ISOLATION OF VARIOUS MILK OLIGOSACCHARIDES AS BIOACTIVE AGENTS

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by

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DECLARATION

I here by **Ms.Tulika Roy** (Enrolment No- 11081090036) thesis entitled **"Isolation of various milk oligosaccharides as Bioactive agents"**Submitted in fulfilment of the requirements for the degree of **Doctor of Philosophy**, Department of Biochemistry,School of Dental Sciences, **Babu Banarasi Das University, Lucknow-226028.** This is to certify that the bonafide experimental work presented in thesis was carried out by my self.

I also declare the work embodied in the present thesis is my original work and has not been submitted by me for any other degree or Diploma of any university or institution

Tulika Roy

To
My Father
Shri A.K. ROY

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(**Tulika Roy**)

ABBREVIATIONS USED

AcOH	=	Acetic acid
Ac_2O	=	Acetic anhydride
Ag_2CO_3	=	Silver carbonate
$AgNO_3$	=	Silver nitrate
AgOH	=	Silver hydroxide
Ag_2S	=	Silver sulphide
Anhyd.	=	Anhydrous
Aq.	=	Aqeous
$BaCO_3$	=	Barium carbonate
BuOH	=	n-Butanol
Br_2	=	Bromine
CC	=	Column chromatography
$CDCl_3$	=	Deuterated chloroform
$CHCl_3$	=	Chloroform
CH_2Cl_2	=	Dichloromethane
CH_3CHO	=	Acetaldehyde
CH_3CN	=	Acetonitrile
$C_6H_5CH_3$	=	Toluene
conc.	=	Concentration
Dd	=	Double doublet
D_2O	=	Deuterated water
DTH	=	Delayed type hypersensitivity
2D	=	Two dimensional
ESMS	=	Electron Spray mass spectrometry
EtOH	=	Ethanol
Et_2O	=	Diethyl ether = solvent ether
EtOAc	=	Ethyl acetate
FABMS	=	Fast atom bombardment mass spectrometry
$FeCl_3$	=	Ferric chloride
$FeCl_3$	=	Ferric chloride
$Fe_2(SO4)_3$	=	Ferric sulphate
Fr.	=	Fraction(s)
Fuc	=	Fucose
Gal	=	Galactose
GalNAc	=	2-Acetamido-2-deoxy-galactose
Glc	=	Glucose
GlcNAc	=	2-Acetamido-2-deoxy-glucose

HA	=	Haemagglutination titre
HCl	=	Hydrochloric acid
H_2O	=	Water
HPLC	=	High performance liquid chromatography
H_2S	=	Hydrogen sulphide
H_2SO_4	=	Sulphuric acid
Hz	=	Hertz
KOH	=	Potassium hydroxide
LND	=	Lacto-N-difucohexaose
LNF	=	Lacto-N-fucopentaose
LNH	=	Lacto-N-hexaose
LNT	=	Lacto-N-tetraose
LNnT	=	Lacto-N-neotetraose
M	=	Multiplet
MeOH	=	Methanol
Me_2CO	=	Acetone
Mg	=	Milligram
MHz	=	Megahertz
Min	=	Minute
Ml	=	Millilitre
µl	=	Microliter
MLR	=	Mixed lymphocyte reaction
MMI	=	Macrophage migration index
Mol. wt.	=	Molecular weight
Mp	=	Melting point
Mmp	=	Mixed melting point
N	=	Normal
NaBH4	=	Sodium borohydride
Na_2CO_3	=	Sodium carbonate
$NaIO_4$	=	Sodium metaperiodate
NaOH	=	Sodium hydroxide
$NaOCH_3$	=	Sodium methoxide
Na_2SO_4	=	Sodium sulphate
NMR	=	Nuclear magnetic resonance spectroscopy
OAc	=	Acetyl
OH	=	Hydroxyl
ORD	=	Optical rotatory dispersion
$Pb(OH)_2$	=	Lead hydroxide
PC	=	Paper chromatography
PFC	=	Plaque-forming cells
P_2O_5	=	Phosphorus penta-oxide

Pyr	=	Pyridine
Q	=	Quartet
R P	=	Reverse phase
S	=	Singlet
S D	=	Standard deviation
SEM	=	Standard error of mean
SiO_2	=	Silica
SRBC	=	Sheep red blood cells
T	=	Triplet
TBA	=	Thiobarbituric acid
TDW	=	Triple distilled water
TLC	=	Thin layer chromatography
TMS	=	Tetra methyl silane
UV	=	Ultra violet
[α]D	=	Rotation/specific rotation
α	=	Alpha
β	=	Beta

PREFATORY NOTE

The work presented in the thesisentitled **"Isolation of various milk oligosaccharides as bioactive agents"**began in January, 2010 under the able guidance of Dr. Aditya Bhushan Pant, Senior Scientist, Indian Institute of Toxicology Research Lucknow, and Dr Desh Deepak Department of Chemistry, Lucknow University It was started with a view to study the nutritional, medicinal and therapeutic properties of various kinds of ruminant milks by detailed chemical investigations of Milk Oligosaccharides and macronutrients present in milk. The Milk of following ruminants i.e. Cow, Buffalo, Camel, Sheep, Chauri-cow, Goat and Mare were studied.

Milk is the first intake taken by any mammalian infant. Milk is mainly constituted proteins, lipids, vitamins, minerals most importantly complex carbohydrates. All cell surfaces are coated with complex carbohydrates which act as recognition site molecules for other cells, functional molecules, and pathogens. Consequently, they are involved in disease indications as diverse as inflammation, cancer, and infectious diseases.Carbohydrates are crucial in mediating essential biological process, and their biosynthesis is an essential aspect to develop a global view of their biological functions. They are present as free monosaccharides, oligosaccharides, polysaccharides, and are essential components of glycoconjugates, including glycolipids, glycoproteins or glycopeptides, and glycosylated natural products. Glycosylated natural products have been commonly used as antimicrobial drugs and now as emerging anti-cancer drug which is undergoing trials. The sugar moieties in many bioactive natural products do not only increase water solubility thus the bioavailability of the compounds, but also decrease toxicity. Some glycans are also the essential components for the bioactivity of the natural products. Carbohydrates are a relatively untapped natural source of new

drugs and therefore offer exciting new therapeutic opportunities.Oligosaccharide and oligosaccharide containing moieties are an important class of bioactive natural products and are emerging as potent drugs against fatal diseases like cancer and AIDS. Various developments on medicinal and pharmaceutical researches have revealed the importance of these compounds. It has been observed that a number of higher plants, fungi, algae, lichen, milk and bacteria serve as rich sources of bioactive oligosaccharides and many oligosaccharides isolated from these natural sources and have exhibited high potent biological activities which are used in pharmaceutical industry such as anti-tumor, anti-oxidant, anti-cancer, anti-inflammatory, anti-coagulant, hypoglycemic and antiviral activities. Number of biologically active oligosaccharides has been isolated from human, buffalo, donkey, cow, mare, sheep and goat milk. The oligosaccharide isolated from various milk sources are categorized in two classes i.e. sialylated oligosaccharide and non-sialylated oligosaccharide.

OBJECTIVES:

- Isolation of Milk oligosaccharides from various Ruminants
- Purification of Milk oligosaccharides
- Assessment of Milk oligosaccharides
- Assessment of Macronutrients of various ruminant milks
- Analysis of Milk oligosaccharides as Bioactive agents
- To explore Antioxidant properties in various ruminant milks
- Various milks as potent Neutraceuticals

Hence various ruminant milks were collected from species like Indian cow, Buffalo, Camel, Sheep, Chauri-cow, Goat, Mare and analyzed for their various biological activities such as immunostimulant and antioxidant properties. Camel milk , Mare milk and Chauri –cow milk showed variable bioactive results , amongst which chauri-cow milk oligosaccharides showed potent antioxidative properties along with Camel , Sheep, Mare milk Oligosaccharides

Milk in bulk were collected and analysed for their natural macronutrient content in them. Lipid estimation, Protein estimation, Lactose estimation, Total soluble sugars were estimated and reported.

Various milks were processed by Kobata and Ginsburg method for obtaining the crude mixture of oligosaccharides present in them. Bioactivities of various milk oligosaccharides were analysed and after obtaining the mixture of oligosaccharides, they were purified in analytical chemical techniques mentioned in the work presented in the thesis.

After subjecting the various milk oligosaccharides for bioactivity it was found that milk oligosaccharides were health promoting agents having antioxidant and immunostimulating properties. Milk oligosaccharides are an excellent source of natural antioxidants and natural prebiotic making various ruminant milks as highly beneficial beverage and Milk Oligosaccharides as potent neutraceutical.

Conclusion of milkoligosaccharides analysis:

- Chauri cow's milk contained higher relative amounts of both sialylated and the more complex neutral fucosylated oligosaccharides
- Abundance of smaller and simpler neutral oligosaccharides was observed in Camel and buffalo's milk
- Chauri cow's milk contains levels of fucosylated oligosaccharides significantly higher than that of Indian Cow, Gaddi sheep, Camel, Mare and buffalo's milk.
- Chauri cow's milk also possesses oligosaccharides with a higher degree of complexity and functional residues (fucose and sialic acid), suggesting it may therefore offer advantages in term of a wider array of bioactivities.

CHAPTER I

INTRODUCTION

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1.1 REVIEW OF LITERATURE

Milk is one of the most precious gifts of nature to its mankind. Milk is the first intake taken by any mammalian infant. It provides the first energy required for all the vital biological functions of any infant, it gives strength to fight out any infections which try to prevail in the newly born. It has the properties of easy digestion and instant energy. Besides this, it has a property to develop the undeveloped pulmonary, neural and secretary systems of infants. The most important property of the milk is the development of brain which controls all the systems of the body[1]. Basically, milk is constituted of simple and complex carbohydrates, proteins, vitamins and most of the nutritional requirements needed for the development of the body systems. Milk can be described as a complete planned complete food. It provides a range of essential nutrients in particular protein and a wide range of vitamins and minerals. Therefore, the milk is the important source of nutrients. One Pint (600 ml. of Milk supplies 7/8th of calcium, 1/3rd of Riboflavin, over 1/4th of proteins and 1/5th of the daily vitamin A requirement of a normal adult person. The proportion of various nutrients in milk also makes it an ideal diet for the neonates. Milk is also called the liquid fountain of nutrition. It is an indispensable part of an infant's diet. The composition of milk is given below.

The human and other ruminant milks have about 87% of water. The major carbohydrate present in milk is lactose, which is a disaccharide composed of the monosaccharides D-glucose and D-Galactose. The chemical name of the Lactose is 4-O-β-D-Galactopyranosyl-D-Glucopyranose. Lactose is essentially unique to milk and it plays a major role in milk synthesis. It plays a major role in milk and the process of synthesis of lactose is responsible for drawing water into milk,it is being formed in the mammalian epithelial cells. Lactose is not as sweet as other Disaccharides such as sucrose and monosaccharides such as fructose and glucose. Lactose is cleaved in the intestines to glucose and galactose. By an enzyme called lactase. Some other carbohydrates which are found free in milk are amino-sugars, sugar phosphates, neutral/acidic oligosaccharides and neucleotide sugars. Some complex oligosaccharides are important in establishing the microflora of neonatal intestine such as Bifidus factor identified in milk[2].

The fat component of the milk is composed of a complex mixture of lipids. Triglycerides which are the major types of lipids in milk are composed of these fatty acids covalently bound to a glycerol molecule by ester linkage. Milk fat is the major source of lipids used by neonate mammal for accumulating body adipose tissues in the initial days after birth. Milk fat is easily digested by the new born and the composition is best suited to them. The total protein component of milk is composed of many specific proteins. The primary group of milk proteins are the caseins. Caseins have an amino-acid composition which is important for the growth and development of the nursing young. Caseins are easily digestible in the intestine compared to many other food proteins available. Caseins reacts with the acid found in the stomach and also with

an enzyme called rennin and form a soft curd. The protein caseins can be separated from the whole milk with the action of acids. The reaction is similar to what happens in the stomach when milk is consumed. Once caseins are removed from the milk, all other proteins left are known as whey proteins. There are many whey proteins in the milk and the specific set of whey proteins found in mammary secretions varies with the species, stage of lactation, the presence of any intra-mammary infections and other factors. The major whey proteins in the milk are α-Lactalbumin[7] and β-Lactglobulin. α-Lactalbumin is an important protein in the synthesis of lactose and it plays a key role in the process of milk synthesis. Other whey proteins are important immunoglobulins which have anti-infective factors especially high in colostrums[3-4]. Whey proteins also include various enzymes, hormones, growth factors, disease resistant factors, iron lactoferrin and various nutrient transporters. The major minerals found in milk are calcium and phosphorous. These minerals are of prime importance for growing neonates and children. They are specially required for bone and teeth growth and development of soft tissues. Milk also has all the vitamins which are required by the human body. The fat soluble vitamin A, D, E, K are found in the milk fat while the B-complex vitamins are found in the aqueous phase of the milk. Milk also contains some bioactive factors such as hormones, enzymes and cellular proteins etc. Milk becomes important food stuff in every stage of life cycle and even in various clinical conditions due to variety of nutrients, it supplies and even larger variety of products one gets from it. Milk is the only naturally occurring food stuff that contains lactose which is made up of glucose and galactose. Glucose is the form in which sugar is absorbed by the body and galactose is utilized for the synthesis of myelin sheath, the insulating cover of the nerve fibres. Lactose also favours calcium absorption, firstly by increasing the permeability of the

small intestine for influx of calcium ions and secondly, by lowering the pH of the intestine consequently favouring calcium absorption. Milk also supplies all the essential amino-acids. Milk also contains essential fatty acids that are good for the body system. The calcium phosphorous ratio of milk is most suited for bone development. Iron content of milk is rather low but the absorption of iron from milk is high and so it is highly utilized by the body. Milk is rich in riboflavin, thiamine and also has high amounts of vitamin A and D. It also has high moisture content and to some extent it relieves the constipation tendency in new-borns and immobilized patients. Water content of milk is dependent upon the synthesis of lactose, without some water in it milk would be a viscous solution of lipids and proteins and would be extremely difficult to remove from the gland. Upon birth the mammalian neonate is not able to meet its own water supply and would dehydrate rapidly without the water component of milk. In pregnant ladies, a balanced diet rich in proteins, minerals and vitamins is essential for the proper growth and health of mother and foetus. Milk can provide all these nutrients in adequate amounts. Milk also helps the pregnant ladies for the following:

(a). rapid growth of the foetus

(b). enlargement of uterus, mammary glands and placenta

(c). increase in maternal circulating blood volume

(d). formation of amniotic fluid

(e). storage reserve for delivery and lactation

(f). transfer of amino acids from the mother to foetus.

Apart from supplying the essential nutrients, it also helps to overcome some common problems of the pregnant ladies-

(a). helps relieve heartburn,

(b). lack of appetite,

(c). constipation,

(d). gastric ulcers,

(e). anaemia.

In the next stage lactating women require a high protein, high calorie balanced diet. The newborn depends entirely on milk for first few months of his life and the quality and quantity of milk is dependent on mother's food intake. Milk can help the lactating women to a great extent in getting proper nutrition for herself and her infant. In infancy the infant doubles its weight in six month and his weight becomes three times by the time he is one year old. The requirement for high protein and various other nutrients is easily met by milk.

During adulthood nutrients are required for the purposes of energy, for replacement of worn out tissues and maintenance of body functions. To meet these requirements an adult needs to take at least 250 ml. of milk everyday. In old age, milk is not only required for its nutrients but is also taken because of its buffering action and is best suited to many physiological changes. These include alterations in taste and smell sensations, decrease in saliva secretion, lowered gastrointestinal functioning, loss of teeth and decreased metabolic rate. It is also important to improve the cognitive abilities of the elderly[20] supplying them with important essential fatty acids. Milk acquires an

important and irreplaceable place in the balanced diet and is also equally important in diseased conditions. The nutrient configuration, bland flavor and taste and neutral action of milk makes it therapeutically very important. In gastrointestinal diseases[5] milk products like curd, butter milk and butter are highly preferred. Milk itself is a bland food and it does not irritate the intestinal diet and they can be given to the patients of peptic ulcer, gastritis, colitis.

In mucosa Milk and milk products are indispensable especially in a vegetarian post-surgical and burn conditions where patients require a high protein and high calorie diet for formation of new tissues and replacement of various body fluids milk is very important. In various hepatic diseases like jaundice and infective hepatitis, milk is given in skimmed form to provide the patient with the required high protein, high carbohydrate and moderate fat diet. Patients of Coronary Heart Disease are advised low calorie, low saturated fat, low carbohydrate and normal protein, mineral and vitamin diet. Skimmed milk and its products can help in providing these required nutrients. In some kidney diseases like Glomerulonephritis and nephritic syndrome, proteins are easily passed in urine and there is low serum albumin. High protein and normal balanced diet is advised in these conditions. In diabetes mellitus, a balance diet high in protein (20% of the daily calorie intake) is good for health because it supplies the essential amino acids needed for tissue repair. In patients of NIDDM consumption of protein along with carbohydrate lowers the blood glucose concentrations due to amino acid stimulation of insulin secretion. Milk has a low glycaemic index and is therefore important. In Cancer patients, milk is given as an important part of diet to meet the increased metabolic demand of the diseased, to prevent catabolism as much as possible

and to prevent excessive weight loss. The primary cause of protein-calorie malnutrition in children between ages 1 to 6 years is inadequate and faulty diet. Malnutrition weakens the child immunity and regularly occurring infections make him more malnourished. In disease of Kwashiorkor and Marasmus milk is important to provide good quality protein and calories.

1.2 Milk oligosaccharides and their biological activities

In recent years milk has emerged as prodigious source of new and structurally complex carbohydrates which are promising therapeutic agents evaluating against various disease like AIDS and tumour. Milk of various origins is prescribed for different ailment in old and traditional system of medicine like Ayurveda and Unani system of medicine. A number of oligosaccharides having biological importance have been isolated from milk of different origin like human, goat Buffalo, Donkey etc., that have shown anti-tumor, anticancer, antigenic and immunostimulant activities. There are numerous other beneficial biological effects of glycoconjugates found in milk e.g. lactoferrin, the major iron-binding protein in human milk, appears to function in the process of iron absorption in infants through interaction with a small intestine receptor, fucosylatedglycan's on the carbohydrate chain of lactoferrin are necessary for receptor recognition. A histone rich glycoprotein has been identified in human milk that binds copper and zinc with high capacity. The glycoprotein in human and pig milk containing fucosegalactose, mannose, galactosamine, glucosamine and sialic acid that bind vitamin B12, and ensure its bioavailability. HMFG membrane glycoproteins in breast milk express blood group related determinants primarily on mucin like epithelial membrane

antigens. Breast milk and its glycoconjugates are also important for normal development and function of the biliary system, e.g. human IgA and breast milk stimulate bile duct growth in an animal model, suggesting a possible role in the developing neonate. Milk is a rich source of glycoconjugates such as oligosaccharides and many have been extensively characterized. The oligosaccharides typically contain residues of most of the necessary glycoconjugate sugars, including fucose, sialic acid (N-acetylneuraminic acid. NANA) galactose, manose, N-acetyglucosamine and N-acetylgalactosamine. Human milk oligosaccharides bind to a wide range of lectins on the surface of epithelial cells lining the mouth, oesophagus and stomach and throughout the gastrointestinal system in the new borne baby. This is turn prevents opportunistic infection while the baby's immune system is developing. Oligosaccharides lectin binding has also been used to target therapeutic agents to diseased cells which express high densities of specific lectin on their surface e.g. GalNAc clusters have been used to target antisensenucleotides to hepatocytes to potentially allows treatment of hepatitis A. A trivalent clusters have been utilized in approach because they are able to able to bind to the required lectins as strongly as galactose terminated multiantenary oligosaccharides isolated from milk has also been used as lead compound for various anti-infective and antiadhesive drugs e.g. bacterial infection caused by the MS fimbrial such as E.coli015H7 and certain salmonella strains oligosaccharide receptors in vivo that contain mannose units. Hence anti-infective oligosaccharides containing mannose could potentially inhibit the infective process. A large number of heterogeneous N containing oligosaccharides of various sizes have been isolated and characterized from milk. All the milk oligosaccharides terminate with lactose or N-actety1 lactosamine at the reducing end and can be classified as acidic or neutral based upon the presence or

absence of NANA. Many milk oligosaccharides contain the basic lacto N tetraose sequence (Gal-GlcNac-Gal-Glc) or one of its derivatives, which has been shown to be a particularly potent bifidus factor. In addition to this NANA may play an important development role in early infancy. Studies in rats have demonstrated that significant amount of brain ganglioside synthesis occurs during the suckling period and that the absolute amount of NANA in brsingangliosides can be modified by delivery availability of NANA.

Human Milk N-Acetyl Neuraminic acid content at different lactational intervals

Lactational Stage (weeks)	**NANA (mg/dl)**	**NANA-nitrogen (mg/dl)**	**%NPN**
0-2	113+8.6	5.16	10.9
2-4	70+7.9	3.20	7.3
4-6	34.4+6.3	1.58	4.0
6-8	25.8+3.4	1.17	3.0
10-28	13.5+1.6	0.61	1.5

Table1.1

Supply of NANA from human milk, which can be hydrolyzed by neuraminidase in the intestinal epithelium, may contribute significantly to glycoprotein and glycolipid synthesis in young mammals especially if NANA synthesis is not yet fully developed. Brain ganglioside development in humans occurs predominantly in third trimester and during the initial postnatal period suggesting that the nutritional availability of high levels of NANA, as found in early breast milk.

1.2.1 Physiological functions and nutritional implications of milk oligosaccharides

Free oligosaccharides are natural constituents of all placental mammals' milk and also found in bacteria, fungi, plants etc. The composition of milk progressively changes post parturition to meet the changing and specific requirements of the suckling neonate. During the first few days post parturition, the "early milk" (colostrums) has a composition quite different from that of "mature milk". Colostrums contain many other biologically active constituents; these include growth factors, antimicrobial compounds and immune enhancing components. The role of milk in these first few days in the life of a newborn is not only to provide nutrition but also to provide protection against infection while the immune system is still developing[6].

Variation of oligosaccharide structure in Human Milk indicates that oligosaccharidesare involved in many functional effects related to the gastrointestinal tracts as well as to systematic processes[7]. There is considerable evidence that oligosaccharide affect intestinal flora and bowel habit. The oligosaccharide might affect mineral absorption[8] and lipid metabolism and end-products of bacterial metabolism can play a role in colon-cancer prevention. Further prebiotic oligosaccharide directly or via, modulating the intestinal flora can influence the immune system[9]. Additionally dietary oligosaccharide influence on the brain development. Oligosaccharide[10]and glycoconjugates in milk have a direct inhibitory effect on certain virulence related abilities of pathogenic microorganisms[11]. Human milk oligosaccharide inhibit monocyte, lymphocyte and neutrophill adhesion to endothelia cells and acts as anti

inflammatory agents. Human milk derived oligosaccharides stimulates cytokine production of cord blood T-cells and exert anti inflammatory properties and also increase immunomodulatory effect.

Recently Human milk oligosaccharide has shown that some oligosaccharide on cell surfaces such as sialyl Lewisx play important role in cell-cell interaction[42]. Artificial mimics of these oligosaccharides are potentially useful for the treatment of inflammation, cancer metastasis, autostasis and autoimmune diseases. So now Human milk oligosaccharides are used for· studying the biosynthesis of antigen I, antigens i. Oligosaccharide present in individual sample of milk can vary with ABO or Lewis blood type of donor as the enzymes involved in their synthesis are also responsible for the formation of structural determinants of these blood types. Immunological study showed that blood group' determinants are expressed in sugar chains and the expression is stronger in mucin type sugar chains than in the asparagines linked sugar chains[12]. Application of a finger printing method to the analysis of milk oligosaccharide led to elucidate the basis of blood types in human[13-14].

