was added to 100 ml distilled water.

1x TGS Running Buffer (2.5 L)

7.5 g of Trisma Base, 36 g of glycine and 25 ml of 10% SDS was added to 1 liter distilled water and stirred. Then, another 1.5 liter was added making a total volume of 2.5 liter. The reagent was stored at 4ºC.

15% Resolving Gel (20ml)

10.6 ml of 30% Acrylamide, 4.2 ml of 1.88 M Tris and 4.2 ml of 10% SDS was added to 2.2 ml distilled water and stirred gently. Before pouring the gel in the casket, 200 µl of TEMED and 50 µl of APS were added.

Stacking Gel

0.8 ml Acrylamide, 1.0 ml of 0.6 M Tris and 1.0 ml of 10% SDS was added to 2.2 ml distilled water and stirred gently. Before pouring the gel in the casket, 10 µl of TEMED and 50 µl of APS were added.

Staining (120 ml)

10 ml of acetic acid, 40 ml of methanol and 0.13 g of Comassie Blue was added to 70 ml of distilled water. The reagent needed filtration and was stored at room temperature.

Destaining (200 ml)

20 ml of acetic acid, 20 ml of methanol was added to 160 ml of distilled water and stored at room temperature.

Appendix- III

Instruments

Instrument	Manufacturer
Weighing Machine	Adam equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
NanoDrop 2000 Spectrophotometer	Thermo Scientific, USA
UV Transilluminator, Model: MD-20	Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
Vortex Machine	VWR International
Microwave Oven, Model:MH6548SR	LG, China
pH Meter: pHep Tester	Hanna Instruments, Romania
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Refrigerator (4^{O}C) Model: 0636	Samsung

Appendix- IV

List of abbreviation

ml	Milliliter
µl	Microliter
mg	Milligram
gm	Gram
Kg	Kilogram
e.g.	For example
et al.	And others
pH	Negative logarithm of hydrogen ion concentration
%	Percentage
°C	Degree Celsius
Sec	Second
mm	Millimeter
µm	Micrometer
rpm	Rotation per minute
PBS	Phosphate Buffer Solution
kDa	Kilo Dalton
L	Liter
M	Molar
V	Volt