1.2.2 Milk oligosaccharides as non-specific defense mechanism

Milk oligosaccharides and glycans are made by the same type of glycosyl transferases that are responsible for the synthesis of human cell surface glycans, so they have common structural moieties[15]. Most enteric pathogens use cell surface glycans to identify and bind to their target cell as the critical first step in pathogenesis therefore soluble glycans from milk act as competitive inhibitors against important pathogens at

the intestinal surface and thereby protect the breastfed infant from diarrhea. Since human milk contains more than 80 oligosaccharides and these milk oligosaccharides are not digested in the small intestine,so a large range of oligosaccharides are available to inhibit pathogen adhesion to the intestinal epithelium. Human milk oligosaccharides inhibit adhesion of Pneumococci and influenza virus to, pharyngeal or bucca epithelial cells and also inhibit binding of streptococcus pneumonia and Haemophilus influenza virus. Similarly, sialylatedoligosaccharides inhibit binding of pathogenic strains of E. coli. In neonates' neutral oligosaccharide from human milk protect the intestinal tract of neonates from Vibrio cholera and fucosylated oligosaccharides inhibit the interaction of an enterotoxin of several viruses with cells of intestine. Sialylated oligosaccharides also inhibit adhesion of ulcer causing human pathogen Helicobacter pylori to epithelial cells[17-19]. Campylobacter is a bacterium causing bacteria induced diarrhea,the main intestinal ligand for this bacterium is the HH2 histo blood group antigens and milk fucosyl oligosaccharide containing α-I-2- linked fucose[20-21]. Thus 'α-I, 2-linked fucosylated glycoconjugates in milk strongly protect against campylobacter infection.

Noroviruses[22] are a major cause of diarrhea in humans and especially in infants. Most milk oligosaccharides contain glycans that specifically block Noroviruses binding to histo blood group antigen receptors which provide protection to infants from noroviruses infection[23]. Human milk oligosaccharides also inhibit adhesion of Neisseria meningitides, a human specific pathogen causing meningitis and septicemia[24]. Thus naturally occurring human milk oligosaccharides are the major components of an innate immune system of human milk that plays a significant role in the ability of the mother to confer protection to her infant against disease[25]. Human milk oligosaccharide have

similar function in non- human species as- the Gal α (1-3) Gal β (1-4) GlcNAC sequence in rabbit glycolipid acts as receptor for the binding of Clostridium difficile toxin A and a trisaccharide Gal α (1-3) Gal β (1-4) Glc found in Bovine, ovine caprine colostrum's, bear and elephant milk are inhibitor of this toxin in intestinal mucosa. Glycolipid containing N-glycosyl neuraminic acid in the form of trisaccharide sequence, Neu 5-Glc α-(2-3) Gal β (1-4) Glc, acts as receptor for binding of E.coli which cause life threatening diarrhea in piglet[26].

1.2.3 Milk oligosaccharides as prebiotic

The human intestine lacks enzymes able to hydrolyze β-glycosidic linkage with exception of lactose. Thus human milk oligosaccharide and most animal milk oligosaccharide as well as non-milk oligosaccharides are considered to be indigestible[27-28]. These dietary non- digestible human milk oligosaccharides reach the colon and are utilized by health promoting colonic bacteria and are known as prebiotic. So prebiotic is non-digestible food ingredients that beneficially affecting the host by selectively affect the growth and activity of bacteria in colon that can improve the host health. Milk oligosaccharides are neither digested nor absorbed in the upper intestinal tract of humans but are delivered intact into the colon where they act as nutrients for colonic microflora. The neutral oligosaccharides present in human milk act as growth enhancers for bacteria of the genus *bifid bactehum* in infants. So the population of bifid bacterium increased in breast fed infants[29-30]. This increased metabolic activity of larger population of bifid bacteria in the lumen and will decrease the intestinal pH and this in turn inhibit proliferation of pathogenic Gram-negative bacteria such as-*Shigella flexneri*

and *E. coli*. Milk oligosaccharides also act as growth promoting factor for *Lactobacillus bifidus*, the predominant intestinal flora of breast fed infants[30-34]. So the incidence of infection in breast-fed infants is reduced in comparison to bottle-fed infants

1.2.4 Immunological Activities Associated with Milk

Milk is a complex biological fluid which has been viewed primarily as a food that provides energy and essential nutrients for optimal health, growth and development of the young mammal. However it is now realized that milk also contains a variety of bioactive components, usually occurring in small quantities, that includes a mix of cells, bioactive proteins, hormones, growth factors, and other immunological factors.

Levels of immunoactive compounds parallel the development of the immune system of the newborn animal, which is functionally immature at birth and undergoes extensive differentiation and recognition during the early postnatal period. For example, levels of complement components, neutrophil activity macrophage activation by interferon-y, production of secretary Ig-A (sIgA), numbers of T cells displaying the CD45RO effector/memory phenotype and production of IgG antibody to T cell-dependent immunogens all are low during early infancy. In addition, relative to adults, newborn infants produce lower quantities of cytokines, e.g., granulocyte/macrophage colony-stimulating factor, interferon-y, interleukin(IL)-3, IL-4, IL-6 and tumor necrosis factor (TNF)- □. Consequently, the capacity of infants to respond to an infectious challenge is low, and provision of immune and immunomodulating factors by way of milk may be critical to the health and survival of the newborn offspring.

This provides functional evidence that infants receiving diets rich in immunomodulating compounds are better suited to protect themselves from infectious agents. An additional consideration is the role that milk-associated immunological factors play in the protection of the mammary gland and ultimately in the mother-infant dyad, itself. Without a protective "food source", i.e., the mother and her milk supply, the young mammal would certainly perish. This multifunctional role of milk-borne immunological factors certainly plays a role in the evolution of mammals. Therefore, not only are immunological active milk components essential for the protection of the suckled newborn, but they are also critical for the protection of the mammary gland and, therefore, for the survival of the species.

Breast-feeding protects human infants against respiratory tract infections, otitis media, botulism, urinary tract infections, and necrotizing enterocolitis[35-38]. Moreover, while environmental exposure of newborns to bacteria, viruses and other micro-organisms is an important concern, it has recently become evident that there is also risk of transmission of human immunodeficiency virus (HIV, cytomegalovirus (CMV) and other viruses through the milk of infected mothers. Hence, the immunologically active components in milk are involved in protecting the young animal against both external pathogens and pathogens of maternal origin.

1.2.5 Milk oligosaccharides as Secretary Antibodies

Human milk contains significant amount of secretary antibodies. When significant amounts of secretary antibodies (sIgA) were found in human milk in 1961,

they were assumed to be the major agents whereby milk protected nursing infants. The very high concentrations of sIgA in colostrums seemed consistent with human milk being a medium through which the maternal adaptive immune system could transmit mucosal protection to the infant gut, augmenting the gift of maternal antibody through prenatal transfer of serum antibodies across the human placenta. This seemed an effective mechanism to protect infants from pathogens to which the mother had prior exposure.

1.2.6 Immunomodulatory effects of milk oligosaccharide

Human Milk oligosaccharide structures like" lacto-N-fucopentaose 111 (LNFPIII) and lacto-N-neotetraose (LNneoT) showed an effect on murine IL-IO production.The human milk derived acidic oligosaccharide fraction 198 is also found to enhance the production of certain cytokines after long-term exposure (20 d) in vitro in the CD4+ as well as in the CD8 + T-cell subfraction[39-40].

1.2.7 Anti-inflammatory effects of milk oligosaccharide

Human milk oligosaccharide show anti-inflammatory property. Excessive leukocyte infiltration causes severe tissue damage in a variety of inflammatory diseases. The initial step in leukocyte extravasations is mediated by selectins and oligosaccharide on their glycoconjugates ligands. The human milk oligosaccharides contain binding determinants for the selectins. They are also able to affect leukocyte rolling and adhesion to endothelial cells under dynamic conditions. 3'-sialyl- lactose and 3'-

sialyll3-fucosyl-lactose present in human milk serves as anti inflammatory components by inhibition of monocyte, lymphocyte and neutrophil adhesion to endothelial cells. Lacto-N- Fucopentaose III (LNFPIII) is a Human milk sugar containing the biologically active Lewis- X (LeX) trisaccharide. LNFPIII LeX is also expressed by immunosuppressive helminthes parasites, by bacteria and on a number of tumor/cancer cells. LNFPIII activates macrophages in vitro' as indicated by up regulation of Gr-l expression on F 4\80 (+)cells and F 4/80 (+) cells is able to activate natural killer cells, inducing up regulation of CD69 expression and gamma interferon production. LNFPIIII stimulated macrophages secrete prostaglandinEinterleukin-10 (IL-10) and tumor necrosis factor alpha. An oligosaccharide fraction isolated from goat milk reduces intestinal inflammation in a rat model of dextran sodium sulfate- induced colitis and contribute to the recovery of damaged colonic mucus [41-43]

1.2.8 Milk oligosaccharides as Tumor Marker

Monoclonal antibodies of several tumor cell lines or carbohydrate antigens have provided evidence that membrane glycoprotein or glycolipid which may function as differentiation antigens or tumor- associate antigens occur as free oligosaccharide in milk. The sialyl- Le structure in glycolipid or glycoprotein has been defined as gastrointestinal tumor associated antigen. Two newly isolated oligosaccharides B-1 and B-2 have both thesialyl Le^a and Le^x or Le-1 structure respectively. These structures have been found in mucin type glycoprotein and glycolipid. In a variety of human cancer Oligosaccharides having the SialylLe^a and blood group H structure or with both

sialyl Le and difucosyl Le-Le structure also occur in milk and Le^a-Le^x structure exhibits high affinity to an antibody directed to a human squamous lung carcinoma.

1.2.9 Milk Oligosaccharides and Brain development

In addition to the biological significance of the unique oligosaccharide composition of human milk, their possible functions are in neonatal host defense and inflammatory events. Oligosaccharides, along with lactose, may play a role in postnatal brain development. Many newborn mammals undergo a period of rapid postnatal brain development that requires large amounts of glycolipid, which are components of the cell membranes of neurons and myelin.

Galactocerebroside, with Gal as its polar head group, is the predominant glycolipid in myelin. The liver may not be capable of providing the entire Gal needed by the young mammal during this period of myelination and brain development. Thus, a possible role of milk oligosaccharides in which Gal is a main component is ensuring that Gal levels in the infant is not become limiting during this time. A prerequisite for this mechanism is that oligosaccharides are not completely excreted via faeces, but are to some extent absorbed in the digestive tract. The conclusion that can be drawn from these studies is that some oligosaccharides were absorbed without being digested. Therefore, one cannot exclude the possibility that beside their local action, oligosaccharides may have systemic effects as well, e.g., on brain glycoconjugate composition. This suggestion was confirmed by comparing the effects of intraperitoneal and intragastric applications of NeuAc on rat brain composition. It was found that both

the oral and intraperitoneal routes resulted in significantly more cerebral and cerebellar glycolipid and glycoprotein NeuAc than did glucose injection. Furthermore, the advantage of oral dose of NeuAc-lactose, the major acidic fraction in human milk, over application of free NeuAc for brain composition in rats has been shown by Witt et al. A further indication that dietary carbohydrates may be important for normal brain composition is the observation that in patients with classic galactosemia, exogenous Gal may be important for the maintenance of a correct ratio' of UDP-Glc to UDP-Gal in some cells. The impairment of UDP-Gal concentration in affected subjects could be, in parts, responsible for the altered biosynthesis of brain glycolipids in these subjects. Because the oligosaccharide pattern in the milk of elephants is even more complex than that of human milk, it is fascinating that the two species show similar patterns of postnatal ontogeny; they grow slowly, have relatively large and highly developed central nervous systems developing mainly after birth, are highly intelligent, and exhibit a high degree of learned behaviour. The degree of cephalisation is considerably higher in humans and elephants than in nonhuman primates (e.g. rhesus monkeys), reflecting the differences in milk oligosaccharide concentrations. Thus, it is speculate that lactose-derived oligosaccharides and in particular their Gal moieties, may play a role in the development of the infant brain. Sialic acid present in human milk also contribute to the increased concentration of NeuAc, present in cerebral and cerebral glycoconjugates of breast fed and thus playa important role in development of the infant brain[44-48].

1.2.10 Effect of milk oligosaccharides on mineral absorption

Human milk oligosaccharides contribute to the high efficiency of Ca absorption of breast fed infants. In adults dietary oligosaccharides also improve Ca absorption.

Fructooligosaccharides are also able to stimulate Calcium absorption. There are also some reports that Magnesium, Iodine and iron metabolism can be improved by dietary oligosaccharide.

1.2.11 Possible Application of Milk Oligosaccharides for Drug Development

By applying a finger-printing method to the analysis of human milk oligosaccharides, several oligosaccharides were found to be deleted in the milk of non-secretor or Lewis negative individual[48]. This finding afforded a clue to elucidate the enzymatic basis of blood types in humans. Furthermore, disappearance of some major oligosaccharides led to the finding of five novel minor oligosaccharides, which were hidden under the major oligosaccharides. Later on, structures of more than seventy oligosaccharides were elucidated. These oligosaccharides are derived from eleven core oligosaccharides by sialylation and/or fucosylation. All these oligosaccharides contain lactose at their reducing end. This evidence, together with the deletion phenomena found in the milk of two blood type individuals, suggested that the oligosaccharides are formed by the concerted action of glycosyltransferases, which are responsible for formation of the sugar chains of glycoproteins on the surface of epithelial cells constructing the mucous membrane. The elongation may start by the action of iGnT.This enzyme is responsible for the addition of a N-acetylglucosamine residue to the C-3 position of the galactose moiety constructing the N-acetyllactosamine group of the sugar chains of glycoconjugates. Therefore, oligosaccharides in human milk may include many structures, starting from the N-acetyllactosamine residues in the sugar chains of various glycoproteins. Many evidences, which indicate that virulent enteric

bacteria and viruses start their infection by binding to particular sugar chains of glycoconjugates on the surface of their target cells, were presented recently. Therefore, milk oligosaccharides are expected to be useful to inhibit the infection of these bacteria and viruses. Studies of human milk oligosaccharides are expected to be useful for the development of drugs, which are effective for protection of babies from harmful intestinal infections and milk oligosaccharides as future anti-adhesion drugs for bacterial diseases[49-52].

1.2.12 Factors Responsible For Biological Activity Of Oligosaccharides

- Oligosaccharide bear structure homology to cell surface glycoconjugates used as receptors by pathogens, thus protecting nursing infants. Human milk oligosaccharide containing**,2- linked fucose** inhibits the stable toxin-producing Escherichia coli in vitro[53-54] and its toxin induced secretory diarrhea in vitro and in vivo. Glycoconjugate found in human milk also inhibit binding by Campylobacter jejuni in vitro and in vivo and also inhibit binding by calciviruses in vitro. Thus specific fucosyl oligosaccharides of human milk have been observed to inhibit specific pathogens. Some important enteric pathogens, for example- rotavirus, are inhibited by human milk oligosaccharide or other glycoconjugates that are not fucosylated. Thus the association described here addresses only one possible set of enteric pathogens that may be inhibited by one family of milk oligosaccharide; other oligosaccharides that inhibit other pathogens are probable. Finally it can be concluded that the family of l,2-linked

fucosylated oligosaccharide, probably in conjugation with other families of oligosaccharide, constitute a powerful innate immune system of milk[55].

- Infection by rotavirus is responsible for much of the diarrhea in infants around the world. The ability of rotavirus to infect MA- 104 cells in culture is inhibited by human milk, and this inhibition is due to a mucin-associated 46 kDa milk glycoprotein named lactadherin. Furthermore, after sialic acid is removed from lactadherin, its ability to inhibit rotavirus is essentially lost, which suggests that the glycan portion of the molecule is responsible for inhibition and that specific terminal sialic acid is required for inhibition. Lactadherin from human milk also inhibits rotavirus (EDIM strain) gastroenteritis in mice[56].

- Due to presence of sialic acid in milk, they serves as anti-inflammatory components and reduce platelet-neutrophill complex formation leading to a decrease in neutrophill B2 integrin expression .While neutral human milk oligosaccharide fraction had no effect. Sialylated human milk oligosaccharide also inhibit binding of pathogenic strains of Escherichia coli and ulcer-causing human pathogen H. py1ori on the other hand neutral human milk oligosaccharide may protect the intestinal tract of neonates from Vibriocholera[57].

- Prebiotic is non-digestible food ingredients that beneficially affect the host by selectively affecting the growth and activity of bacteria in colon that can improve the host health.Milk oligosaccharides are non-digested due to the

presence of β-glycosidic linkage. So this β-glycosidic linkage plays a important role for its prebiotic activity[58-59].

- N-and O-linked oligosaccharide causes the release of histamine and other mediators of the allergenic, response which then lead to the development of allergeic symptoms[60].

- Oligosaccharide mimics containing galactose and fucose specifically label tumour cell surfaces and- inhibit cell adhesion to fibronectin[61].

- Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro flora and fermentation in term infants[62].

- Galactose and sialic acid present in milk oligosaccharide are required for optimal development of the infant's brain.[63]

1.2.13 Other contents of Milk having Biological Activities Antimi crobial Protein and Peptides

Lactoferrin, an iron-binding protein present in human milk at remarkably high concentrations (1-2 g/L or 10-20% of total protein content) and in bovine milk at lower concentrations, has been reported to exert a bacteriostatic effect against *E. coli*, and to be bactericidal against several pathogens to which infants may be exposed, e.g., *Vibro cholerae, Salmonella* and *Staphylococcus*. Recently, a peptide called lactoferrin has been isolated from human and bovine lactoferrins. These peptides contained 47 and 27

amino acids, respectively, and have strong bactericidal effects in *vitro* and in animal models. Lactoferrin is located at the N-terminal end of lactoferrin and does not contain an iron-binding region. Lysozyme is present in breast milk in unusually high concentrations (0.1-0.3 g/L) and is found in bovine milk at lower levels. This enzyme can degrade the cell walls of Gram-position bacteria and has strong antimicrobial activity in *vitro.* Ellison and Giehlrecently have demonstrated that lactoferrin can bind to, and remove, lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, thereby allowing lysozyme access to the underlying proteoglycan matrix to cause lysis. Whether this potent synergism occurs*in vivo* remains to be explored.

Many other proteins and peptides in milk have been reported to exert antimicrobial and/or immunostimulatory actions, although it should be recognized that these activities have been demonstrated only *in vitro,* or in animal models.

Nucleotides

Nucleotides are present in human milk in significant concentrations but, until recently, these compounds have not been added to infant formulas. In animal models, nucleotides have been reported to prevent malnutrition-and starvation-induced immunosuppression, to aid resistance to *Staphylococcus aureus* and *Candida albicans*[141,142], to enhance T-cell maturation and functionand to stimulate gastrointestinal growth and maturation. Recently, Tanaka have shown that individual nucleotides, particularly AMP, can induce apoptosis in human fetal intestine in culture. These authors suggest that AMP, and possibly other nucleotides, may reinforce intestinal mucosal integrity by increasing cellular proliferation and differentiation via enhanced apoptosis.

Immunoglobulin Anti-Idiotypic Antibodies and Hyper immunization

Milk contains high concentrations of antibodies, IgG being the predominant class in cow's milk and sIgA the predominant class in human milk. The sIgA antibodies in milk are known to recognize a wide variety of microorganism that are found in the respiratory tract and intestine, e.g., bacterial pathogens such as *E. coli, V. cholerae, H. influenzae, S. pneumoniae, C. difficile* and *Salmonella*, viruses including rotavirus, CMV, HIV, influenza virus and RSV, and yeasts such as *Candida* albicans.

Immunomodulating Agents including Anti-Inflammatory Components: Cytokine and Non-Cytokine Factors

Milk contains a wide variety of cytokines, some of which are found in concentrations that suggest physiological activity. The discovery of cytokines in milk opened new possibilities for effects of breast milk components on diverse cellular targets. Cytokines are polypeptides that act in an autocrine, paracrine or endocrine fashion by binding to specific cellular receptors, thereby affecting cell protein synthesis and/or other cellular activities. Human milk was early found to be anti-inflammatory as it protected against infection in the absence of clinical evidence of inflammation. One possible explanation for this is that human milk contains soluble cytokine/chemokine receptors and receptor antagonists. For example, breast milk at all stages of lactation contain soluble TNF-α receptors I and II, as well as IL-I receptor antagonist.

The Role of Milk Secretory IgA in Protecting Infants from Bacterial Enteritis

In most of the world diarrhea is the leading cause of death in infancy. The risk of bacterial enteritis include acute life-threatening fluid losses, renal failure, and impaired growth. Exposure to bacterial enteropathogens varies geographically. In areas where sanitation is poor variety of pathogens occurs at high frequency. In more developed countries a limited range of pathogens is found with only organisms easily spread direct contact or through contaminated food being common. Milk is generally believed to provide the suckling infant with protection from bacterial enteritis.The basis for this defense mechanism is complex. Breast-feeding serves as an uncontaminated source of food so that there is decreased exposure to enteropathogens. It contains pathogen-specific Secretory IgA (sIgA) and non-specific protective factors including cell receptor analogs (glycolipids, glycoproteins, oligosaccharides), and anti-infectives such as lactoferrin and lysozyme. Breast-feeding lowers stool pH so that acid-intolerant enteropathogens fail to thrive in the intestinal milieu. The best studied of the milk factors is sIgA

Vibrio cholera

Cholera is a clinical syndrome caused by intestinal infection with specific serotypes of Vibrio cholerae in which severe, often life-threatening, water and electrolyte losses occur in stool. In much of the world cholera is a major cause a childhood death. The organism has the ability to cause watery diarrhea because it adheres to intestinal epithelial cells and delivers a toxin, cholera toxin that causes ADP

ribosylation of an adenylate cyclase regulatory protein. Milk has been shown to contain sIgA to choleratoxin and to V.cholerae antigen. The O antigen also called somatic antigen or lipopolysaccharide (LPS) has repeating carbohydrate units linked that from part of the outer membrane lipopolysaccharide of Gram negative bacteria.

Shigella spp.

Shigella spp. infections are among the most serious that occur in infants and toddlers. These infections are characterized by high fever, watery or bloody diarrhea, and toxicity. The clinical features reflect the fact that the fundamental virulence trait of these organisms is their ability to invade the intestinal epithelium.There is evidence that breast-feeding may decrease the risk and severity of illness due to *Shigella.* Clemens related breast-feeding to illness severity and showed that breast-feeding was protective.

Escherichia coli

Breast-feeding has been correlated with protective against diarrhea caused by ETEC. A community-based prospective study by Long *et. al.* in urban Mexican children revealed that strictly formula-fed infants colonized by ETEC-LT were symptomatic with diarrhea nearly three times as often as strictly breast-fed infants and twice as often as infants receiving mixed diet.

Salmonella and Non-Typhoidal Salmonella

Infections with the various *Salmonella spp.* is associated with a variety of intestinal and extraintestinal manifestations including typhoid fever, gastroenteritis, sepsis, and focal infections.Several studies have suggested that breast-feeding protects infants from *Salmonella* infections. Retrospectively, France*et.al.*found that of 253 infants less than 12 months of age who were infected with *Salmonella* (based on microbiology records of three institutions) only one was breast-fed at the time of study and only twelve had ever been breast-fed.

Campylobacter jejuni and C. Coli

Campylobacter spp. are important intestinal pathogens particularly in the first four years of life. They cause both watery diarrhea and bloody diarrhea. IN many places they are among the most common causes of bacterial diarrhea. Some strains are invasive and others produce a heat labile toxin related to cholera toxin. Breast feeding can be protective against these pathogens.

Antimicrobial and antibacterial Actions of Lactoferrin

Human milk provides infants with nutrients, harmones, enzymes and growth factors. These substances contribute to the child's health and offer protection against infectious diseases. Passively transferred components such as IgA antibodies, lysozyme

and lactoferrin exert protective role against a wide variety of microorganisms.Lactoferrin (Lf) is an iron-binding glycoprotein, formerly called red milk protein, first isolated from bovine and human milk. The protein is found in many other secretions such as tears, saliva, pancreatic juice and secondary granules of neutrophils.Lactoferrin is present in the milk of most species. Human milk is particularly rich in Lf, which represents about 20% of total milk protein.A large number of studies has indicated that Lf plays an important role in host defense. In particular, it inhibits antimicrobial activities (including bacteriostatic and in some cases, bactericidal effect) against a wide range of microorganisms.

Antimicrobial Functions of Milk Lipids

Milk is not only a source of nutrients for the newborn but also a source of immunoglobulins and non-immunoglobulins secretary products similar to those of other parts of the secretary immune system, which play an important role in protecting mucosal surfaces from infection. Infants who are breast-fed have been found to have a lowered incidence of gastrointestinal infections then infants fed formula or cow's milk. The incidence of any infection in very low birthweight infants is significantly lower in infants fed human milk than in infants fed formula.

Antimicrobial and Immunomodulating Actions of Milk Leukocytes

Leukocytes, the main cellular component of mammary secretions, include mononuclear phagocytes (macrophages), polymorphonuclear neutrophils (PMN) and

lymphocytes. These cell types normally make up more than 90% of the somatic cells in colostrums and milk. The remaining fraction may contain a varying number of epithelial cells. Leukocytes and epithelial cells in milk are termed body, or somatic, cells to differentiate them from contaminant microbial cells. Milk somatic cells counts (MSCC) range from several hundred to several million cells per ml, depending on the species, the stage of lactation and, above all, the level of inflammation[64]

1.3 Isolation of Milk Oligosaccharides

Besides the protein the other important content of the milk is oligosaccharides starting from lactose which is major constituent of milk and is a disaccharide it also contains a number of branched and liner oligosaccharide chains containing three to ten monosaccharides in them. These oligosaccharide (O.S.) play a definite role in immunological processes and other diseases. Milk of different origin contains different oligosaccharide chains and is used for cure of different ailments in Ayurvedic and Unani System of medicine. For procuring these oligosaccharide contents of different kinds of milk, number of Scientist used different methods for the carbohydrate isolation of the milk which are as under:

1. KOBATA AND GINSBURG METHO

Milk was collected and stored at -20 ^{0} C. It was centrifuged for 15 minutes at 5000 rpm at 4 ^{0} C. The solidified lipid layer was removed by filtration through glass wool column in cold. Ethanol was added to the clear filtrate to a final concentration of

68 % and the resulting solution was left overnight at 0^0 C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^0 C. The supernatant and washings were combined and filtered through a microfilter to remove remaining lactose and lyophilized affording crude oligosaccharide mixture. The lyophilized material (mixture of oligosaccharides) was further purified by fractioning it on sephadex G-25 column using glass double distilled water as eluant at a flow rate of 3 ml/min. Each fraction was analysed for sugars by phenol sulphuric acid reagent for presence of neutral sugar.

2. T. URASHIMA ET AL. METHOD

The milk was thawed and then extracted. The chloroform/methanol 2:1 (v/v) emulsion was centrifuged at 4^o C and 3500X g., the lower chloroform layer and the denatured protein were discarded, the methanol was removed from the upper layer by rotary evaporation, and the residue was dissolved in 2 ml water and freeze dried. The resulting white powder was called the 'carbohydrate fraction'. The carbohydrate fraction from the milk was dissolved in 2 ml of water and the solution passed through a Bio Gel p-2 column (2.5 x 100 cm.). Elution was done with distilled water at suitable flow rate. It was further analysed and purified by HPLC.

3. ISOLATION METHOD BY EGGE ET AL

The frozen milk was thawed and concentrated at 3000 RPM for 1 hr. at 4^oC. The thin layer was skimmed and the aqueous phase was decanted filtered through glass wool

to remove detached fat flakes and other clotted material. The protein was precipitated by adding an equal volume of pre-cooled acetone under vigorous stirring 4^{o}C. After settling over night the clear yellowish supernatant was siphoned off and the sediment was centrifuged at 3000 RPM for 30 min. the combined supernatant was concentrated on a rotary evaporator about 1/10 of its original volume. The major amount of lactose was removed by repeated crystallization, centrifugation and concentration. The final supernatant was applied to a Sephadex G-10 column and eluted with water. Carbohydrate containing fractions were identified. Uncharged oligosaccharides were separated from the sialic acid containing compounds by ion exchange chromatography on a DEAE-sephadex. Anthron positive but Ehrlich-negative fractions were pooled desalted on the Sephadex G-10 columns (35x9 cm) and lyophilized.

4. ISOLATION PROCEDURE BY SMITH ET AL

A crude oligosaccharides fraction from human milk was obtained. One litre of milk was centrifuged (100g) for 15 min at 2^{o}C. The solidified lipid was removed and the solution was clarified by filtration through loosely packed glass wool. Ethanol was added to the filtrate with stirring to a final concentration of 68%. After 18 hours at 10^{o}C the suspension was centrifuged for 15 min at 0^{o}C, and the resulting prepitate was washed twice with 200 ml portions of 68% ethanol at 0^{o}C. The supernatant solution and washing were combined and evaporated to syrup under reduced pressure. The syrup was dissolved in 100 ml of water and insoluble material was removed by centrifugation (1000g) for 15 min at 2^{o}C. The clear supernatant solution was applied to a column of Sephadex G-25 (8x160 cm). as previously described for the partial separation of

oligosaccharides by gel filtration. The sialic acid containing fractions were detected with resorcinol reagent, pooled and lyophilized.

1.4 ISOLATION AND PURIFICATION OF MILK OLIGOSACCHARIDES

After obtaining the crude oligosaccharide mixture from the above described methods, the mixture was purified by using different chromatographic techniques starting from old classical methods like thin layer chromatography, column chromatography and paper chromatography. The different Scientists used recent techniques like Gel Chromatography, Pressure Chromatography and High Performance Liquid Chromatography for purification of oligosaccharides chains. In the following paragraphs all the chromatographic techniques used for oligosaccharide purification are described;

1. PAPER CHROMATOGRAPHY

Paper chromatography is the oldest method for separation and qualitative determination of oligosaccharides. This is the analytical technique that can be applied to a large variety of separations. For milk oligosaccharides the descending paper chromatography was performed with the following solvents:

Paper chromatography was performed on Whatman no.1 paper by the descending method for 24-48 h, using following systems-[177]

A- ethyl acetate – pyridine-water (2:1:2)

B- ethyl acetate – pyridine-acetic acid-water (5:5:1:3)

C- ethyl acetate-acetic acid-water (3:1:1)

D- ethyl acetate-1-proponal-water (1:6:3)

E- 2-proponal-water (4:1)

F- 1-butanol-1-proponal-aceticacid-water (1:2:1:1)

Oligosaccharides were located with $AgNO_3$ reagent[178], while oligosaccharides containing sialic acid were visualized with Thiobarbituaric acid (TBA) reagent. The neutral fraction and the slowest moving acidic fraction were eluted from the paper with water and applied separately as streaks on Whatman No. 3MM paper (1mg of sugar per cm.). The papers were than developed with solvent 1 for 10 days; lacto-N-difucohexaose 1 (LND-1) was used as a standard for the neutral fraction and disialyllacto-N-tetraose (DLN) was used as a standard for the acidic fraction. From the paper containing the neutral fraction, N-2 (R_{LND-1}=0.79) and N-3 (R_{LND-1}=0.50) were eluted separately with H_2O. From the paper containing the acidic fraction, S-5 (R_{DLN-1}=0.77) and S-6 (R_{DLN-1}=0.61) were eluted separately with water and lyophilized. The hexasaccharides, LND-1, runs faster than other oligosaccharides indicated that the separation is sensitive not only to chain length but also to the stereochemistry of the compound.

2. THIN LAYER CHROMATOGRAPHY[65]

Thin layer chromatography is the useful technique for the isolation and purification of natural products. The resolution depends upon the polarity of the solvent. Therefore for better resolution the suitable solvent system should be taken. From

nonpolar single solvent system to highly polar three solvent systems are taken for the Thin Layer Chromatography. The high retention time place an important and decisive role for the purification of natural products. The thin layer chromatography is limited to less polar compounds and it is not very effective for the isolation of highly polar compounds like oligosaccharides etc., however it could be useful after the derivatization of oligosaccharides. The polarity of oligosaccharides could be reduced by converting them into their acetylated form for better resolution on the TLC. In the absence of HPLC this technique could be used as an alternative.

4. ION EXCHANGE CHROMATOGRAPHY

The Ion-exchange chromatography was developed in the early 1950's. This chromatography is based on the principle of attraction between oppositely charged particles. Cation and anion exchangers are used for separation of negatively and positively charged molecules and are called acidic ion exchangers and basic ion exchangers respectively[177]. The neutral and sialylated oligosaccharides of human milk are partially separated by ion exchange chromatography on DEAE-cellulose (Diethyl aminoethyl cellulose) in acetic acid-pyridine buffers, pH 5.4) This is an easier method for large scale isolation of individual sialylated oligosaccharides. It may be purified without the necessity of preparative high voltage paper electropheresis. The mixed sialylated oligosaccharides were purified on DE 52 column using 0.002 M acetic acid-pyridine buffer, pH 5.4 previously equilibrated with the same buffer. For removal of neutral oligosaccharides the column was washed with starting buffer and the sialylated oligosaccharides were eluted with 0.012 M and 0.060 M acetic acid-pyredine buffer at

pH 5.4. Fractions were detected by phenol-sulphuric acid. Oligosaccharides fractions were electrophoresed for 1 hr at 45 V/cm in water-pyridine-glacial acetic acid (3870:30:11:5) (pH 5.4) or chromatographed for 3 days in ethyl acetate-pyridine-acetic acid-water (5:5:1:3). Papers were stained by alkaline $AgNO_3$reagent

5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Recent advances in separation techniques like HPLC have provided new possibilities for purification. HPLC is an alternative method to paper chromatographybecause of its speed of performance and high resolution. The high performance liquid chromatography has replaced the all traditional systems. This chromatographic technique is popular due to its wide applicability, sensitivity. HPLCis commonly used at the last step in purification processes. This technique also helps in the identification of oligosaccharides. HPLC further deals with the normal phase HPLC, reverse phase HPLC, HPAE, Gel filtration etc. The following sequence is followed for better resolution and yield.

1. **Better choice of HPLC system:**The separation of different compound depends on different chemical and physical properties of the solvent. In certain cases, TLC an

2. Analysis of the ample is used as a first indication of the correct operating conditions, silica gel plates for normal-phase column and sialylated silica gel plates for reversed-phase columns.

2. **Optimization of analytical columns of small quantities:**A preliminary analytical search is necessary for the right choice of conditions, which saves time, sample and solvent, required in a HPLC system.
3. **A good analytical HPLC** separation is usually a prerequisite for a successful preparative operation. Relative intention (selectivity, α) is a very important parameter in determining possible sample size and it is necessary to maximize this value.
4. **Scaling of preparative HPLC apparatus:** In many preparative HPLC examples, the column is actually overloaded, nonlinear adsorption isotherm are obtained and peaks are not symmetrical. Scaling-up a successful analytical separation may causeproblems associated with the solubility of the sample. The is especially true for reversed phase HPLC, of the compound under investigation does not dissolve in aqueous solvents. Diluting the sample may help but if the volume injected is too great, separation efficiency decreases. If on the other hand the sample is too concentrated, precipitation on the column may occur. One possible solution is to mix the crushed sample with sorbent and dry pack into a sample column or into a chromatography column itself. Besides all the above-discussed factors choice of specialized column also plays a decisive role in isolation of mixture of compounds.

6. HIGH PERFROMANCE ANION-EXCHANGE CHROMATOGRAPHY (HPAE)

This ion-exchange chromatography is based on the principle of separation of molecules on the basis of their molecular charge. The separation proceeds because ions of opposite

charge are retained to different extents. The use of ion-exchange resins is preferred due to their increased selectivity. Hardy and Townsend first reported separations of neutral oligosaccharides using high-performance anion exchange (HPAE) chromatography with pulsed electrochemical detection. They found that the separations were sensitive to molecular size sugar composition and linkage of the monosaccharide units. Later anion-exchange chromatography at high pH has been shown to be exceptionally useful in separation neutral, sialylatedand phosphorylatedsugars.

High performance anion exchange (HPAE) chromatography under alkaline condition (pH~13) has been used to separate neutral oligosaccharides from human milk. The system used for HPAEC-PAD consisted of Model PAD2 detector (Dionex corp., Sunnyvale, CA). A carbopac PA 1 (4x250) pellicular anion-exchange column equipped with a carbopac guard column was used at ambient temperature. The Dionex eluant degas modulant was employed to sparge and pressurize the eluant with Helium. In these experiments eluant 1 was 200 ml/NaOH, eluant 2 was water, eluant 3 was 15mM NaOH solution. Sample injection was vic-Dionix microinjection valve equipped with a 25-μL sample loop operated by a controlled helium source of 100-120 psi. LNH and mono and difuco LNH were separated by 200ml/NaOH, whereas LNF-1, 2, 3, LNT, LNnT, and LND-1 were separated using 150mM NaOH. All the oligosaccharides alditols and monosaccharides alditols were eluted with 15mM NaOH isocratically. Unless otherwise mentioned the flow rate was 1ml/min. The column was regularly checked by measurements of the retentions times of glucose (4.2 I o.1 min) fructose (4.7 + 0.1 min) and sucrose (9.2 + 0.1 min) eluted with 150mM NaOH at 1 ml/min at ambient

temperature. Detection of the separated oligosaccharides was performed by PAD using a gold electrode.

7. GEL PREMEATION CHROMATOGRAPHY

Gel permeation chromatography is the technique used for the separation of biological macromolecules and polymers. The separation of compounds is based on the size and solutes are eluted in order of decreasing molecular size. Gel filtration with Sephadex has occupied a key position in the purification of thousands of enzymes, oligosaccharides, nucleic acids, proteins and other biological macromolecules. This gel permeation chromatography is useful for obtaining a sample of lacto-N- and lacto-N-neohexose derivatives. For preparative purposes, a large column of Sephadex G-25 roughly fractioned the milk oligosaccharides fraction and fraction was then concentrated and subjected to Bio-Gel P-4 gel permeation chromatography. The presence of a GlsNAc residue is reflected in the behaviour during gel chromatography, since one such esidue behaves, as do two residue of hexose on Bio-Gel P-4, resulting in a higher rate of elution. Bio-Gel P-4 column is superior to Sephadex G-25 column for the separation of milk oligosaccharides in two respects:

(1). Firstly a better group separation can be obtained, and

(2). The N-acetylglucosamine residue behaves as two hexaose units.

The second characteristic of the column is useful for the effective separation of lacto-N-hexaose from lacto-N-difucohexaoses I and II, and also of monofucosyl derivatives lacto-N-octaose and lacto-N-neooctaose from difucosyl derivatives of lacto-

N-hexaose and lacto-N-neohexaose Bio-Gel P-4, resulting in a higher rate of elution. A column (2x1cm) of Bio-Gel P-4 (under 400 mesh) equipped with a water jacket. During operation, the column was kept at 55^{o}C by circulating warm water in the jacket. Differential refractometers R-402 (Water Associates, Inc., Framingham, Mass) was used for monitoring sugars that eluted from the column. Permeation of free sialic acid was not observed when 3'-sialyl lactose was subjected to the chromatography.

7. ELECTROPHORESIS[66]

The biological molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids possess ionisable groups and can therefore be made to exist in solution as electrically charged species, either as cations (+) or anions (-). Similarly even substances like carbohydrates can be given work charges by derivatisation as borates or phosphates. Stefanorich investigated the electrophoresis of a group of carbohydrates (mono-, di-, tri- and polysaccharides) on silica gel G. Borate buffers are used to separate carbohydrates since they produce charged complexes with carbohydrates.A series of fucosyl and sialyl derivatives of lacto-N-hexaose and lacto-N-neohexaose were found to occur in human milk, however, preliminary studies showed that they are all mixtures of isomeric oligosaccharides. However, conclusive results cannot be obtained when too many components are included in one sample. Oligosaccharides Fuc-α-1→2 Gal grouping (H-haptens) were separated easily from other isomeric sugars by paper electrophoresis using borate buffer. High voltage paper electrophoresis was performed in pyridine-acetate buffer pH 5.4 (pyridine-acetic acid-

water) (3:1:387), or in 0.06 M borate buffer, pH 9.5. Sugars were located by radiochromatogram.

8. IMMUNOAFFINITY CHROMATOGRAPHY[67]

Immunoaffinity chromatographic technique is a useful technique in comparison to others since it is theoretically capable of giving absolute purification of even more complex mixtures in a single process. This process is used for separation and purification because of its unique property of extremely specific biological interactions. Monoclonal antibody one-M26 (Igm) was dialyzed extensively against coupling buffer (0.5m Nacl, 0.5m NaOAc 1mM $CaCl_{2,}$ and 1mM $MgCl_{2,}$ pH-7.6), and the protein concentration was adjusted to 0.5-1mg/ml by dilution with the same buffer. Selectispher-10-con A column (10cmX 5mm i.d. and 25cmX 5mm i.d., Pierce) containing concanavalin. A covalently bonded to a 10-µm macroprous silica matrix was mounted in a Dionex 4000i system. The columns were first conditioned with coupling buffer and then loaded by passing the antibody solution through the columns at a flow rate of 0.5ml/min. The amount of antibody bind was determined by comparing the decrease in A280nm of the antibody solution before and after the coupling step. Loaded columns were extensively with coupling buffer and then with running buffer Sodium sulphate plus 0.02M Sodium phosphate, pH-7.5). All samples were isocratically with running buffer at a flow rate of 0.2-0.3 ml/min with column addition of 0.1 M NaOH at a flow rate of 0.2-0.2 ml/min is a mixing tech to provide the alkaline pH required for detection of oligosaccharides in the PAD (same parameters as described above for detection of monosaccharide).Since complete separation of milk oligosaccharides

isomers by paper chromatography or thin layer chromatography is difficult, some workers have been using antibody and lectin-affinity chromatography to separates oligosaccharides with the least structural dissimilarities.Lectin affinity chromatography which separates oligosaccharides based on their stereochemical structure will be useful method to resolve the complex mixture of sialyl and fucosyl derivatives of oligosaccharides chains found in human milk and in oligosaccharides derived from cell surface glycolipids and glycoproteins. Since the immobilized WGA column had a higher affinity for the neutral hexaose derived from the siallyllacto-N-neohexaose, this column may also be useful in the separation of the high molecular weight neutral oligosaccharides, which contain polylactosamine structures.

9. GAS CHROMATOGRAPHY

Gas liquid chromatography is preferred to HPLC in a no. of instances. It is a more sensitive techniques, allowing the analysis of sub-nanomolar amounts of carbohydrates. Detection is usually by means of a flame ionization detector (FID), which responds to all carbohydrate related molecules over an extremely wide linear range. GLC separation is dependent upon the differential extractive distillation of the component in the mixture. It is fundamental to the technique, therefore, that volatile derivatives of the carbohydrates are prepared. Gas-liquid chromatography (GLC) has provided a sensitive, precise, and rapid method for quantitation and identification of monosaccharides, as well as certain di-and tri-saccharides. Combined gas-liquid chromatography-mass spectrometry (g.l.c. m.s), which has increasingly being used for structural and quantitatively analysis, has proved to be a method of choice when

picomole amounts of carbohydrate are to be quantitated. Liberation of components sugars from glycoconjugates by use of aqueous acid catalysis has been considered to be the main cause of loss in carbohydrate analysis.Methanolysis, where the liberated monosaccharides are converted into the corresponding methyl glycosides, results in little sugar decomposition, and makes possible the analysis of sialic acid and uronic acids. Because, simultaneously, amino sugars and sialic acid lose their N-acetyl groups, an N-reacetylation step is employed for good separation of amino sugars in GLC. This, however, restores their marked polarity, and increases their tendency towards adsorption, making quantitative analysis of them in small amounts difficult. G.l.c.-m.s was performed with a combind Hewlett Packard 5710A gas chromatograph/Jeol JMS-D300 mass spectrometer/Jeol JMA-2000 mass data analysis system. Mass spectra (70ev) were recorded with an ion source temperature of 200^0, an accelerating voltage of 3 kv, and an ionizing current of 300μA. After cleavage from the sialyl-lactose, the identify of the sialic acid was established by a Glc-ms micro-technique, using a series of characteristic fragments-ions which indicate the number, type, and position of the O-acyl substituents and the type of N-acyl group in N, O-acyl neumeric acids. The sialyl lactose was treated with dilute acid, and the resulting sialic acid fraction was isolated by ion-exchange chromatography and derivatised with diazomethane and hexamethyldisilazane-chlorotrimethyl silane. In g.l.c. -ms., a main component (70%) having the same retention time (R_{ncu5Ac} 1.18) and the same mass spectrum as the trimethylsilylated methyl ester derivative of 4-0 acetyl-N-acetylneuraminic acid (Neu4, 5Ac2), and a minor one corresponding to the derivative of N-acetylneuraminic acid (Neu5Ac) were detected.

STRUCTURE DETERMINATION

This is the important part, which deals with the composition, constitution and configuration aspects or stereochemistry of the oligosaccharides. Traditionally, carbohydrates structures were determined by chemical degradation/transformation methods which includes methylation or acetylation analysis etc., followed by hydrolysis. Sequential digestions with Exoglycosidases were also used. but in recent years the structure information can re-obtained by chemical methods and other spectroscopic methods such as NMR (^{1}H, ^{13}C and 2D) and mass spectroscopy (EI, ES and FAB), give much information on composition and structure. The various techniques used in structure determination of oligosaccharides are as under.

ACID HYDROLYSIS

Hydrolysis analysis forms a vital part of structure determination of oligosaccharides since it provides the identification and confirmation of constituent's monosaccharides units of the oligomer. In practice different condition of acid hydrolysis i.e. from strong to very mild is generally used depending on the nature of oligosaccharides.

METHYLATION/ACETYLATION ANALYSIS

Permethylation/Peracetylation is the main chemical transformation of the

carbohydrates which is performed to enhance certain analytical objectives i.e. increasing the volatility and hydrophobicity, of carbohydrates. Permethylation is generally carried out by using the sodium hydroxide (NaOH) / methyl iodine or (DMF) / methyl iodide or using Hakomori's procedure and peracetylation by AC2O/pyridine or sodium acetate or zinc chloride with AC_2O.

DIGESTION WITH EXOGLYCOSIDASES

The specificity of the exoglycosidases has also made them useful tools for oligosaccharides sequencing. Judicious use of the enzymes of known specificity can give insight into the glycosylation of purified oligosaccharides. Sequential exoglycosidase digestion, could be combine with the chromatographic (gel permeation or size exclusion) or electrophoretic techniques for exploring the "sequence" of an oligosaccharide.

NMR SPECTROSCOPY[68-70]

In this modern era NMR is used as an important tool in the structure determination. Due to revolutionary developments in high resolution instruments, incorporation of computers and introduction of multidimensional NMR spectroscopy is the principal components that have been used in the study of isolated milk oligosaccharides. This non-destructive technique gives information about molecular structures in terms of spectra of various dimensions. NMR spectroscopy has the major advantage of being a

non-destructive and easy to perform method for quick retrieval of important structural and conformational data. NMR spectroscopy has made it more popular amongst chemist. On the basis of the NMR experiments, the information that can be obtained is basically chemical shifts (δ), coupling constants (*J*), and NOEs. Geminal and vicinal scaler coupling constants are normally used for determination of conformations. Combination of ^{1}H, ^{13}C and 2D NMR experiment clearly defined all the aspects i.e. configurational and conformational, of oligosaccharides isolated from milk. It also defines the inter sugar linkages and configuration of glycosidic bonds.

ONE DIMENSIONAL NMR SPECTROSCOPY ^{1}H NMR[71-72]

The introduction of high-field proton NMR spectroscopy has brought improved criteria of precision to the structural identification of the complex oligosaccharides of related structures. This non-destructive method can be used simultaneously to verify the purity of an oligosaccharides separation and to identify the residues, their anomeric configuration and linkage, as well as their position in the oligosaccharides chain.There are several ways to perform a primary structural analysis of monosaccharides, oligosaccharides or polysaccharides by NMR spectroscopy.In case of oligosaccharides the chemical shift of the anomeric proton of various sugars and various methane protons of different sugars are confined to the regions δ 4.2-6.3and δ 3.0-4.0respectively hence it requires expert interpretation of spectra for monosaccharide identification. The anomeric protons of α-glycosidic linkages usually resonate 0.3-0.5 ppm downfield from those of corresponding β-glycoside.The splitting of anomeric doublet depends on the stereochemistry of H-1 and H-2 of that particular monosaccharide. If the H-2 is axial (as

in the case of gluco and galacto stereochemistry), $J_{1,2}$is relatively small (2-4 Hz) for an α-glycosidic linkage, whose H-1 is equatorial.In β-anomers of sugars with gluco and galacto configuration H-1 and H-2 are trans-diaxial which results in a larger (8-10 Hz) coupling constant.In sugars having the manno-configuration such as rhamnose, where H-2 is equatorial the small dihedral angle gives rise to small values of $J_{1,2}$for both α and β-anomers.Vligenthart et al introduced the "structural reporter group" concept, which is based on signals outside the bulk region (3-4 ppm) in the ^{1}H spectra of carbohydrates such as anomeric protons (4.3-5.9 ppm), equatorial protons, deoxy protons such as methyl protons of 6-deoxy sugars at 1.1-1.3 ppm, and those of distinct functional groups such as amides that is methyl singlets of acetomido groups in the range of 2.0-2.2 ppm. This approach is used to identify individual sugars or sequences of residues and can be used to identify moieties or specific sugars and linkage positions. A good starting point for a structural analysis is the anomeric proton chemical shift. Integration of the anomeric resonances offers an initial estimate on the number of different monosaccharides residues present.In case of milk oligosaccharides the anomeric proton resonances are found in the shift range 4.4-5.5 ppm. The remaining ring protons resonances are found in the range 3-4.2 ppm in unprotected oligosaccharides. But in the case of acetylated oligosaccharides, the acetyl group induce a strong downfield shift of the signals of proton linked to hydroxiated carbons. Understandably, the signals of methane protons occur further downfield (4.8-5.5 ppm) than those of methyline protons (~4-4.8 ppm).The resonances of protons linked to the non-acetoxylated carbons at the site of glycosidic linkage and at the ring C-5 occupy the interval between 3.5 and 3.9 ppm.The H-2 signal of 2-acetyl amine-2-deoxy glucose occur in the highest field (δ 2.9-3.5). In contradistinction what is typical for native oligosaccharides, the resonances of

the anomeric protons of their peracetylated derivatives do not occur separately at the low field end of the spectrum but rather are intermingled with the methane and methyline resonances. The sites of glycosidic linkages can unmistakably be recognized by the dramatic upfield shift of the signals of protons at those sides exceeding several times other shifts induced simultaneously. In summary, the above findings show that proton NMR spectroscopy of peracetylated oligosaccharides is an effective technique for elucidation of their primary of other complex carbohydrates seems recommendable.The ^{1}H NMR spectra show the typical features of reducing oligosaccharides ending in lactose. The reducing Gle residue is characterized by the H-1 signals for its α and β anomers at δ5.221 ($^{3}J_{1,2}$3.7 Hz) and 4.668 ($^{3}J_{1,2}$ 8.0 Hz) respectively, in the intensity ratio of 7:10. The Gal moiety (δ H-1 4.434; $^{3}J_{1,2}$8.0 Hz) of the lactose unit. Chain.4-substituted reducing glucose is evident from the signals of H-1 at 5.219 ppm for the α anomer and at 4.663 ppm for the β anomer and H-2 β at 3.281 ppm.Normally the α anomer resonates downfield compared to the β anomer in D-pyranoses in $^{4}C_{1}$ confirmation. A doublet due to H-2 of the β anomer of the glucosyl residue may be assigned by selective decoupling.The methyl protons of C-6 resonate near 1.2 ppm with the precise chemical shift being governed by the linkage of the α-fucose residue. Similarly the fucose H-5 and H-1 resonances are also sensit6ive t6o linkage positions.The chemical shift values are sensitive to conformational changes due to long through space contacts or crowded situations at highly substituted residues. Upon substitutions, the signals from the substituted residue will be affected.2-Acetamido-2-deoxyglucose, glucose and galactose and reduces were identified on the basis of their coupling patterns, which are characteristic of the stereochemistry of the type of carbohydrate.Besides these informations, presence of some other moieties like

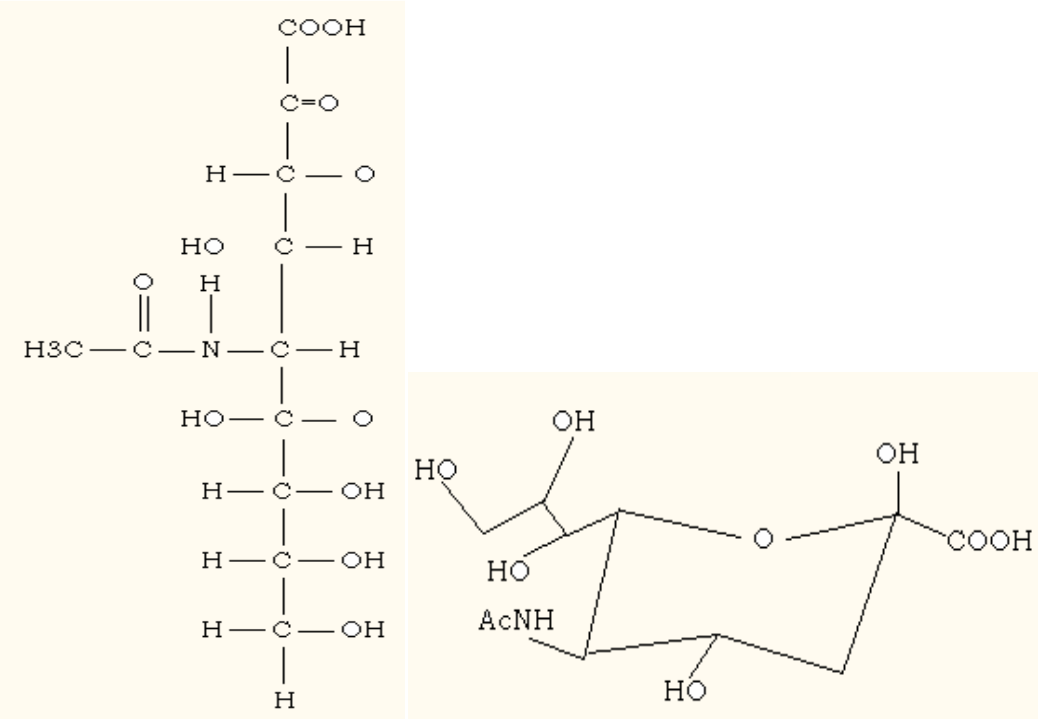
sialic acid residues with the oligosaccharide units can be ascertained by the ^{1}H NMR spectroscopy. The presence of sialic acid residue could be ascertained by the characteristic resonances of H-3 axial and equatorial protons of sialic acid at &1.78 and & 2.75 respectively. Besides the other signals of methane protons which appear between & 3.52-3.90.The difference Nuclear Overhauser Effect (NOE) measurements can decisively prove the position of attachment of sugar moiety of milk oligosaccharides. Assignments of the anomeric and some of the other proton resonances are confirmed with data obtained from decoupling and NOE experiments. An effective way of knowing the glycosidic linkage between two monosaccharide residues is by monitoring the Nuclear Overhauser Effect (NOE) from signal for an anomeric reporter group to the hydrogen of the substituted position in the adjacent ring.

^{13}C NMR

The ^{13}C NMR acts as an important tool in the structure determination of the isolated oligosaccharide. ^{13}C NMR Spectra not only give information of the anomeric configuration of the carbohydrate residues but also provide information of the composition of monosaccharides, their sequences and the overall conformation. The number, sequence and linkage of a sugarcould by assigned by the ^{13}C NMR data and identify of monosaccharide has also been established by the comparison of chemical shift of anomeric carbon with the reported values. The ^{13}C chemical shifts are quite informative in the structural elucidation of homologous compounds. The carbon carrying substituent normally shows a downfield shift to a higher ppm values. The magnitude of the shift depends on the stereochemistry around the glycosidic bond and

the type of monosaccharide involved. The presence of number of monosaccharide units in milk oligosaccharide could be assigned by counting the anomeric carbons present in the ^{13}C NMR of that particular compound. The anomeric signals generally appear between the range of & 95-110.The nature of the monosaccharide unit could be determined on the basis of the chemical shift of the anomeric carbons. It also provides regarding the nature of glycosidic linkages.The signals due to β -linkages usually appear 2.0-6.0 ppm downfield from their α-counterpart.The stereochemistry produced by the glycosylation is an important factor in determining the chemical shift of he axially substituted β-carbon atom. The C-2 atoms adjacent to the linkage position give resonances that undergo a small (0.2ppm) upfield shift. The resonances of $–CH_3$ of 6-deoxy sugars, methoxy functions, $-CH_2OH$ of normal hexoses and ring carbons generally appear in the region δ 16-19,δ 55-62,δ 60-64 and δ 65-85 respectively. Typically a carbon substituted by a hexose or an Nacetylhexosamine is shifted downfield by 6-10 ppm, which is twice as much as for a substituent of an α-linked Neu5Ac (3-4ppm). Carbons adjacent to a substitution position usually show an upfield shift by 2-4ppm. The presence of sialic acid residue could also be well determined by ^{13}C NMR spectroscopy. [125] The anomeric signals (C-2) appear at δ 100-101 ppm while signals for-COOH group appears δ 174 ppm. In ^{13}C-NMR spectra, substitution at a primary or secondary carbon by α Neu5 Ac will desgield the carbon and shift the signals by 2-3.5 p.p.m, which is less than half the value obtained by substitution with a neutral monosaccharide residue. ^{13}C NMR data show symmetry in the molecule with the identical shifts for the NeuAc α2-6 Gal sequences. The downfield shifts of C-4 of the GlcNAc residues (81.3 respectively 81.6 ppm) verify the NeuAc α2-6 Gal β 1-4 GlcNac sequence exemplifying the long-range effect of about 3 ppm field shift for C-4 of the

GlcNAc residue.The other important signals of sialic acid are given in the following table.



N-ACETYL NEURAMINIC ACID[208]
(5-amino-3, 5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid)

Table
CHEMICAL SHIFTS OF SIALIC ACID IN ^{1}H AND ^{13}C NMR

Sialic acid residue		Chemical shifts of NMR	
		^{1}H	^{13}C
A-Neuraminic-5Ac	1	-	174.0-174.6
	2	-	100.2-101.0
	3ax	1.693-1.801	40.5-41.0
	3eq	2.668-2.762	-
	4	3.56-3.68	69.0-69.3
	5	3.79-3.85	52.5-52.7
	6	3.63-3.71	73.3-73.7
	7	3.55-3.65	69.0-69.3
	8	3.86-3.90	72.5-72.7

	9	3.64	63.3-63.9
	9’	3.87-3.88	-
	C=O	-	175.7-175.8
	CH_3	22.8-22.9	2.025-2.038

Table1.3.1

TWO DIMENSIONAL NMR SPECTROSCOPY

The proton resonances of oligosaccharide are too overlapping to be disentangled by homonuclear correlation alone. In such case two-dimensional spectroscopy separates signals with different chemical shifts from their coupling constants and enable the assignment of ^{1}H resonances because in such a spectrum the connectivites between ^{1}H and ^{13}C is observed. The chemical shifts are observed along one frequency axis and the associated spin-spin couplings along the other frequency axis. This technique has made it possible to perform a much more complete analysis of the overlapping signals in oligosaccharides. By 2D NMR we can also measure

- Internuclear distance and
- Scalar coupling constants in molecules, which was not possible by 1D experiment.

CORRELAED SPECTROSCOPY (COSY)

The COSY was invented by Belgian Physicist Jeener and is also known as JEENER experiment and is one of the important experiments of 2D NMR spectroscopy. In 2D COSY experiments both frequency axes contain chemical shifts and spin-spin

coupled nuclei are indicated by cross peaks.COSY experiments and its RELAY extensions not only give shift information but also coupling patterns, which allow each monosaccharide residue to be designated α and β. The coupling patterns of the cross peaks made it possible to distinguish between axial and equatorial hydrogens and thereby determine the anomeric configuration and the identity of each monosaccharide residue.The assignment of the ring hydrogens are readily complished, at least up to H-4, from a relayed COSY experiment. When the shifts and coupling constants of all ring hydrogens are considered, usually sufficient information about sugar identity and substitution pattern is obtained.

In 2D COSY experiments both frequency axis contain chemical shifts and spin-spin coupled nuclei are indicated by cross peaks.The correlation between similar nuclei i.e. either ^{1}H-^{1}H or ^{13}C-^{13}C shows homonuclear shift correlation 2D experiments and termed as HOMOCOSY. The normal NMR spectra are plotted on a two frequency axes and the conventional 1D spectrum appears along the diagonal. The clear representation of 2D NMR spectrum is obtained as contour plots of mutual coupling which exists between two nuclei (^{1}H-^{1}H, ^{13}C-^{13}C), cross peak, appears at the chemical shift coordinates (X, Y) and (Y, X). 2D ^{1}H-^{1}H COSY is the homonuclear shift correlation through J-coupling. After the removal of several artifacts by proper phase cycling and choosing suitable experimental parameters make the COSY spectrum an elegant approach for making connection through bonds.

HETEROCOSY

The correlation of ^{1}H and ^{13}C is known as heteronuclear chemical shift correlation 2D experiments. Since due to the excellent power of decoupled ^{13}C NMR with ease of interpretation of proton chemical shifts HETEROCOSY is considered as one of the important experiments of 2D NMR. The two pairs of ^{1}H and ^{13}C Shifts are likely to be identical only when the chemical environments are similar. In normal use, 2D shift correlation produces an intensity map in which one peak appears for each distinct CH group in a molecule, the frequency coordinates are the ^{1}H and ^{13}C chemical shift frequencies in the F1 and F2 dimensions respectively.The ^{13}C corresponding resonances were reported from the ^{1}H-^{13}C heteronuclear COSY. The assignment of the ^{13}C resonances made by proton carbon correlated experiments are quite informative. The ^{1}H and ^{13}C NMR shift values help in assigning the monosaccharide residues.

Signals for two α–linked Fuc residues of H-1 at 5.031 ppm and 5.150ppm verified their presence, one 1-2 linked to a Gal and another 1-4 linked to a GlcNAc residue.A sequence of Fuc α1-2Galβ1-3[Fucα1-4] GlcNAcβ1- is deducted from the typical spread of shift for H-2, H-3, and H-4 of the GlcNAc residue at 3.848, 4.130 and 3.744 ppm. The fuc linked to the Gal in this branch sequence causes a downfield shift of H-1 of the Gal residue to 4.657ppm. The chemical shift for C-1 of this 3, 4 disubstituted GlcNAc residue at 104.0 ppm locates this fucosylated branch to the 3 and 4 position of the 3, 6 disubstituted Gal residue. The other GlcNAc residue with C-1 at 101.7 ppm and H-1 at 4.66 ppm shows that this residue is linked to the 6-position of the same branched

Gal residue. The ^{13}C long range effects from sialic acid deshield C-4 of this GlcNAc by 2.4ppm is seen.

MASS SPECTROMETRY[73]

Mass spectrometry is a technique to determine and confirm the structure of the oligosaccharides. The Electron impact mass spectroscopy was used in the initial stages for the structure elucidation of oligosaccharides but its limitation was that only the lower fragments were observed.Later the field desertion mass spectrometry was introduced due to which this problem was solved but the information obtained from FDMS was limited to the higher fragments only and for complete interpretation of mass spectrometry data chemists have to couple the results of EIMS and FDMS. Further there was the innovation of the FAB mass techniques that gave complete information regarding the lower and higher mass fragment into one spectrum.

FABMS

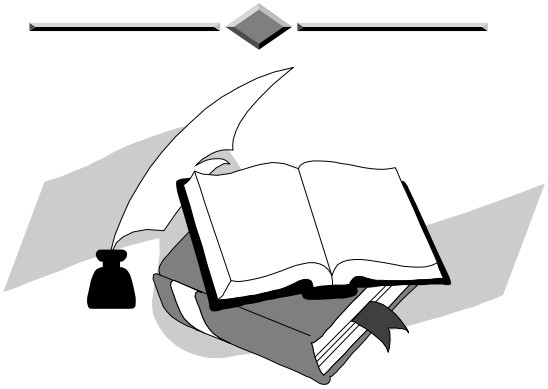
In FABMS and abundant molecular ion, or its protonated species ($M+H^+$) or a cationic species ($M+Na^+$), ($M+K^+$) is obtained. It plays a decisive role in the structure elucidation of milk oligosaccharides. Recently it has been seen that the FABMS not only fixed the molecular weight of the oligosaccharides but also ascertained the sequence of the monosaccharide units. The molecular ion was fragmented into the fragment units were formed by the decomposition pathways in which repeated H

transfer in the oligosaccharides is accompanied by the elimination of terminal sugars less water,such fragmentation goes on until the monosaccharide is left. Negative ion fast atom bombardment-mass spectrometry has been an important tool in the structure elucidation of milk oligosaccharides and the results have also been found to be consistent with structures proposed on the basis of the results of 500 MHz1H NMR spectroscopy. The presence of acetamido containing monosaccharides, a fucosylated and substituted fucose branching could be identified by FABMS.The molecular weight is determined from the from the protonated molecular ion, (M+1). The monosaccharide sequence, from the non-reducing terminal, is deduced from the primary sequence ions, which are formed by cleavage of the glycosidic bonds. The most abundant sequence ions are formed by cleavage of 2-acetamido-2-deoxyhexosy1 (HexNAc) linkages. Secondly fragments, formed by eliminations, determine the positions of substitution of the HexNAc residue. These secondary fragments are preferentially formed by elimination of the substituent in the 3-position of the HexNAc residue-1. The secondary ions m/z 196 and m/z228, which always are present in HexNAc containing oligosaccharides, are results of eliminations from the 3-and-4 positions of the HexNAc residue. Sequences ions, however less abundant, from the reduced terminal are also seen. These ions are formed by cleavage of the bond between a hexose or deoxyhexose and a HexNAc residue with charge retention at C-3 or C-4 of the HexNAc residue.Negative ion FABMS of milk oligosaccharides gave molecular ions (M-H)FABMS has also been found to be very useful in assigning the monosaccharide sequence and some linkage positions. In the analysis of peraxcetylated oligosaccharides mass spectrometry using FD or FAB proved to be highly efficient.The spectra of peracetylated oligosaccharides shows pseudomolecular ions M+Na and $M+H^+$ together

with daughter ions formed by the elimination of one or two CH_2o=C=O units, i.e. 42 or 84 m.u. Excellent FAB spectra could be obtained with 1-mercapto-2,3- propanediol and the addition of sodium acetate to the target. Besides very intense pseudomolecular ions $M+Na^+$ and $M+K^+$, often constituting the base peak, Fragment ions characteristic for the carbohydrate constituent are also present. The exact carbohydrate composition of each fraction can be determined with hitherto unknown precision from the molecular of pseudomolecular ion. This rule is true for permethylated derivatives and for native oligosaccharides can be analyzed by FAB-MS preferably in the negative ion mode. In comparision to conventional methods of determination of carbohydrate constituents, this method is highly reliable, sensitive, and specific.

CHAPTER II

MATERIALS AND METHODOLOGY



CHAPTER II
MATERIALS AND METHODS

2.1 ISOLATION AND METHODOLOGY

The basic aim of this study was to know the nutritional constituents and biological activities of various kinds of milk. For this purpose we have selected seven milks of following animals

1. Cow Milk
2. Buffalo Milk
3. Sheep Milk
4. Camel Milk
5. Chauri Milk
6. Goat Milk
7. Mare Milk

For ascertaining the nutritional constituents, the protein, fat, lactose, oligosaccharide and total soluble sugars of these various milks were estimated. For the estimation of protein, fat, lactose, oligosaccharide and total soluble sugars, the following methodologies were used

1. Micro Kjeldal's Method for protein estimation.[74]
2. Rose Gottlieb method for fat estimation.[75]
3. Lane Eynon's Method for fat estimation.[76]
4. Ferricynide Method for estimation of total soluble sugar.[77]

5. Kobata and Ginsberg method for estimation of oligosaccharide.[78]

Determination of milk protein by Micro-Kjeldahl's method

Principle

The amino nitrogen in various nitrogenous compounds is converted to ammonium sulphate on digestion with concentrated sulphuric acid in presence of K_2SO_4 and $CuSO_4$. K_2SO_4 is included in digestion mixture to raise the boiling temperature whereas $CuSO_4$ acts as a catalyst. Alternatively, selenium dioxide can be used as a catalyst. On distillation of the digested sample with NaOH, NH_3 is liberated and trapped in boric acid containing a mixture of bromocresol green and methyl red as an indicator. Ammonia reacts with boric acid to form ammonium borate which is then estimated volumetrically by titrating against standardized HCl and the amount of nitrogen is determined. Since proteins contain about 16% nitrogen, the protein content of the sample is calculated by multiplying its nitrogen content by 6.25.

Sample Preparation– 100 ml. milk sample was taken. 15 gm K_2SO_4 and 1 ml $CuSO_4$. $5H_2O$ solution was added to it. K_2SO_4 acts as boiling point elevator to release nitrogen from protein and retains nitrogen as ammonium salt. $CuSO_4$. $5H_2O$ acts as a catalyst. The milk was warmed at 38 ^{o}C and was placed in digestion tube. 25 ml H_2SO_4 was added to it and the solution was digested on heater till the solution became clear. After cooling it was transferred to the volumetric flask. The digestion flask was rinsed several

times with small amounts of water and the washings were poured into the volumetric flask.

Procedure

1. 1 gm of sample was taken in a long necked digestion flask. 10 ml of Conc H_2SO_4 and 200 mg of catalyst mixture was added and the sample was digested on heater till the solution became clear. After cooling it was transferred to 50 ml volumetric flask. The digestion flask was rinsed several times with small amount of water and the washings were poured into the volumetric flask. Finally the volume was made to 50 ml with distilled water.
2. 10 ml of boric acid solution was taken in 100 ml conical flask. The receiving flask was placed in such a way that the condenser of Micro-Kjeldahl's distillation apparatus dips into the boric acid solution.
3. 5 ml of digested sample was transferred to the steam chamber of Micro Kjeldahl's apparatus. 6 ml of 40% NaOH was added to the aliquot of the digested sample. Immediately the stopcock was closed and the steam was passed through the steam chamber to distill ammonia till about 30-40 ml of distillate was collected in the receiving boric acid containing flask.
4. The receiving flask was removed and the condenser outlet tip was rinsed into the receiving flask with water.
5. The contents of receiving flask were titrated against 0.01 N HCI till the bluish green colour changes to pink.
6. A blank preparation was run which was identically prepared except that it did not contain the sample.

Calculations: According to the findings of the titration, the calculations were done as follows.

1 ml of 0.01 N HCI = 0.00014 g nitrogen.

Since average nitrogen content of most proteins is 16%

I g of nitrogen = 100/16 g of protein.

Volume of 0.01 N HCI used for blank = *v* ml.

Volume of 0.01 N HCI used for sample = *y* ml.

Titre volume of sample = *y* - *u* ml

Vol. of sample taken for distillation = 5 ml.

Total volume made of the digested sample = 50 ml.

Nitrogen present in 5 ml of digested sample = 50 ml.

Nitrogen present in 5 ml. of digested sample = (*y* - *u*) 0.00014 g

$$\text{Nitrogen present in 50 ml digested sample} = \frac{(y\text{-}v)\ x\ 0.00014\ x\ 50\ g}{5}$$

$$\text{Nitrogen present in 1 g sample} = \frac{(y\text{-}v)\ x\ 0.00014\ x\ 50\ g}{5}$$

$$\text{Amount of nitrogen present in 100 g sample} = \frac{(y\ -v)\ x\ 0.00014\ x\ 50\ x\ 100\ g}{5}.$$

$$\text{\% Protein content in sample} = \frac{6.25\ x\ (y\ -v)\ x\ 0.00014\ x\ 50\ x\ 100\ g}{5}.$$

2.1.2 Rose Gottlieb Method for Estimation of Fat Content

The milk sample was treated with ethanol and ammonia. The separated fat was extracted with combination of mixed ethers. Ammonia helps soften the curd of milk and alcohol breaks of milk emulsion and separates fat from proteins. It also enhances

contact between solvent and fat. The petroleum used, reduces solubility of alcohol and solvent ether in water. The extracted fat was weighed. The extraction was carried out in a Mojoinner tube. Ammonia also neutralizes the free acid of the sample which would be extracted by ether. The petroleum ether decreases the solubility of milk, sugar and other non-fat solids soluble in ether.

5 gms. of the prepared sample was weighed and put into an extraction tube. The sides of the tube were washed with 2 ml hot water and mixed by gentle swirling. 2 ml concentrated ammonia was added and mix thoroughly. The tube was heated on a water bath for 20 minutes at 60^{o}C with occasional shaking. 10 ml alcohol was added and mixed. Mixture was transferred to a separating funnel. In a beaker 25 ml ether and 5 ml petroleum ether were rinsed and added to the funnel.

After each reagent addition shaking was done for 5 minutes, till a clear upper layer was obtained. The ether layer was transferred into a tared flask. The extraction tubes were washed with 1:1 mix of titre solvents and added to the flask. The liquid in the separating funnel were re-extracted twice and collected into a tared flask. The solvent was distilled on a hot plate or a steam bath at 60^{0}C. The residue of fat was dried in an oven at 100^{0}C. The flask was cooled and weighed. The fat was removed in the flask with 15-20 ml petroleum ether, then it was dried and weighed as before.

Loss in wt. of flask = wt. of fat

$$\text{Percent fat} = \frac{100[(\text{wt. of flask} + \text{dry fat}) - (\text{wt. of empty flask})]}{\text{wt. of sample}}$$

2.1.3 Lane Eynon's Method for Estimation of Lactose Content

10 gm of milk was accurately weighed and transferred into a 250 ml volumetric flask with 50 ml water. 5 ml. each of $K_3Fe(CN)_6$ and Zinc Acetate were added as clearing agents. The volume was make up to 250 ml with distilled water and filtered. Fehling's Solution was titrated against this solution by Lane Eynon's method.

1 ml. of Fehling's solution = 0.00645 gms. of anhydrous lactose.

Isolation of milk oligosaccharides by Kobata and Ginsberg Method

1000 ml of milk was collected and stored at -20^0C. It was centrifuged for 15 minutes at 5000 rpm at 4^0C. The solidified lipid layer was removed by filteration through glass wool column in cold. Ethanol was added to the clear filtrate to a final concentration of 68 % and the resulting solution was left overnight at 0^0C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^0C. The supernatant and washings were combined and filtered through a microfilter to remove remaining lactose and lyophilized affording crude oligosaccharide mixture. The lyophilized material (mixture of oligosaccharides) was further purified by fractioning it on sephadex G-25 column using glass double distilled water as eluant at a flow rate of 3 ml/min. Each fraction was analysed for sugars by phenol sulphuric acid reagent for presence of sugars.

2.1.4 Ferricyanide Method for Estimation of Total Soluble Sugar

To prepare the milk sample 1 litre milk was taken, 500 ml of alcohol was added to it. Further it was filtered through Watman No.1 filter paper. The supernatant was concentrated in Rotary Evaporators. Further 1 gm of this sample was suspended in 40 ml of distilled water and heated on boiling water bath for 30 minutes. It was then centrifuged at 3000 rpm for 20 minutes. The supernatant was collected. 2 ml of saturated lead acetate was added to this extract. Proper mixing was done for 15 minutes and the extract was filtered through fine filter paper. The volume was made upto 250 ml by adding distilled water. Excess of lead acetate was then precipitated with solid calcium oxalate.

For estimation of total soluble sugar 5 ml of this extract was taken in a test tube and 5 ml of potassium ferricynide was added to it. Excess of lead acetate was precipitated out with solid sodium oxalate. For estimation 25 ml of this extract was hydrolized with 5 ml of concentrated Hcl for 8 minutes at 68^0 C. After cooling the hydrolysate was neutralized with solid sodium carbonate and the volume was made up to 100 ml with distilled water. For estimation 5 ml of this extract was taken in a test tube and 5 mls of potassium ferricynide was added to it. It was heated in a boiling water bath for 15 minutes and then cooled. 5 ml of iodine-solution was added to this extract followed by 3 ml of 5% glacial acetic acid. The excess iodine was titrated against 0.01 N $Na_2S_2O_3$ till the colour of the solution turned pale yellow. After that starch indicator solution was added upon which the colour changed to blue. The titration was completed till disappearance of blue colour. For estimation purposes once a blank was also run taking water instead of sugar extract. Volume of $Na_2S_2O_3$ used for the sample is deducted from that consumed for the blank.

Calculations

The amount of total soluble sugars is calculated from the following relationship:

mg of total soluble sugars in 5 ml of sample extract=u (x + 0.05)

Where

μ = 0.338

x = vol. of 0.01 N na2 S2O3 used for sample, i.e. vol. of $Na_2S_2O_3$ used in blank-vol. used in sample.

COW

Classification

Kingdom- Animalia
Phylum Chordata
Class Mammalia
Order Bovidae
Family Bovinae
Genus Bos
Species Bos indicus

2.2 Estimation of Protein Content of Cow's Milk byMicro Kjheldal's Method

The estimation of protein in Cow's milk was done by Micro Kjheldal's Method. The procedure was same as described earlier. The formula used for estimation of protein was as under:

% of protein is equal to $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$gms.

Sl. No.	Volume of 0.01 N H Cl used for blank in ml. (v)	Volume of 0.01 N H Cl used for sample in ml. (y)	N_2 present in 1 gm. digested sample $\frac{(y-v) \times 0.00014 \times 50}{5}$	N_2 present in 100 gm. Sample $\frac{(y-v) \times 0.00014 \times 50 \times 100}{5}$	% Protein content in sample $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$
1.	2.18	6.63	0.0062	0.56	3.9
2.	2.18	6.52	0.0060	0.60	3.8
3.	2.18	6.63	0.0062	0.62	3.9
4.	2.18	7.09	0.0069	0.69	4.3
5.	2.18	6.75	0.0063	0.59	4.0
6.	2.18	6.63	0.0062	0.62	3.9
7.	2.18	6.98	0.0067	0.67	4.2
8.	2.18	6.96	0.0067	0.64	4.2
9.	2.18	6.86	0.0065	0.65	4.1
10.	2.18	6.53	0.0060	0.57	3.8
				Maximum Value	**4.3**
				Minimum Value	**3.8**
				Mean Value	**4.1**

Table 2.2

2.2.1 Estimation of Fat Content of Cow's Milk by Rose Gottlieb's Method

The estimation of fat in Cow's milk was done by Rose Gottlieb Method with the procedure described earlier. The formula used for estimation of fat was

Table 2.2.1

$$\text{Percent fat} = \frac{100[(\text{wt. of flask} + \text{dry fat}) - (\text{wt. of empty flask})]}{\text{wt. of sample}}$$

Sl. No.	**Weight of empty flask (A)**	**Weight of flask + dry fat (B)**	**Weight of Fat (B - A)**	**Percentage Fat** % Fat = 100 [(wt. of flask + dry Fat) – wt. of empty flask] / wt. of sample
1.	250.000	250.273	0.273	5.46
2.	250.000	250.283	0.283	5.66
3.	250.000	250.281	0.281	5.62
4.	250.000	250.283	0.283	5.66
5.	250.000	250.274	0.274	5.49
6.	250.000	250.275	0.275	5.51
7.	250.000	250.277	0.277	5.55
8.	250.000	250.285	0.285	5.70
9.	250.000	250.274	0.274	5.48
10.	250.000	250.286	0.286	5.72
			Maximum Value	**5.72**
			Minimum Value	**5.46**
			Mean Value	**5.58**

Table 2.2.1

2.2.2 Estimation of Lactose content in Cow's Milk by Lane Eynon's Method

The estimation of lactose content in Cow's milk was done by Lane Eynon's Method with the procedure described earlier. The formula used for estimation of lactose content was

1 ml. of Fehling's solution = 0.00645 gms. of anhydrous lactose.

Lactose content = 0.00645 x mls of Fehling's solution used

Sl. No. (A)	Quantity of milk sample (in ml.) (B)	Quantity of Fehling's Solution for 10 ml. of milk sample (C)	Calculated amount of Lactose in 10 ml. of Milk sample using quantity of Fehling solution as shown in column (C) (D)	Calculated amount of Lactose in 100 ml. of milk (E)
1.	10.00	75.90	0.49	4.9
2.	10.00	72.82	0.47	4.7
3.	10.00	71.36	0.46	4.6
4.	10.00	79.10	0.51	5.1
5.	10.00	80.16	0.51	5.1
6.	10.00	74.00	0.47	4.7
7.	10.00	75.30	0.48	4.8
8.	10.00	86.80	0.55	5.5
9.	10.00	77.50	0.49	4.9
10.	10.00	74.44	0.48	4.8
			Maximum Value	**5.5**
			Minimum Value	**4.6**
			Mean Value	**4.9**

Table 2.2.2

2.2.3 Estimation of Total Soluble Sugar in Cow's Milk by Ferricyanide Method

The estimation of total soluble sugar in Cow's milk was done by Ferricyanide Method with the procedure described earlier. The procedure was same as well as stated before. The formula used for estimation of total soluble sugar was

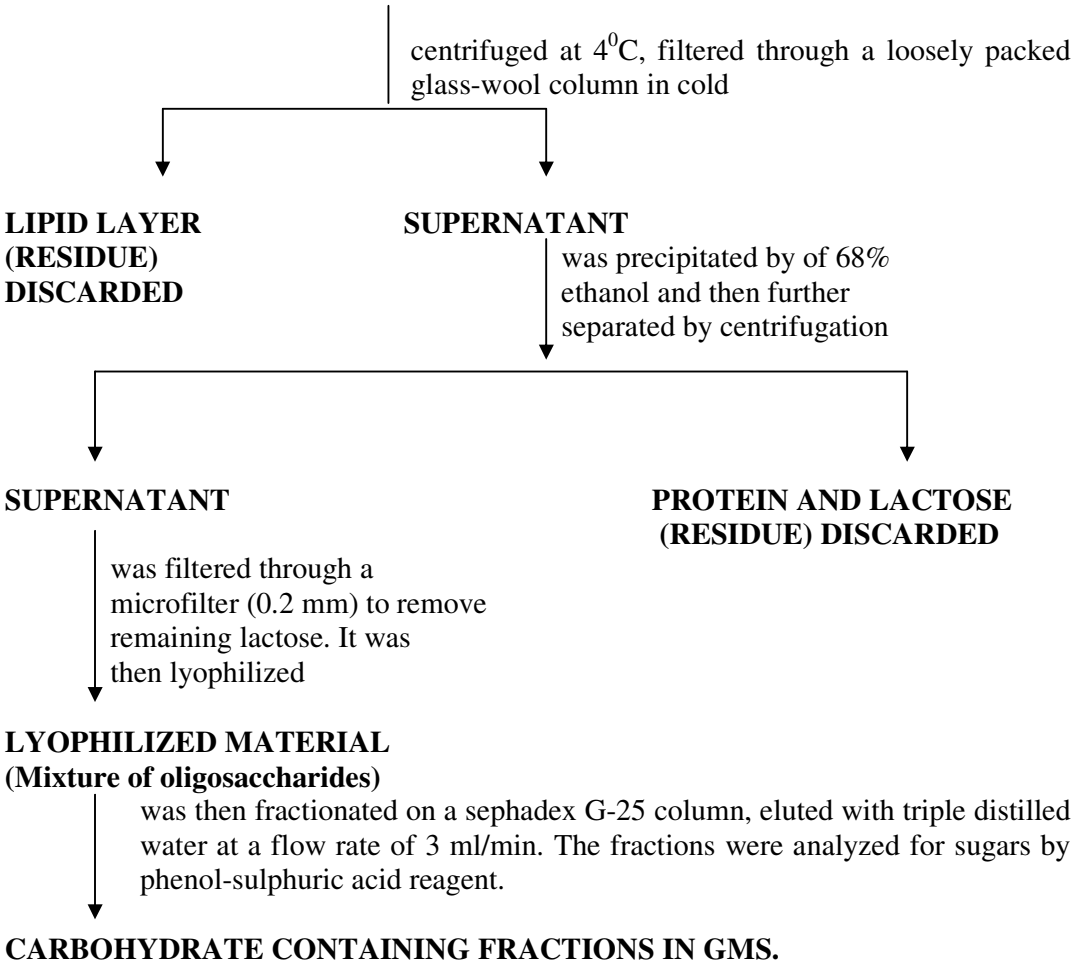
Totat soluble sugar = u(x+0.05) x 20

x = (vol. of 0.01 N $Na_2S_2O_3$ used for sample, i.e. vol. of $Na_2S_2O_3$ used in blank) – (vol. used in sample).

Sl. No.	**Volume of 0.01 $Na_2S_2O_3$ used in blank (A)**	**Volume of 0.01 $Na_2S_2O_3$ used in sample (B)**	**X = (A - B)**	**u = 0.338**	**mg of Total Soluble Sugar in 100 ml. of sample = u (x + 0.05) x 20**
1.	11.6	10.57	1.03	0.338	7.36
2.	11.6	10.56	1.04	0.338	7.37
3.	11.6	10.55	1.05	0.338	7.44
4.	11.6	10.57	1.02	0.338	7.28
5.	11.6	10.57	1.02	0.338	7.27
6.	11.6	10.57	1.03	0.338	7.31
7.	11.6	10.54	1.05	0.338	7.48
8.	11.6	10.56	1.04	0.338	7.42
9.	11.6	10.53	1.07	0.338	7.57
10.	11.6	10.53	1.06	0.338	7.54
				Maximum Value	**7.57**
				Minimum Value	**7.27**
				Mean Value	**7.40**

Table 2.2.3

ISOLASTION OF COW'S MILK OLIGOSACCHARIDES
BY
KOBATA AND GINSBURG METHOD



2.2.4 Oligosaccharide Contents of Cow's Milk

Sl. No.	Method used	Volume taken	Oligosaccharide (Gms.)
1	Kobata& Ginsberg Method	1 Liter	0.86
2	Kobata& Ginsberg Method	1 Liter	0.88
3	Kobata& Ginsberg Method	1 Liter	0.84
4	Kobata& Ginsberg Method	1 Liter	0.85
5	Kobata& Ginsberg Method	1 Liter	0.87
6	Kobata& Ginsberg Method	1 Liter	0.84
7	Kobata& Ginsberg Method	1 Liter	0.83
8	Kobata& Ginsberg Method	1 Liter	0.84
9	Kobata& Ginsberg Method	1 Liter	0.86
10.	Kobata& Ginsberg Method	1 Liter	0.82
	Maximum Value		**0.88**
	Minimum Value		**0.82**
	Average Value		**0.85**

Table 2.2.4

2.2.5 SEPHADEX G-25 GEL FILTRATION OF' COW'S MILK OLIGOSACCHARIDE MIXTURE

The repeated gel filtration was performed by Sephadex G-25 chromatography of crude cow milk oligosaccharide mixture. Cow milk oligosaccharide mixture was packed in a column (1.6 x 40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 h to settle down. The material was applied on a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide (low molecular weight component). Presences of neutral sugars were monitored in all eluted fractions by phenol-sulphuric acid test. In this U.V. monitored Sephadex G-25 chromatography of oligosaccharide mixture showed four peaks i.e. I, II. III and IV. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive coloration with p-dimethyl aminobenzaldehyde and phenol-sulphuric acid reagent. Fractions that gave a positive

phenol-sulphuric acid test for sugars, showed the presence of oligosaccharide mixture in cow milk. These fractions were pooled and lyophilized together.

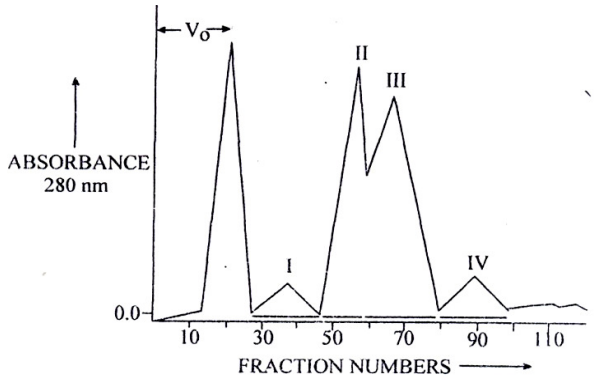


Figure 2.1
Sephadex G-25 chromatography of Cow's milk oligosaccharide (12.0 g) detected by Phenol-sulphuric acid method. Elution was made with glass-triple distilled water; 3 ml were collected.

Fraction No.	SOLVENT	Fraction No.	PHENOL H_2SO_4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-27	Glass triple distilled H2O		+ve	-
28-46	’’	I	-ve	
47-67	’’	II	+++ve	HPLC monitoring
68-79	’’	III	++ve	
80-98	’’	IV	-ve	

Table 2.2.5

ANALYTICAL HPLC

The carbohydrate fractions were eluted with triple distilled water (containing 0.1 % TFA and CH_3CN) at a flow rate 1 ml/min, to check the homogeneity of the milk oligosaccharides. The elution monitored by UV absorbance at 220 nm.

CONFIRMATION OF HOMOGENEITY OF COW'S MILK OLIGOSACCHARIDE BY REVERSE PHASE HPLC

Pooled fractions (peaks II, III) obtained from Sephadex G-25 column, containing oligosaccharide mixture were qualitatively analysed by reverse phase HPLC. The HPLC system was equipped with Shimadzu CLASS-VP V6.13 solvent delivering system, 235-diode array detector and G.P. 100 printer plotter. The column used for this purpose was C18 Purosphere 25 cm x 0.4 cm x 5-µ m (from E. Merck). A binary gradient system of acetonitrile : 0.5% trifluro-acetic acid (5:95) in triple distilled water (TDW) to CH3CN : 0.5% TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 220 nm. Twelve peaks were noticed in the sample (pooled fraction II and III) at the varied retention times from 1.092 min to 20.675 in, for convenience the peaks were numbered in their increasing order of retention time i.e. 1.925 min (R_1), 3.250(R_2), 4.225(R_3), 4.950(R_4), 5.450(R_5), 6.725(R_6), 8.200(R_7), 10.233(R_8), 13.250(R_9), 15.433(R_{10}), 19.000(R_{11}) and 19.892(R_{12}).

2.6.6 HPLC TABLE OF COW 'S MILK OLIGOSACCHARIDE

Detector A-1 (220 nm)			
Pk#	**Retention Time**	**Area %**	**Height**
1	1.925	7.210	25646
2	3.250	15.484	222236
3	4.225	10.887	83985
4	4.950	5.578	96702
5	5.450	7.343	50398
6	6.725	14.781	86464
7	8.200	10.127	39140
8	10.233	5.480	44421
9	13.250	5.575	92688
10	15.433	1.961	16513
11	19.000	5.455	171444
12	19.892	0.566	11938

Table 2.6.6

Buffalo

Classification

Kingdom- Animalia
Phylum Chordata
Class Mammalia
Order Bovidae
Family Bovinae
Genus Artiodactyla
Species Bovini

2.3 Estimation of Protein Content of Buffalo's Milk by Micro Kjheldal's Method

The estimation of protein in Buffalo's milk was done by Micro Kjheldal's Method. The procedure was same as described earlier.The formula used for estimation of protein was as under:

% of protein is equal to $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$gms.

Sl. No.	Volume of 0.01 N H Cl used for blank in ml. (v)	Volume of 0.01 N H Cl used for sample in ml. (y)	N_2 present in 1 gm. digested sample $\frac{(y-v) \times 0.00014 \times 50}{5}$	N_2 present in 100 gm. Sample $\frac{(y-v) \times 0.00014 \times 50 \times 100}{5}$	% Protein content in sample $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$
1.	2.18	8.78	0.0092	0.92	5.8
2.	2.18	8.69	0.0091	0.91	5.7
3.	2.18	9.03	0.0096	0.96	6.0
4.	2.18	9.26	0.0099	0.99	6.2
5.	2.18	8.92	0.0094	0.94	5.9
6.	2.18	8.92	0.0094	0.94	5.9
7.	2.18	9.49	0.0102	1.02	6.4
8.	2.18	8.99	0.0095	0.96	6.1
9.	2.18	8.79	0.0093	0.92	5.8
10.	2.18	9.15	0.0097	0.97	6.1
				Maximum Value	**6.4**
				Minimum Value	**5.7**
				Mean Value	**5.9**

Table 2.3

2.3.1 Estimation of Fat Content of Buffalo's Milk by Rose Gottlieb's Method

The estimation of fat in Buffalo's milk was done by Rose Gottlieb Method with the procedure described earlier. The formula used for estimation of fat was

$$\text{Percent fat} = \frac{100[(\text{wt. of flask} + \text{dry fat}) - (\text{wt. of empty flask})]}{\text{wt. of sample}}$$

Sl. No.	**Weight of empty flask (A)**	**Weight of flask + dry fat (B)**	**Weight of Fat (B - A)**	**Percentage Fat** % **Fat = 100 [(wt. of flask + dry Fat) – wt. of empty flask] wt. of sample**
1.	250.000	250.528	0.528	10.56
2.	250.000	250.516	0.516	10.33
3.	250.000	250.530	0.530	10.61
4.	250.000	250.526	0.526	10.52
5.	250.000	250.520	0.520	10.40
6.	250.000	250.522	0.522	10.44
7.	250.000	250.519	0.519	10.38
8.	250.000	250.521	0.521	10.42
9.	250.000	250.518	0.518	10.37
10.	250.000	250.523	0.523	10.46
			Maximum Value	**10.61**
			Minimum Value	**10.37**
			Mean Value	**10.44**

Table 2.3.1

2.3.2 Estimation of Lactose content in Buffalo's Milk by Lane Eynon's Method

The estimation of lactose content in Buffalo's milk was done by Lane Eynon's Method with the procedure described earlier. The procedure is same as well as stated before. The formula used for estimation of lactose content was

1 ml. of Fehling's solution = 0.00645 gms. of anhydrous lactose.

Lactose content = 0.00645 x mls of Fehling's solution used

Sl. No. (A)	Quantity of milk sample (in ml.) (B)	Quantity of Fehling's Solution for 10 ml. of milk sample (C)	Calculated amount of Lactose in 10 ml. of Milk sample using quantity of Fehling solution as shown in column (C) (D)	Calculated amount of Lactose in 100 ml. of milk (E)
1.	10.00	65.11	0.42	4.2
2.	10.00	65.00	0.41	4.1
3.	10.00	62.67	0.40	4.0
4.	10.00	59.32	0.39	3.9
5.	10.00	65.16	0.42	4.2
6.	10.00	68.20	0.43	4.3
7.	10.00	65.11	0.41	4.1
8.	10.00	69.76	0.45	4.5
9.	10.00	58.81	0.38	3.8
10.	10.00	68.85	0.44	4.4
			Maximum Value	**4.5**
			Minimum Value	**3.8**
			Mean Value	**4.15**

Table 2.3.2

2.3.3 Estimation of Total Soluble Sugar in Buffalo's Milk byFerricyanide Method

The estimation of total soluble sugar in Buffalo's milk was done by Ferricyanide Method with the procedure described earlier. The procedure is same as well as stated before. The formula used for estimation of total soluble sugar was

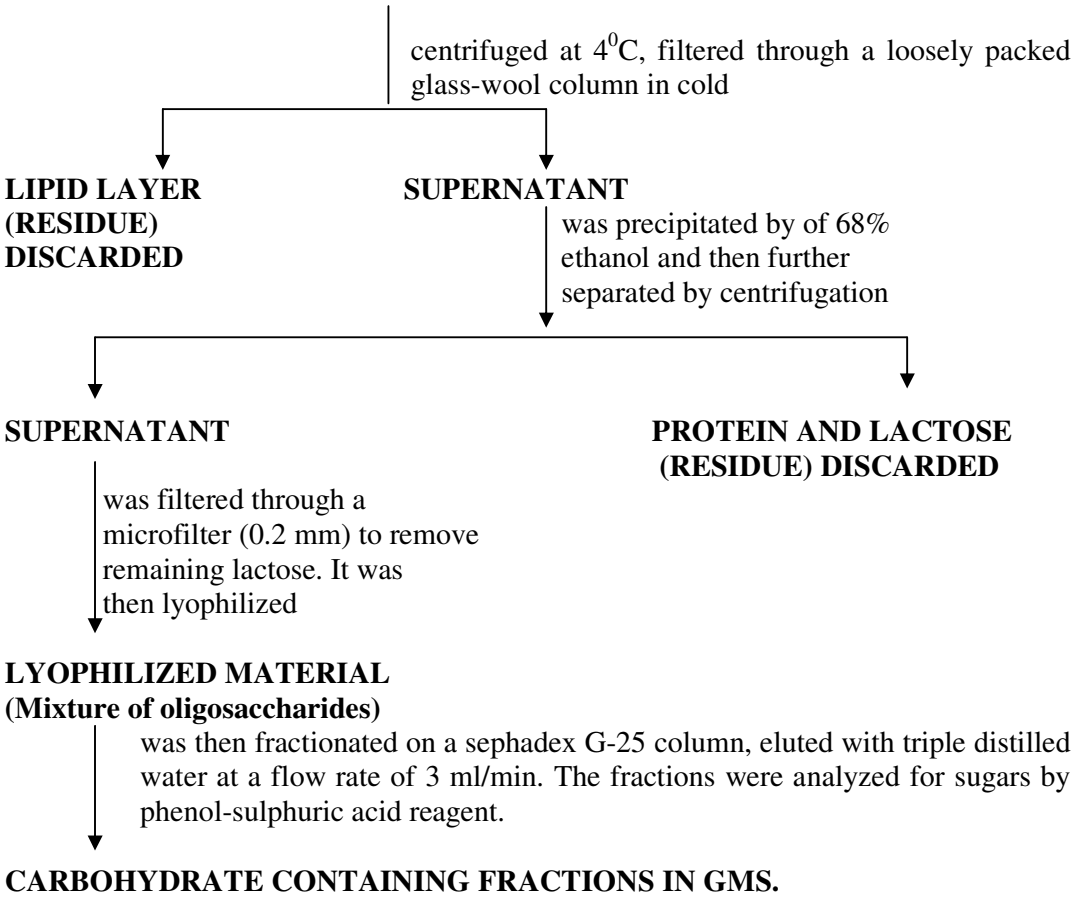
$$\mu = 0.338$$

x = vol. of 0.01 N $Na_2S_2O_3$ used for sample, i.e. vol. of $Na_2S_2O_3$ used in blank-vol. used in sample.

Sl. No.	**Volume of 0.01 $Na_2S_2O_3$ used in blank (A)**	**Volume of 0.01 $Na_2S_2O_3$ used in sample (B)**	**X = (A - B)**	**u = 0.338**	**mg of Total Soluble Sugar in 100 ml. of sample = u (x + 0.05) x 20**
1.	11.60	10.69	0.91	0.338	6.49
2.	11.60	10.68	0.92	0.338	6.57
3.	11.60	10.71	0.89	0.338	6.39
4.	11.60	10.66	0.94	0.338	6.69
5.	11.60	10.70	0.90	0.338	6.42
6.	11.60	10.72	0.88	0.338	6.29
7.	11.60	10.70	0.90	0.338	6.44
8.	11.60	10.65	0.95	0.338	6.78
9.	11.60	10.64	0.96	0.338	6.84
10.	11.60	10.62	0.98	0.338	6.97
				Maximum Value	**6.97**
				Minimum Value	**6.29**
				Mean Value	**6.58**

Table 2.3.3

ISOLASTION OF BUFFALO'S MILK OLIGOSACCHARIDES
BY
KOBATA AND GINSBURG METHOD



2.3.4 Oligosaccharide Contents of Sheep's Milk

Sl. No.	Method used	Volume taken	Oligosaccharide (gms.)
1	Kobata& Ginsberg Method	1 Liter	0.90
2	Kobata& Ginsberg Method	1 Liter	0.85
3	Kobata& Ginsberg Method	1 Liter	0.88
4	Kobata& Ginsberg Method	1 Liter	0.86
5	Kobata& Ginsberg Method	1 Liter	0.92
6	Kobata& Ginsberg Method	1 Liter	0.87
7	Kobata& Ginsberg Method	1 Liter	0.91
8	Kobata& Ginsberg Method	1 Liter	0.89
9	Kobata& Ginsberg Method	1 Liter	0.88
10.	Kobata& Ginsberg Method	1 Liter	0.85
	Maximum Value		**0.92**
	Minimum Value		**0.85**
	Average Value		**0.88**

Table 2.3.4

2.3.5 SEPHADEX G-25 GEL FILTRATION OF' BUFFALO'S MILK OLIGOSACCHARIDE MIXTURE

Fraction II obtained from Sephadex G-25 column appeared to be the most dominant fraction was used for analytical analysis by reverse phase (RP) HPLC. The solvent used for the analytical HPLC (WATERS) was a binary gradient system. TDW containing 0.1% TFA (pH2) and acetonitrile as the organic modifier, and the solvent programme involved a linear gradient of 0.60% acetonitrile over 45 min at a flow of 1 ml/min. A μ-BONDAPAK 18 column (3 x 300mn) was used for this purpose. The effluent monitored by U.V. absorbance at 220 nm gave three peaks, two peaks I and II are

retention times 3.00 and 3.47 min were quite overlapped and peak III was obtained at retention time 23 mi

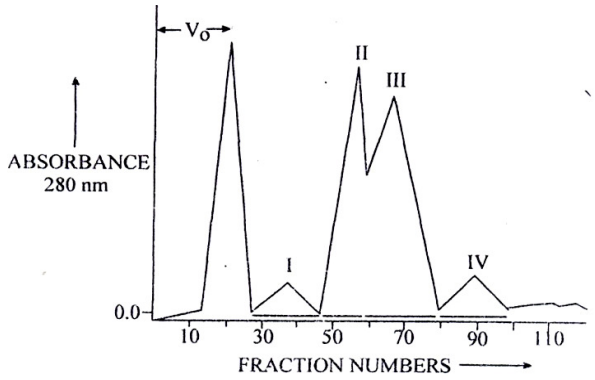


Figure 2.3

Sephadex G-25 chromatography of Buffalo milk oligosaccharide (520 mg) detected by Phenol-sulphuric acid method. Elution was made with glass-triple distilled water; 3 ml were collected. Fractions indicated by a bar (-) in each peak were pooled.

Fraction No.	SOLVENT	Fraction No.	PHENOL H_2SO_4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-24	Glass triple distilled H2O		+ve	-
25-44	’’	I	-ve	
45-67	’’	II	+++ve	HPLC monitoring
68-78	’’	III	++ve	

Table 2.3.5

CONFIRMATION OF HOMOGENEITY OF BUFFALO’S MILK OLIGOSACCHARIDE BY REVERSE PHASE HPLC

The oligosaccharide contents were analyzed by HPLC (High Performance Liquid Chromatography). The solvent used for the analytical HPLC (WATERS) was binary

gradient system. TDW containing 0.1% TFA (pH2) and acetonitrile as the organic modifier and the solvent programme involved a linear gradient of 0.60% acetonitrile over 45 min at a flow of 1 ml/min. The effluent monitored by U.V. absorbance at 220 nm gave three peaks, two peaks I and II are retention times 3.00 and 3.47 min were quite overlapped and peak III was obtained as retention time 23 min.

Sheep

Classification

Kingdom- Animalia
Phylum Chordata
Class Mammalia
Order Bovidae
Family Bovinae
Genus Artiodactyla
Species Ovis aries

2.4 Estimation of Protein Content of Sheep's Milk by Micro Kjheldal's Method

The estimation of protein in Sheep's milk was done by Micro Kjheldal's Method. The procedure was same as described earlier. The formula used for estimation of protein was as under:

% of protein is equal to $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$gms.

Sl. No	Volume of 0.01 N H Cl used for blank in ml. (v)	Volume of 0.01 N H Cl used for sample in ml. (y)	N_2 present in 1 gm. digested sample $\frac{(y-v) \times 0.00014 \times 50}{5}$	N_2 present in 100 gm. Sample $\frac{(y-v) \times 0.00014 \times 50 \times 100}{5}$	% Protein content in sample $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$
1.	2.18	8.81	0.0092	0.92	5.8
2.	2.18	9.02	0.0095	0.96	6.0
3.	2.18	8.92	0.0094	0.94	5.9
4.	2.18	8.12	0.0083	0.83	5.2
5.	2.18	8.46	0.0087	0.87	5.5
6.	2.18	8.25	0.0084	0.85	5.3
7.	2.18	9.03	0.0096	0.96	6.0
8.	2.18	8.35	0.0086	0.86	5.4
9.	2.18	8.23	0.0085	0.85	5.3
10.	2.18	8.36	0.0087	0.86	5.4
				Maximum Value	**6.0**
				Minimum Value	**5.4**
				Mean Value	**5.7**

Table 2.4

2.4.1 Estimation of Fat Content of the Sheep's Milk by Rose Gottlieb Method

The estimation of fat in Sheep's milk was done by Rose Gottlieb Method. The formula used for estimation of fat was

$$\text{Percent fat} = \frac{100[(\text{wt. of flask} + \text{dry fat}) - (\text{wt. of empty flask})]}{\text{wt. of sample}}$$

Sl. No.	**Weight of empty flask (A)**	**Weight of flask + dry fat (B)**	**Weight of Fat (B - A)**	**Percentage Fat % Fat = 100 [(wt. of flask + dry Fat) – wt. of empty flask] wt. of sample**
1.	250.000	250.269	0.269	5.39
2.	250.000	250.270	0.270	5.40
3.	250.000	250.276	0.276	5.52
4.	250.000	250.280	0.280	5.56
5.	250.000	250.269	0.269	5.39
6.	250.000	250.273	0.273	5.46
7.	250.000	250.271	0.271	5.43
8.	250.000	250.272	0.272	5.45
9.	250.000	250.272	0.272	5.44
10.	250.000	250.269	0.269	5.38
			Maximum Value	**5.56**
			Minimum Value	**5.38**
			Mean Value	**5.44**

Table 2.4.1

2.4.2 Estimation of Lactose content in Sheep's Milk by Lane Eynon's Method

The estimation of lactose content in Sheep's milk was done by Lane Eynon's Method with the procedure described earlier. The formula used for estimation of lactose content was

1 ml. of Fehling's solution = 0.00645 gms. of anhydrous lactose.

Sl. No.	**Quantity of milk sample (in ml.)**	**Quantity of Fehling's Solution for 10 ml. of milk sample**	**Calculated amount of Lactose in 10 ml. of Milk sample using quantity of Fehling solution as shown in column (C)**	**Calculated amount of Lactose in 100 ml. of milk**
(A)	**(B)**	**(C)**	**(D)**	**(E)**
1.	10.00	75.90	0.49	4.9
2.	10.00	72.82	0.48	4.8
3.	10.00	71.36	0.49	4.9
4.	10.00	79.10	0.51	5.1
5.	10.00	80.16	0.51	5.1
6.	10.00	75.28	0.53	5.3
7.	10.00	75.30	0.48	4.8
8.	10.00	86.80	0.55	5.5
9.	10.00	77.50	0.49	4.9
10.	10.00	74.44	0.48	4.8
			Maximum Value	**5.5**
			Minimum Value	**4.8**
			Mean Value	**5.0**

Table 2.4.2

2.4.3 Estimation of Total Soluble Sugar in Sheep's Milk byFerricyanide Method

The estimation of total soluble sugar in Sheep's milk was done by Ferricyanide Method with the procedure described earlier. The procedure was same as well as stated before. The formula used for estimation of total soluble sugar was

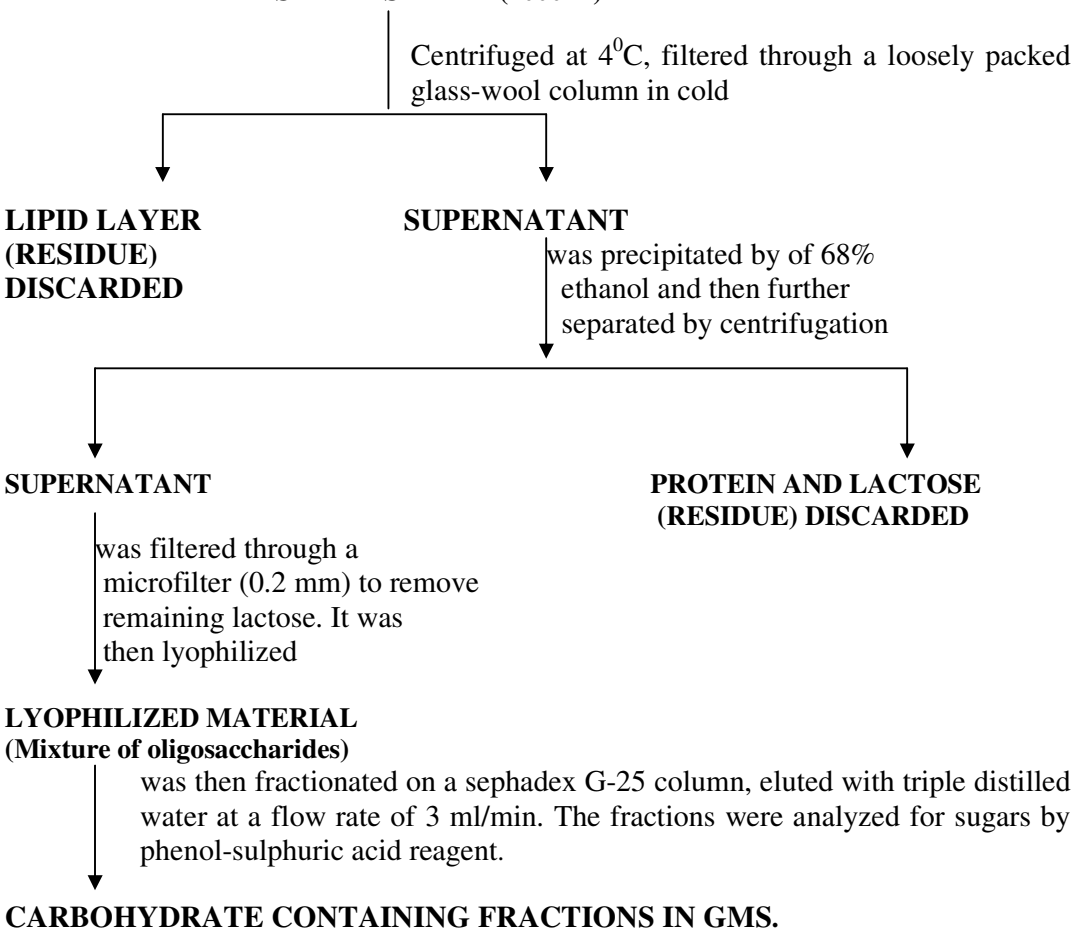
$\mu = 0.338$

x = vol. of 0.01 N $Na_2S_2O_3$ used for sample, i.e. vol. of $Na_2S_2O_3$ used in blank-vol. used in sample.

Sl. No.	**Volume of 0.01 $Na_2S_2O_3$ used in blank (A)**	**Volume of 0.01 $Na_2S_2O_3$ used in sample (B)**	**X = (A - B)**	**u = 0.338**	**mg of Total Soluble Sugar in 100 ml. of sample = u (x + 0.05) x 20**
1.	11.60	10.62	0.97	0.338	6.94
2.	11.60	10.60	1.00	0.338	7.11
3.	11.60	10.59	1.01	0.338	7.19
4.	11.60	10.57	1.02	0.338	7.29
5.	11.60	10.61	0.98	0.338	7.19
6.	11.60	10.61	0.98	0.338	6.98
7.	11.60	10.60	1.01	0.338	7.23
8.	11.60	10.63	0.96	0.338	6.87
9.	11.60	10.57	1.03	0.338	7.32
10.	11.60	10.63	0.97	0.338	6.93
				Maximum Value	**7.32**
				Minimum Value	**6.87**
				Mean Value	**7.11**

Table 2.4.3

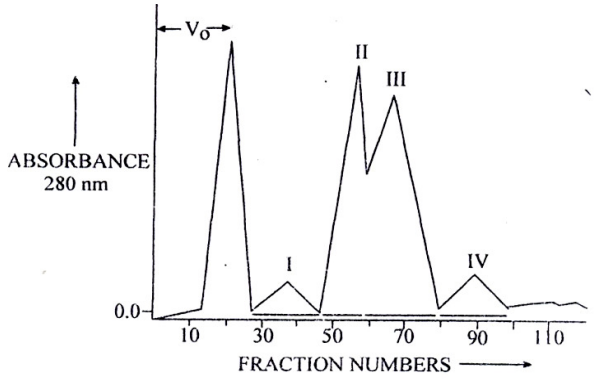
ISOLASTION OF SHEEP'S MILK OLIGOSACCHARIDES
BY
KOBATA AND GINSBURG METHOD



2.4.4 SEPHADEX G-25 GEL FILTRATION

OLIGOSACCHARIDE MIXTURE

The repeated gel tiltration was performed by Sephadex G-25 chromatography of crude sheep milk oligosaccharide mixture. Sheep milk oligosaccharide mixture was packed in a column (1.6 x 40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 h to settle down. The material was applied on a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide Presence of' neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test. In this U.V. monitored Sephadex G-25 chromatography of sheep milk oligosaccharide mixture showed four peaks i.e. I, II, III and IV. A substantial amount of proteins, glycoprotein's and serum albumin were eluted in the void volume, which was concluded by positive coloration with p-dimethylaminobenzaldehyde reagent and phenol-sulphuric acid reagent. Fractions under peaks II and III gave a positive phenol-sulphuric acid test for sugars, which showed the presence of oligosaccharides mixture in sheep milk. These fractions (peaks II and III) were pooled and lyophilized together.



Fraction No.	SOLVENT	Fraction No.	PHENOL H_2SO_4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-24	Glass triple distilled H2O		+ve	-
25-47	’’	I	-ve	-
48-70	’’	II	+++ve	Analyzed by
71-82	’’	III	++ve	HPLC
83-100	’’	IV	-ve	-

Table 2.4.4

2.4.5 Oligosaccharide Contents of Sheep's Milk

Sl. No.	Method used	Volume taken	Oligosaccharide (gms)
1	Kobata& Ginsberg Method	1 Liter	0.80
2	Kobata& Ginsberg Method	1 Liter	0.82
3	Kobata& Ginsberg Method	1 Liter	0.80
4	Kobata& Ginsberg Method	1 Liter	0.83
5	Kobata& Ginsberg Method	1 Liter	0.80
6	Kobata& Ginsberg Method	1 Liter	0.78
7	Kobata& Ginsberg Method	1 Liter	0.82
8	Kobata& Ginsberg Method	1 Liter	0.83
9	Kobata& Ginsberg Method	1 Liter	0.81
10.	Kobata& Ginsberg Method	1 Liter	0.84
	Maximum Value		**0.84**
	Minimum Value		**0.78**
	Average Value		**0.81**

Table 2.4.5

ANALYTICAL HPLC

The carbohydrate fractions were eluted with triple distilled water (containing 0.1 % TFA and CH_3CN) at a flow rate 1 ml/min, to check the homogeneity of the milk oligosaccharides. The elution monitored by UV absorbance at 220 nm.

CONFIRMATION OF HOMOGENEITY OF SHEEP'S MILK OLIGOSACCHARIDE BY REVERSE PHASE HPLC

Pooled fractions (peaks II and III) obtained from Sephadex G-25 column, containing oligosaccharide mixture were qualitatively analysed by reverse phase HPLC. The HPLC system was equipped with Perkin-Elmer 250 solvent delivering system, 235-diode array detector and G.P. 100 printer plotter. The column used for this purpose was C_{18}Purosphere 25 cm x 0.4 cm x 5-μ m (from E. Merck). A binary gradient system of acetonitrile : 0.5% trifluro-acetic acid (5:95) in triple distilled water (TDW) to CH_3CN : 0.5% TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 220 nm. Twelve peaks were noticed in the sample (pooled fraction II and III) at the varied retention times from 2.842 min to 23.358 min, for convenience the peaks were numbered in their increasing order of retention time i.e. 2.842(R_1), 4.175(R_2), 6.850(R_3), 8.308(R_4), 9.367(R_5), 10.358(R_6), 11.117(R_7), 18.983(R_8), 19.892(R_9), 20.917(R_{10}), 21.892(R_{11}), 23.358(R_{12}) as described in the Table below.

2.4.6 HPLC TABLE OF SHEEP'S MILK OLIGOSACCHARIDE

Detector A-1 (220 nm)			
Pk#	**Retention Time**	**Area %**	**Height**
1	2.842	31.831	139012
2	4.175	4.257	30652
3	6.850	34.917	54511
4	8.308	4.896	39926
5	9.367	9.892	29796
6	10.358	3.767	23045
7	11.117	6.795	18107
8	18.983	2.231	57103
9	19.892	0.392	6945
10	20.917	0.436	3831
11	21.892	0.225	7143
12	23.358	0.360	2703
Total		**100.0001**	**4127741**

Table 2.4.6

Camel

Classification

Kingdom- Animalia
Phylum Chordata
Class Mammalia
Order Artiodactyla
Family Camelidae
Genus Camelus
Species Camelini

2.5 Estimation of Protein Content of the Camel's Milk by Micro Kjheldal's Method

The estimation of protein in Camel's milk was done by Micro Kjheldal's Method. The procedure is same as well as stated before. The formula used for estimation of protein is as under :

% of protein is equal to $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$gms.

Sl. No.	Volume of 0.01 N H Cl used for blank in ml. (v)	Volume of 0.01 N H Cl used for sample in ml. (y)	N_2 present in 1 gm. digested sample $\frac{(y-v) \times 0.00014 \times 50}{5}$	N_2 present in 100 gm. Sample $\frac{(y-v) \times 0.00014 \times 50 \times 100}{5}$	% Protein content in sample $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$
1.	2.18	6.54	0.0060	0.60	3.8
2.	2.18	6.64	0.0063	0.63	4.0
3.	2.18	6.63	0.0062	0.62	3.9
4.	2.18	6.86	0.0065	0.65	4.1
5.	2.18	6.93	0.0067	0.67	4.2
6.	2.18	6.55	0.0061	0.61	3.8
7.	2.18	7.09	0.0069	0.69	4.3
8.	2.18	6.52	0.0060	0.60	3.8
9.	2.18	6.84	0.0066	0.66	4.1
10.	2.18	6.95	0.0068	0.68	4.2
				Maximum Value	**4.3**
				Minimum Value	**3.8**
				Mean Value	**4.0**

Table 2.5

2.5.1 Estimation of Fat Content of the Camel's Milk by Rose Gottlieb's Method

The estimation of fat in Camel's milk was done by Rose Gottlieb Method with the procedure described earlier. The formula used for estimation of fat was

$$\text{Percent fat} = \frac{100[(\text{wt. of flask} + \text{dry fat}) - (\text{wt. of empty flask})]}{\text{wt. of sample}}$$

Sl. No.	**Weight of empty flask (A)**	**Weight of flask + dry fat (B)**	**Weight of Fat (B - A)**	**Percentage Fat % Fat = 100 [(wt. of flask + dry Fat) – wt. of empty flask] wt. of sample**
1.	250.000	250.244	0.244	4.88
2.	250.000	250.245	0.245	4.89
3.	250.000	250.256	0.256	5.12
4.	250.000	250.253	0.253	5.06
5.	250.000	250.248	0.248	4.96
6.	250.000	250.245	0.245	4.91
7.	250.000	250.259	0.259	5.18
8.	250.000	250.245	0.245	4.89
9.	250.000	250.248	0.248	4.96
10.	250.000	250.258	0.258	5.16
			Maximum Value	**5.18**
			Minimum Value	**4.88**
			Mean Value	**5.00**

Table 2.5.1

2.5.2 Estimation of Lactose content in Camel Milk by Lane Eynon's Method

The estimation of lactose content in Camel milk was done by Lane Eynon's Method with the procedure described earlier. The formula used for estimation of lactose content was 1 ml. of Fehling's solution = 0.00645 gms. of anhydrous lactose.

Sl. No.	**Quantity of milk sample (in ml.)**	**Quantity of Fehling's Solution for 10 ml. of milk sample**	**Calculated amount of Lactose in 10 ml. of Milk sample using quantity of Fehling solution as shown in column (C)**	**Calculated amount of Lactose in 100 ml. of milk**
(A)	**(B)**	**(C)**	**(D)**	**(E)**
1.	10.00	79.00	0.50	5.0
2.	10.00	75.90	0.49	4.9
3.	10.00	83.76	0.54	5.4
4.	10.00	74.44	0.48	4.8
5.	10.00	77.42	0.49	4.9
6.	10.00	75.00	0.48	4.8
7.	10.00	77.50	0.49	4.9
8.	10.00	82.76	0.53	5.3
9.	10.00	79.60	0.51	5.1
10.	10.00	81.12	0.52	5.2
			Maximum Value	**5.4**
			Minimum Value	**4.8**
			Mean Value	**5.1**

Table 2.5.2

2.5.3 Estimation of Total Soluble Sugar in Camel's Milk byFerricyanide Method

The estimation of total soluble sugar in Camel milk was done by Ferricyanide Method with the procedure described earlier. The procedure is same as well as stated before. The formula used for estimation of total soluble sugar was

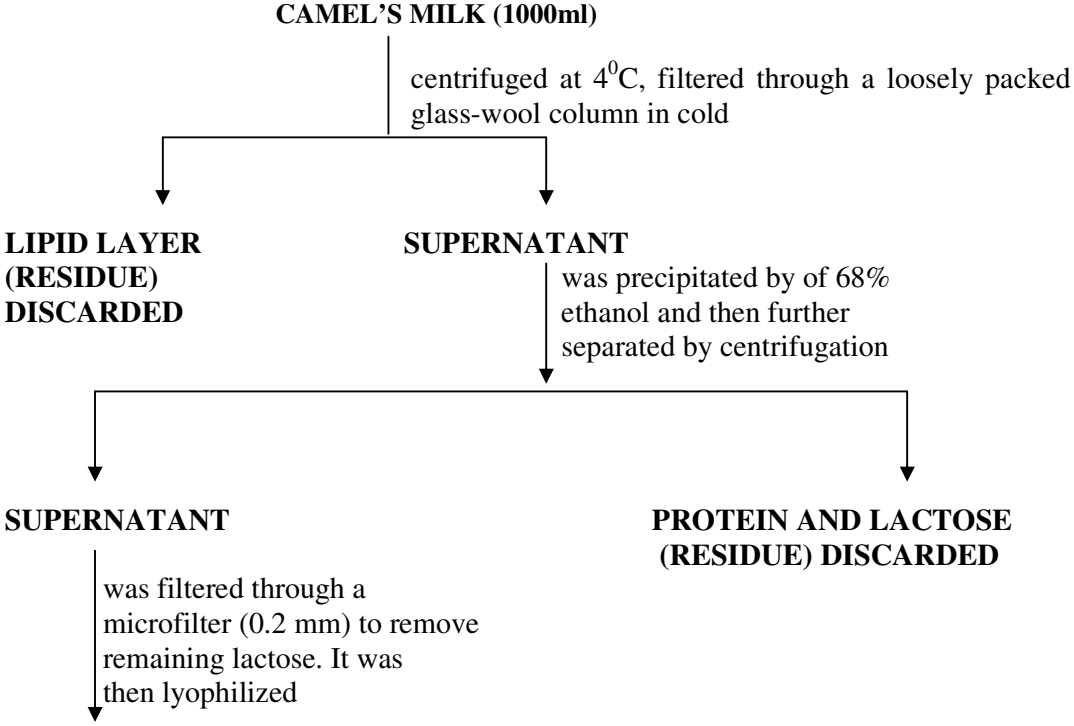
$$\mu = 0.338$$

x = vol. of 0.01 N $Na_2S_2O_3$ used for sample, i.e. vol. of $Na_2S_2O_3$ used in blank-vol. used in sample.

Sl. No.	**Volume of 0.01 $Na_2S_2O_3$ used in blank (A)**	**Volume of 0.01 $Na_2S_2O_3$ used in sample (B)**	**X = (A - B)**	**u = 0.338**	**mg of Total Soluble Sugar in 100 ml. of sample = u (x + 0.05) x 20**
1.	11.60	10.58	1.02	0.338	7.21
2.	11.60	10.62	0.98	0.338	6.98
3.	11.60	10.63	0.97	0.338	6.94
4.	11.60	10.54	1.06	0.338	7.52
5.	11.60	10.58	1.02	0.338	7.24
6.	11.60	10.63	0.97	0.338	6.99
7.	11.60	10.55	1.04	0.338	7.43
8.	11.60	10.56	1.04	0.338	7.38
9.	11.60	10.54	1.05	0.338	7.46
10.	11.60	10.58	1.01	0.338	7.18
				Maximum Value	**7.52**
				Minimum Value	**6.94**
				Mean Value	**7.23**

Table 2.5.3

ISOLASTION OF CAMEL'S MILK OLIGOSACCHARIDES
BY
KOBATA AND GINSBURG METHOD



2.5.4 Oligosaccharide Contents of Camel's Milk

Sl. No.	Method used	Volume taken	Oligosaccharide %
1	Kobata& Ginsberg Method	1 Liter	0.89
2	Kobata& Ginsberg Method	1 Liter	0.86
3	Kobata& Ginsberg Method	1 Liter	0.84
4	Kobata& Ginsberg Method	1 Liter	0.88
5	Kobata& Ginsberg Method	1 Liter	0.87
6	Kobata& Ginsberg Method	1 Liter	0.85
7	Kobata& Ginsberg Method	1 Liter	0.86
8	Kobata& Ginsberg Method	1 Liter	0.90
9	Kobata& Ginsberg Method	1 Liter	0.83
10.	Kobata& Ginsberg Method	1 Liter	0.85
	Maximum Value		**0.90**
	Minimum Value		**0.83**
	Average Value		**0.86**

Table 2.5.4

2.5.5 SEPHADEX G-25 GEL FILTRATION OF' CAMEL'S MILK OLIGOSACCHARIDE MIXTURE

The repeated gel filtration was performed by Sephadex 0-25 chromatography of crude camel milk oligosaccharide mixture. Camel milk oligosaccharide mixture was packed in a column (1.6 x 40 cm) (void volume 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 h to settle down. The material was applied on a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide. Presence of neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test. In this U.V monitored Sephadex G-25 chromatograph' of camel milk oligosaccharide mixture showed four peaks i.e. I, II. III and IV. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive colouration with p-dimethyl amino benzaldehyde reagent and phenol- sulphuric acid reagent. Fractions under Peaks III and IV gave a positive phenol-sulphuric acid test for sugars while showed the presence of oligosaccharide Mixture in camel milk These fractions (Peaks III and IV) were pooled and lyophilized

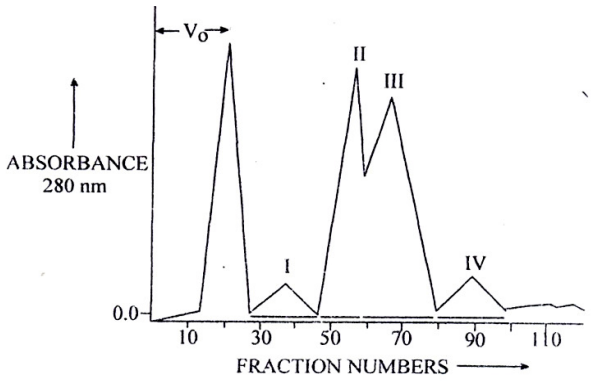


Figure 2.5

Camel milk oligosaccharide mixture chromatographed over sephadex G-25 (1.6 x 40 cms)

Fraction No.	SOLVENT	COMPOUND (gms)	PHENOL H2SO4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-20	Glass triple distilled H2O		+ve	-
21-44	’’	I	-ve	HPLC monitoring
45-70	’’	II	+++ve	-
71-80	’’	III	++ve	
81-98	’’	IV	-ve	

2.5.6 ANALYTICAL HPLC

The carbohydrate fractions were eluted with triple distilled water (containing 0.1 % TFA and CH_3CN) at a flow rate 1 ml/min, to check the homogeneity of the milk oligosaccharides. The elution monitored by UV absorbance at 220 nm.

CONFIRMATION OF HOMOGENEITY OF CAMEL’S MILK OLIGOSACCHARIDE BY REVERSE PHASE HPLC

Oligosaccharide mixture were quantitatively analysed by reverse phase HPLC. The HPLC system was equipped with Perkin Elmer 250 solvent delivering system, 235-diode array detector and G.P. 100 printer plotter. A binary gradient system 0.05% trifluro-acetic acid (5:95) in triple distilled water (TDW) to acetonitrile : 0.05% TFA within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 215 nm. Ten peaks were noticed in the sample at the varied retention times from 2.41 min. to 17.63 min for convenience the peaks were numbered in their increasing order of

retention time i.e. 2.432 min(R_1), 2.997 min(R_2), 3.349(R_3), 3.744(R_4), 4.235(R_5), 5.483(R_6), 8.203 min(R_7), 8.619min(R_8), 10.667min(R_9) and 17.579 min(R_{10}).

2.5.6 HPLC TABLE OF CRUDE CAMEL'S MILK OLIGOSACCHARIDE

Pk	Retention Time	Area %	Height
1	2.432	33.79	5614
2	2.997	26.78	5284
3	3.349	5.15	3595
4	3.744	18.93	3953
5	4.235	0.60	334
6	5.483	7.40	2794
7	8.203	1.09	298
8	8.619	1.87	633
9	10.667	3.17	1406
10	17.579	1.21	140

Table 2.5.6

Chauri Cow

Classification

Kingdom- Animalia
Phylum Chordata
Class Mammalia
Order Theria
Family Bovidae
Genus Bovini
Species Bos grunniens

2.6 Estimation of Protein Content of Chauri's Milk by Micro Kjheldal's Method

The estimation of protein in Chauri's milk was done by Micro Kjheldal's Method. The procedure is same as well as stated before. The formula used for estimation of protein is as under:

% of protein is equal to $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$ gms.

Sl. No.	Volume of 0.01 N H Cl used for blank in ml. (v)	Volume of 0.01 N H Cl used for sample in ml. (y)	N_2 present in 1 gm. digested sample $\frac{(y-v) \times 0.00014 \times 50}{5}$	N_2 present in 100 gm. Sample $\frac{(y-v) \times 0.00014 \times 50 \times 100}{5}$	% Protein content in sample $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$
1.	2.18	4.23	0.0028	0.28	1.8
2.	2.18	4.34	0.0030	0.30	1.9
3.	2.18	4.46	0.0031	0.31	2.0
4.	2.18	4.35	0.0030	0.30	1.9
5.	2.18	4.24	0.0028	0.28	1.8
6.	2.18	4.48	0.0031	0.31	2.0
7.	2.18	4.52	0.0026	0.26	2.1
8.	2.18	4.47	0.0031	0.31	2.0
9.	2.18	4.35	0.0030	0.30	1.9
10.	2.18	4.23	0.0029	0.29	1.8
				Maximum Value	**2.1**
				Minimum Value	**1.8**
				Mean Value	**1.9**

Table 2.6

2.6.1 Estimation of Fat Content of Chauri's Milk by Rose Gottlieb's Method

The estimation of fat in Chauri's milk was done by Rose Gottlieb Method with the procedure described earlier. The formula used for estimation of fat was

$$\text{Percent fat} = \frac{100[(\text{wt. of flask} + \text{dry fat}) - (\text{wt. of empty flask})]}{\text{wt. of sample}}$$

Sl. No.	**Weight of empty flask (A)**	**Weight of flask + dry fat (B)**	**Weight of Fat (B - A)**	**Percentage Fat % Fat = 100 [(wt. of flask + dry Fat) – wt. of empty flask] wt. of sample**
1.	250.000	250.069	0.069	1.38
2.	250.000	250.069	0.069	1.39
3.	250.000	250.071	0.071	1.42
4.	250.000	250.070	0.070	1.40
5.	250.000	250.070	0.070	1.41
6.	250.000	250.069	0.069	1.38
7.	250.000	250.072	0.072	1.45
8.	250.000	250.071	0.071	1.43
9.	250.000	250.070	0.070	1.41
10.	250.000	250.072	0.072	1.44
			Maximum Value	**1.45**
			Minimum Value	**1.38**
			Mean Value	**1.41**

Table 2.6.1

2.6.2 Estimationof Lactose content in Chauri's Milk by Lane Eynon's Method

The estimation of lactose content in Chauri,s milk was done by Lane Eynon's Method with the procedure described earlier. The procedure was same as well as stated before. The formula used for estimation of lactose content was

1 ml. of Fehling's solution = 0.00645 gms. of anhydrous lactose.

Sl. No. (A)	Quantity of milk sample (in ml.) (B)	Quantity of Fehling's Solution for 10 ml. of milk sample (C)	Calculated amount of Lactose in 10 ml. of Milk sample using quantity of Fehling solution as shown in column (C) (D)	Calculated amount of Lactose in 100 ml. of milk (E)
1.	10.00	111.62	0.72	7.2
2.	10.00	105.44	0.68	6.8
3.	10.00	108.57	0.70	7.0
4.	10.00	106.97	0.69	6.9
5.	10.00	116.27	0.75	7.5
6.	10.00	113.17	0.73	7.3
7.	10.00	109.57	0.71	7.1
8.	10.00	106.77	0.69	6.9
9.	10.00	105.42	0.68	6.8
10.	10.00	108.52	0.70	7.0
			Maximum Value	**7.5**
			Minimum Value	**6.8**
			Mean Value	**7.0**

Table 2.6.2

2.6.3 Estimation of Total Soluble Sugar in Chauri's Milk byFerricyanide Method

The estimation of total soluble sugar in Chauri's milk was done by Ferricyanide Method with the procedure described earlier. The procedure is same as well as stated before. The formula used for estimation of total soluble sugar was

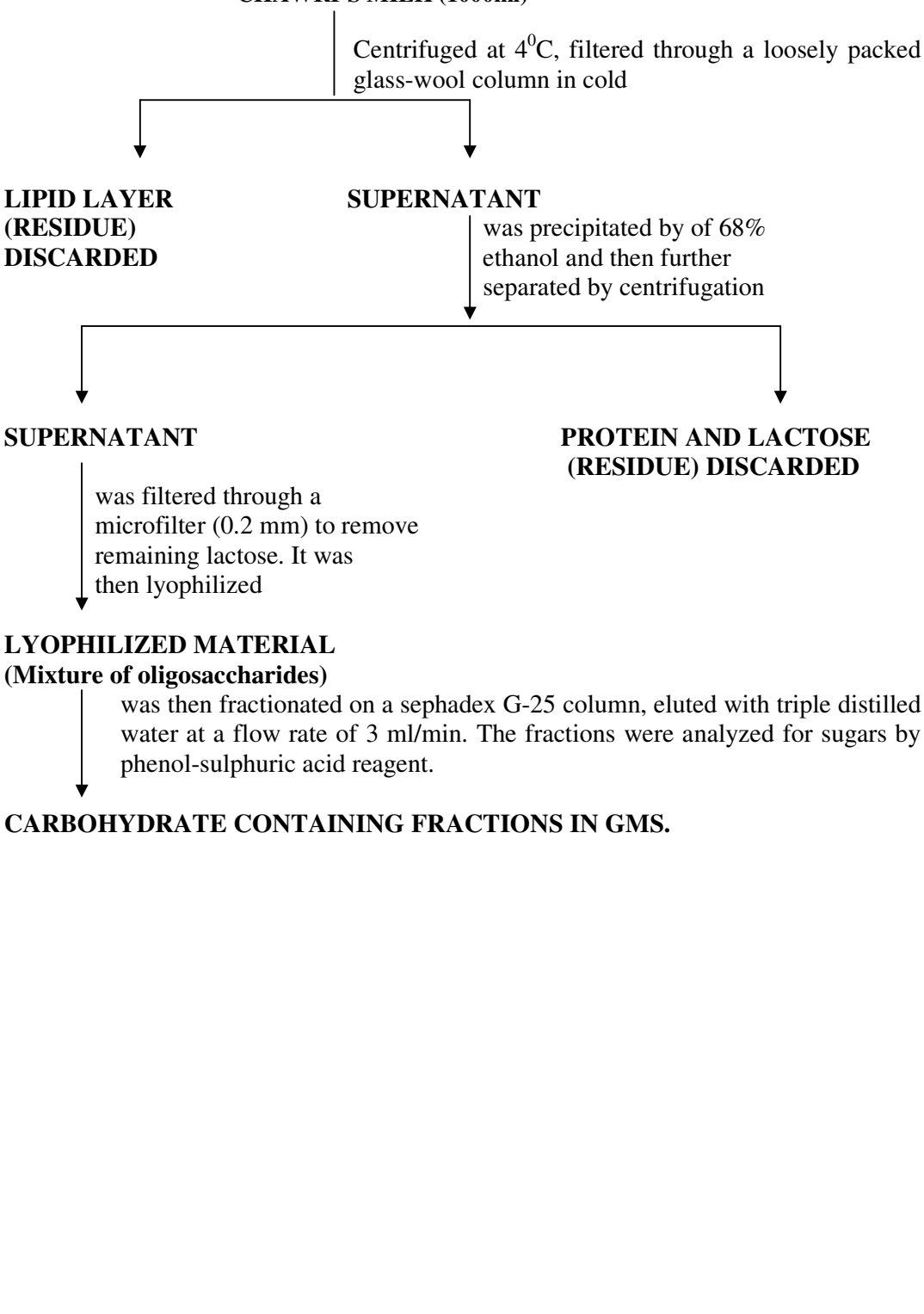
$$\mu = 0.338$$

x = vol. of 0.01 N $Na_2S_2O_3$ used for sample, i.e. vol. of $Na_2S_2O_3$ used in blank-vol. used in sample.

Sl. No.	Volume of 0.01 $Na_2S_2O_3$ used in blank (A)	Volume of 0.01 $Na_2S_2O_3$ used in sample (B)	X = (A - B)	u = 0.338	mg of Total Soluble Sugar in 100 ml. of sample = u (x + 0.05) x 20
1.	11.60	10.34	1.25	0.338	8.78
2.	11.60	10.37	1.23	0.338	8.67
3.	11.60	10.40	1.20	0.338	8.45
4.	11.60	10.33	1.27	0.338	8.99
5.	11.60	10.32	1.28	0.338	9.00
6.	11.60	10.37	1.23	0.338	8.67
7.	11.60	10.34	1.25	0.338	8.81
8.	11.60	10.31	1.29	0.338	9.07
9.	11.60	10.31	1.28	0.338	9.02
10.	11.60	10.37	1.23	0.338	8.67
				Maximum Value	**9.07**
				Minimum Value	**8.67**
				Mean Value	**8.81**

Table 2.6.3

ISOLASTION OF CHAURI'S MILK OLIGOSACCHARIDES
BY
KOBATA AND GINSBURG METHOD



2.6.4 Oligosaccharide Contents of Chauri's Milk

Sl. No.	Method used	Volume taken	Oligosaccharide %
1	Kobata& Ginsberg Method	1 Liter	0.95
2	Kobata& Ginsberg Method	1 Liter	0.94
3	Kobata& Ginsberg Method	1 Liter	0.92
4	Kobata& Ginsberg Method	1 Liter	0.88
5	Kobata& Ginsberg Method	1 Liter	0.87
6	Kobata& Ginsberg Method	1 Liter	0.91
7	Kobata& Ginsberg Method	1 Liter	0.90
8	Kobata& Ginsberg Method	1 Liter	0.89
9	Kobata& Ginsberg Method	1 Liter	0.86
10.	Kobata& Ginsberg Method	1 Liter	0.94
	Maximum Value		**0.95**
	Minimum Value		**0.86**
	Average Value		**0.91**

Table 2.6.4

2.6.5 SEPHADEX G-25 GEL FILTRATION OF CHAURI,S MILK OLIGOSACCHARIDE MIXTURE

The repeated gel filtration was performed by Sephadex G-25 chromatography of crude Chauri milk oligosaccharide mixture. Chauri milk oligosaccharide mixture was packed in a column (16 x 40 cm) (void volume = 25 011) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 h to settle down. The material was applied on a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide (low molecular weight component). Presence of neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test. In this U. V monitored Sephadex G-25 chromatography ofChauri milk oligosaccharide mixture showed four peaks i.e. I, II. III and IV. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive colouration with p-dimethylaminobenzaldehyde reagent and phenol-sulphuric acid reagent. Fractions under peaks II and III gave a positive phenol-sulphuric acid test for sugars which showed the presence of oligosaccharide mixture in Chauri milk. These fractions (peak II and III) were pooled and lyophilized together.

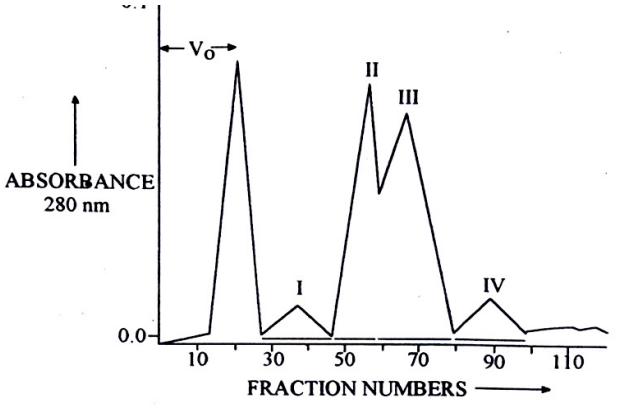


Figure 2.6

Sephadex G-25 chromatography pfChauri's milk oligosaccharide detected by Phenol-sulphuric acid method. Elution was made with glass-triple distilled water, 3 ml were collected. Fractions indicate by a bar (-) in each peak were pooled.

CHAURI'S MILK OLIGOSACCHARIDE MIXTURE CHROMATOGRAPHY OVER SEPHADEX G-25 (1.6 X 40 cm) COLUMN SIZE

FRACTION No.	SOLVENT	COMPOUND (grams)	PHENOL-H2SO4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-25	Glass triple distilled H2O		+ve	-
26-44	,,	I	-ve	-
45-63	,,	II	+++ve	Analysedby HPLC
64-75	,,	III	++ve	-
76-92	,,	IV	-ve	

CONFIRMATION OF HOMOGENEITY OF CHAURI'S MILK OLIGOSACCHARIDE BY REVERSE PHASE HPLC

Pooled fractions (peaks II and III) obtained from Sephadex G-25 column, containing oligosaccharide mixture were qualitatively analyzed by reverse phase HPLC. The HPLC system was equipped with Perkin Elmer 250 solvent delivering system, 235-diode array detector and G.P. 100 printer plotter. The column used for this purpose was C18 Purosphere 25 cm x 0.4 cm x 5-μ m (from E. Merck). A binary gradient system of acetonitrile : 0.5% trifluro-acetic acid (5:95) in triple distilled water (TDW) to CH3CN : 0.5% TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 220 nm. Eighteen peaks were noticed in the sample (pooled fraction II and III) at the varied retention times from 3.50 to 20.32 min, for convenience the peaks were numbered in their increasing order of retention time i.e. 0.83(R_1), 0.658(R_2), 1.933(R_3), 2.633(R_4), 3.25(R_5), 4.467(R_6), 6.85(R_7), 8.4(R_8), 9.333(R_9), 10.475(R_{10}), 11.475(R_{11}),

13.00(R_{12}), 14.425(R_{13}), 15.067(R_{14}), 17.058(R_{15}), 19.85(R_{16}), 20.683(R_{17}) and 22.45(R_{18}).

2.6.6 HPLC TABLE OF CHAURI'S MILK OLIGOSACCHARIDE

Detector A-1 (220 nm)			
Pk#	**Retention Time**	**Area %**	**Height**
1	0.083	0.394	24837
2	0.658	3.857	117768
3	1.933	6.985	44050
4	2.633	10.281	111947
5	3.250	6.353	154406
6	4.467	13,990	50165
7	6.850	19,237	49136
8	8.400	5.736	40291
9	9.333	6.475	36058
10	10.475	4.895	31927
11	11.475	4.128	29962
12	13.000	6.459	24974
13	14.425	1.560	15438
14	15.067	3.116	14087
15	17.058	2.160	10137
16	19.850	2.838	17807
17	20.683	0.707	7335
18	22.450	0.831	6227

Table 2.6.6

Goat

Classification

Kingdom- Animalia
Phylum Chordata
Class Mammalia
Order Theria
Family Caprinae
Genus Capri agragus
Species Capri hircus

2.7 Estimation of Protein Content of Goat's Milk by Micro Kjheldal's Method

The estimation of protein in Goat's milk was done by Micro Kjheldal's Method. The procedure was same as well as stated before. The formula used for estimation of protein is as under:

% of protein is equal to $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$ gms.

Sl. No.	Volume of 0.01 N H Cl used for blank in ml. (v)	Volume of 0.01 N H Cl used for sample in ml. (y)	N_2 present in 1 gm. digested sample $\frac{(y-v) \times 0.00014 \times 50}{5}$	N_2 present in 100 gm. Sample $\frac{(y-v) \times 0.00014 \times 50 \times 100}{5}$	% Protein content in sample $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$
1.	2.18	6.18	0.0056	0.56	3.5
2.	2.18	6.52	0.0060	0.60	3.8
3.	2.18	6.63	0.0062	0.62	3.9
4.	2.18	7.09	0.0069	0.69	4.3
5.	2.18	6.40	0.0059	0.59	3.7
6.	2.18	6.63	0.0062	0.62	3.9
7.	2.18	6.98	0.0067	0.67	4.2
8.	2.18	6.75	0.0064	0.64	4.0
9.	2.18	6.86	0.0065	0.65	4.1
10.	2.18	6.29	0.0057	0.57	3.6
				Maximum Value	**4.3**
				Minimum Value	**3.5**
				Mean Value	**3.9**

Table 2.7

2.7.1 Estimation of Fat Content of Goat's Milk by Rose Gottlieb's Method

The estimation of fat in Goat's milk was done by Rose Gottlieb Method with the procedure described earlier. The formula used for estimation of fat was

$$\text{Percent fat} = \frac{100[(\text{wt. of flask} + \text{dry fat}) - (\text{wt. of empty flask})]}{\text{wt. of sample}}$$

Sl. No.	**Weight of empty flask (A)**	**Weight of flask + dry fat (B)**	**Weight of Fat (B - A)**	**Percentage Fat % Fat = 100 [(wt. of flask + dry Fat) – wt. of empty flask] wt. of sample**
1.	250.000	250.185	0.185	3.70
2.	250.000	250.179	0.179	3.58
3.	250.000	250.181	0.181	3.62
4.	250.000	250.183	0.183	3.66
5.	250.000	250.169	0.169	3.39
6.	250.000	250.167	0.167	3.35
7.	250.000	250.177	0.177	3.55
8.	250.000	250.180	0.180	3.60
9.	250.000	250.175	0.175	3.51
10.	250.000	250.171	0.171	3.42
			Maximum Value	**3.70**
			Minimum Value	**3.35**
			Mean Value	**3.53**

Table 2.7.1

2.7.2 Estimation of Lactose content in Goat's Milk by Lane Eynon's Method

The estimation of lactose content in Goat's Milk was done by Lane Eynon's Method with the procedure described earlier. The procedure was same as well as stated before. The formula used for estimation of lactose content was

1 ml. of Fehling's solution = 0.00645 gms. of anhydrous lactose.

Sl. No. (A)	Quantity of milk sample (in ml.) (B)	Quantity of Fehling's Solution for 10 ml. of milk sample (C)	Calculated amount of Lactose in 10 ml. of Milk sample using quantity of Fehling solution as shown in column (C) (D)	Calculated amount of Lactose in 100 ml. of milk (E)
1.	10.00	68.19	0.44	4.4
2.	10.00	71.30	0.46	4.6
3.	10.00	69.76	0.45	4.5
4.	10.00	68..20	0.47	4.7
5.	10.00	77.54	0.50	5.0
6.	10.00	74.4	0.48	4.8
7.	10.00	69.76	0.45	4.5
8.	10.00	75.98	0.49	4.9
9.	10.00	72.80	0.47	4.7
10.	10.00	68.17	0.44	4.4
			Maximum Value	**5.0**
			Minimum Value	**4.4**
			Mean Value	**4.65**

Table 2.7.2

2.7.3 Estimation of Total Soluble Sugar in Goat's Milk byFerricyanide Method

The estimation of total soluble sugar in Goat's Milk was done by Ferricyanide Method with the procedure described earlier. The procedure is same as well as stated before. The formula used for estimation of total soluble sugar was

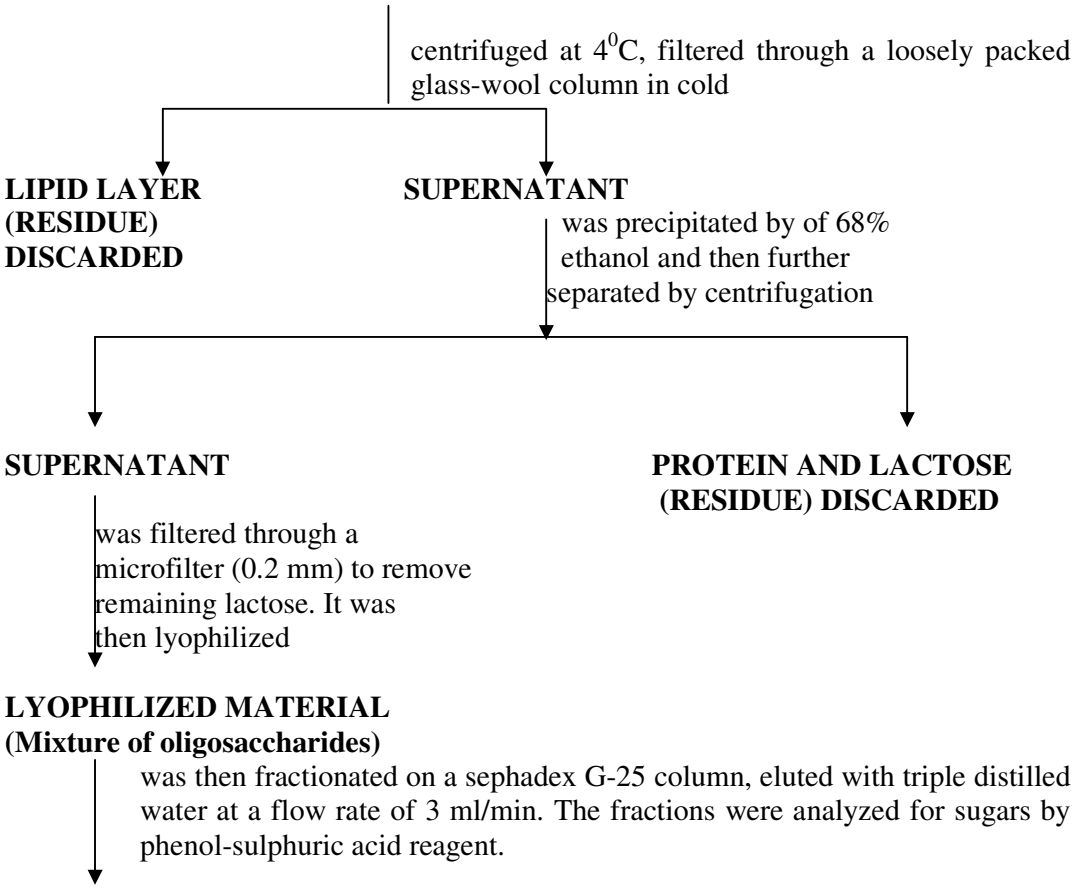
$$\mu = 0.338$$

x = vol. of 0.01 N $Na_2S_2O_3$ used for sample, i.e. vol. of $Na_2S_2O_3$ used in blank-vol. used in sample.

Sl. No.	**Volume of 0.01 $Na_2S_2O_3$ used in blank (A)**	**Volume of 0.01 $Na_2S_2O_3$ used in sample (B)**	**X = (A - B)**	**u = 0.338**	**mg of reducing sugar in 100 ml. of sample = u (x + 0.05) x 20**
1.	11.6	10.68	0.92	0.338	6.58
2.	11.6	10.67	0.93	0.338	6.65
3.	11.6	10.71	0.89	0.338	6.39
4.	11.6	10.63	0.97	0.338	6.90
5.	11.6	10.77	0.83	0.338	5.96
6.	11.6	10.73	0.87	0.338	6.22
7.	11.6	10.69	0.91	0.338	6.49
8.	11.6	10.64	0.96	0.338	6.85
9.	11.6	10.65	0.95	0.338	6.79
10.	11.6	10.63	0.97	0.338	6.90
				Maximum Value	**6.90**
				Minimum Value	**5.96**
				Mean Value	**6.57**

Table 2.7.3

ISOLASTION OF GOAT'S MILK OLIGOSACCHARIDES
BY
KOBATA AND GINSBURG METHOD



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2.7.4 Oligosaccharide Contents of Goat's Milk

Sl. No.	Method used	Volume taken	Oligosaccharide %
1	Kobata& Ginsberg Method	1 Liter	0.80
2	Kobata& Ginsberg Method	1 Liter	0.78
3	Kobata& Ginsberg Method	1 Liter	0.77
4	Kobata& Ginsberg Method	1 Liter	0.79
5	Kobata& Ginsberg Method	1 Liter	0.81
6	Kobata& Ginsberg Method	1 Liter	0.83
7	Kobata& Ginsberg Method	1 Liter	0.76
8	Kobata& Ginsberg Method	1 Liter	0.79
9	Kobata& Ginsberg Method	1 Liter	0.75
10.	Kobata& Ginsberg Method	1 Liter	0.82
	Maximum Value		**0.83**
	Minimum Value		**0.75**
	Average Value		**0.79**

Table 2.7.4

2.7.5 SEPHADEX G-25 GEL FILTRATION OF GOAT MILK OLIGOSACCHARIDE MIXTURE

The lyophilized material (mixture of oligosaccharides) of goat milk was further purified on Sephadex G-25 column chromatography for separation of enzymes, nucleic acids, peptide, glycoproteins, free proteins and other biological macromolecules from oligosaccharide by using glass distilled water as eluant at a flow rate of 5 ml/min. Goat milk oligosaccharide mixture was packed in a column (l.6x40 cm) (void volume = 25

ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 hrs to settle down. Presences of neutral sugars were monitored in all eluted fractions by phenol-sulphuric acid test. In this U.V. monitored Sephadex G-25 chromatography of goat milk oligosaccharide mixture showed seven peaks i.e. I, II, III, IV. V. A substantial amount of proteins, glycoproteins and serum albumin were eluted in void volume that was confirmed by positive colouration with p-dimethylaminobenzaldehyde reagent and phenol-sulphuric acid reagent. Fractions under peaks II and III gave a positive phenol-sulphuric acid test for sugars, which showed the presence of oligosaccharide mixture in goat milk. These fractions (peak II, III) were polled and lyophilized together.

Fraction No.	SOLVENT	Fraction No.	PHENOL H2SO4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-60	Glass triple distilled H2O		-ve	-
61-171	’’	I	-ve	-
172-275	’’	II	+++ve	Analyzed by HPLC
276-343	’’	III	+++ve	-
344-374	’’	IV	-ve	
375-389	’’	V	-ve	

Table 2.7.5

CONFIRMATION OF HOMOGENEITY OF GOAT’S MILK OLIGOSACCHARIDE BY REVERSE PHASE HPLC

Pooled fractions (peaks II, III) obtained from Sephadex G-25 column, containing oligosaccharide mixture were qualitatively analysed by reverse phase HPLC. The HPLC

system was equipped with Shimadzu CLASS-VP V6.13 solvent delivering system, 235-diode array detector and G.P. 100 printer plotter. The column used for this purpose was C18 Purosphere 25 cm x 0.4 cm x 5-μ m (from E. Merck). A binary gradient system of acetonitrile : 0.5% trifluro-acetic acid (5:95) in triple distilled water (TDW) to CH3CN : 0.5% TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 220 nm. Twelve peaks were noticed in the sample (pooled fraction II and III) at the varied retention times from 1.092 min to 20.675 in, for convenience the peaks were numbered in their increasing order of retention time i.e. [.299 (R_1), 1.323(R_2), 2.272(R_3), 3.093(R_4), 3.605(R_5), 3.968(R_6), 4.320(R_7), 5.067(R_8), 5.483(R_9), 5.728(R_{10}), 5.877(R_{11}) and 6.165(R_{12})

2.7.6 HPLC TABLE OF GOAT'S MILK OLIGOSACCHARIDE

Detector A-1 (220 nm)			
Pk#	**Retention Time**	**Area %**	**Height**
1	.299	4.55	15424
2	1.323	0.11	345
3	2.272	1.10	4216
4	3.093	21.25	60946
5	3.605	4.26	10293
6	3.968	0.06	223
7	4.320	9.26	16377
8	5.067	0.07	95
9	5.483	0.03	77
10	5.728	0.02	98
11	5.877	0.01	57
12	6.165	0.07	155

Table 2.7.6

Mare

Classification

Kingdom- Animalia
Phylum Chordata
Class Mammalia
Order Perrisodactyla
Family Equidae
Genus E. Ferrus
Species E. callabus

2.8 Estimation of Protein Content of Mare's Milk by Micro Kjheldal's Method

The estimation of protein in Mare's milk was done by Micro Kjheldal's Method. The procedure is same as well as stated before. The formula used for estimation of protein is as under:

% of protein is equal to $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$gms.

Sl. No.	Volume of 0.01 N H Cl used for blank in ml. (v)	Volume of 0.01 N H Cl used for sample in ml. (y)	N_2 present in 1 gm. digested sample $\frac{(y-v) \times 0.00014 \times 50}{5}$	N_2 present in 100 gm. Sample $\frac{(y-v) \times 0.00014 \times 50 \times 100}{5}$	% Protein content in sample $\frac{6.25 \times (y-v) \times 0.00014 \times 50 \times 100}{5}$
1.	2.18	5.26	0.0043	0.43	2.7
2.	2.18	4.92	0.0040	0.40	2.6
3.	2.18	5.38	0.0045	0.45	2.8
4.	2.18	5.83	0.0051	0.51	3.2
5.	2.18	5.58	0.0047	0.47	3.0
6.	2.18	5.26	0.0043	0.43	2.7
7.	2.18	5.43	0.0046	0.46	2.9
8.	2.18	5.15	0.0041	0.41	2.6
9.	2.18	5.95	0.0052	0.52	3.3
10.	2.18	5.38	0.0044	0.44	2.8
				Maximum Value	**3.3**
				Minimum Value	**2.6**
				Mean Value	**2.8**

Table 2.8

2.8.1 Estimation of Fat Content of Mare's Milk by Rose Gottlieb's Method

The estimation of fat in Mare's milk was done by Rose Gottlieb Method with the procedure described earlier. The formula used for estimation of fat was

$$\text{Percent fat} = \frac{100[(\text{wt. of flask} + \text{dry fat}) - (\text{wt. of empty flask})]}{\text{wt. of sample}}$$

Sl. No.	**Weight of empty flask (A)**	**Weight of flask + dry fat (B)**	**Weight of Fat (B - A)**	**Percentage Fat** % Fat = 100 [(wt. of flask + dry Fat) – wt. of empty flask] / wt. of sample
1.	250.000	250.084	0.084	1.68
2.	250.000	250.089	0.089	1.78
3.	250.000	250.091	0.091	1.82
4.	250.000	250.088	0.088	1.76
5.	250.000	250.094	0.094	1.89
6.	250.000	250.088	0.088	1.76
7.	250.000	250.087	0.087	1.75
8.	250.000	250.089	0.089	1.78
9.	250.000	250.087	0.087	1.74
10.	250.000	250.088	0.088	1.77
			Maximum Value	**1.89**
			Minimum Value	**1.68**
			Mean Value	**1.77**

Table 2.8.1

2.8.2 Estimation of Total Soluble Sugar in Mare's Milk byFerricyanide Method

The estimation of total soluble sugar in Cow's milk was done by Ferricyanide Method with the procedure described earlier. The procedure is same as well as stated before. The formula used for estimation of total soluble sugar was

$\mu = 0.338$

x = vol. of 0.01 N $Na_2S_2O_3$ used for sample, i.e. vol. of $Na_2S_2O_3$ used in blank-vol. used in sample.

Sl. No.	**Volume of 0.01 $Na_2S_2O_3$ used in blank (A)**	**Volume of 0.01 $Na_2S_2O_3$ used in sample (B)**	**X = (A - B)**	**u = 0.338**	**mg of reducing sugar in 100 ml. of sample = u (x + 0.05) x 20**
1.	11.6	10.44	1.16	0.338	8.19
2.	11.6	10.43	1.17	0.338	8.27
3.	11.6	10.48	1.12	0.338	7.92
4.	11.6	10.51	1.09	0.338	7.72
5.	11.6	10.45	1.15	0.338	8.13
6.	11.6	10.46	1.14	0.338	8.10
7.	11.6	10.49	1.11	0.338	7.86
8.	11.6	10.45	1.15	0.338	8.15
9.	11.6	11.47	1.13	0.338	8.01
10.	11.6	10.43	1.17	0.338	8.28
			Maximum Value	**1.89**	**8.28**
			Minimum Value	**1.45**	**7.72**
			Mean Value	**1.65**	**8.06**

Table 2.8.2

2.8.3. Estimation of Lactose content in Mare's Milk by Lane Eynon's Method

The estimation of lactose content in Mare's Milk was done by Lane Eynon's Method with the procedure described earlier. The procedure is same as well as stated before. The formula used for estimation of lactose content was

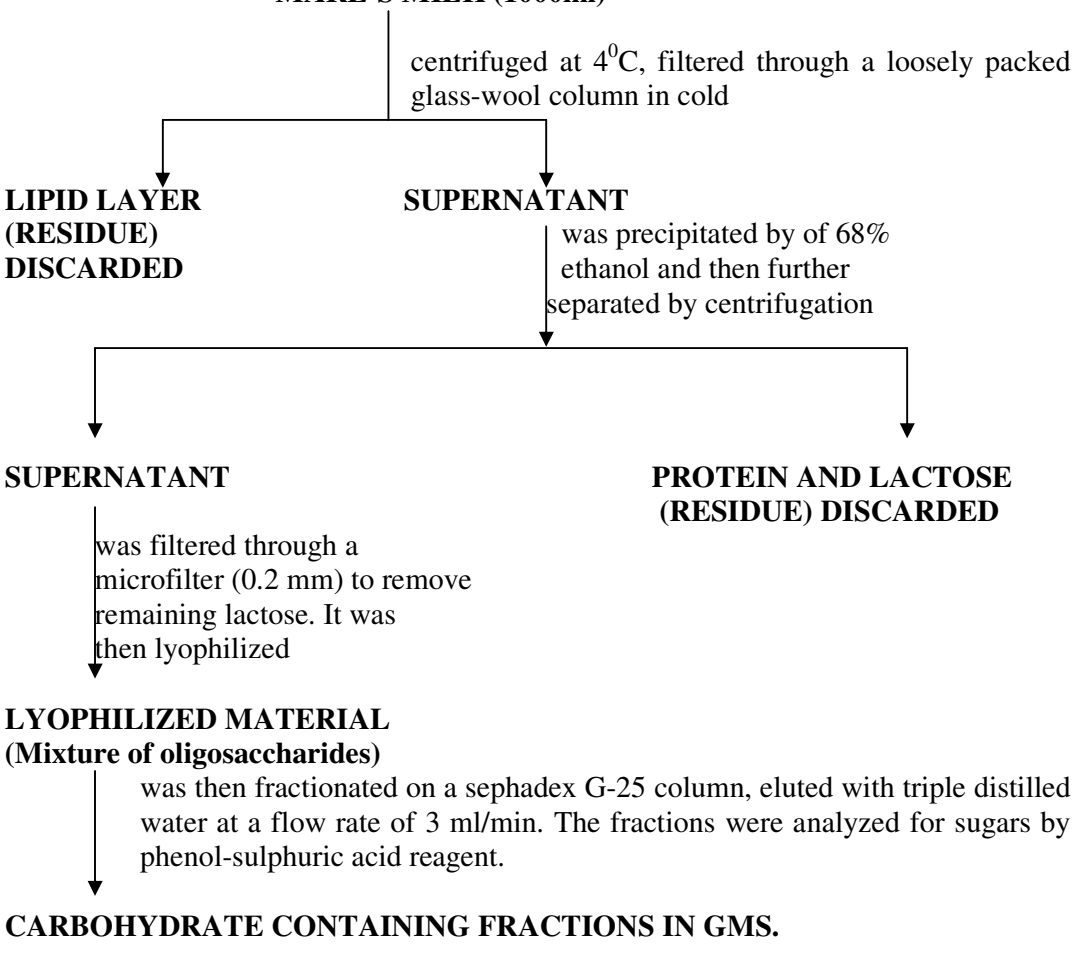
1 ml. of Fehling's solution = 0.00645 gms. of anhydrous lactose.

MARE'S MILK

Sl. No. (A)	Quantity of milk sample (in ml.) (B)	Quantity of Fehling's Solution for 10 ml. of milk sample (C)	Calculated amount of Lactose in 10 ml. of Milk sample using quantity of Fehling solution as shown in column (C) (D)	Calculated amount of Lactose in 100 ml. of milk (E)
1.	10.00	89.91	0.58	5.8
2.	10.00	94.57	0.61	6.1
3.	10.00	97.67	0.63	6.3
4.	10.00	99.22	0.64	6.4
5.	10.00	93.02	0.60	6.0
6.	10.00	96.12	0.62	6.2
7.	10.00	102.32	0.66	6.6
8.	10.00	106.97	0.69	6.9
9.	10.00	96.15	0.62	6.2
10.	10.00	105.42	0.68	6.8
			Maximum Value	**6.9**
			Minimum Value	**5.8**
			Mean Value	**6.3**

Table 2.8.3

ISOLASTION OF MARE'S MILK OLIGOSACCHARIDES
BY
KOBATA AND GINSBURG METHOD



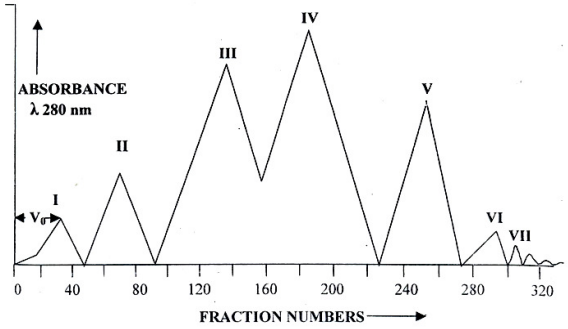
2.8.4 Oligosaccharide Contents of Mare's Milk

Sl. No.	Method used	Volume taken	Oligosaccharide %
1	Kobata& Ginsberg Method	1 Liter	0.82
2	Kobata& Ginsberg Method	1 Liter	0.79
3	Kobata& Ginsberg Method	1 Liter	0.80
4	Kobata& Ginsberg Method	1 Liter	0.81
5	Kobata& Ginsberg Method	1 Liter	0.83
6	Kobata& Ginsberg Method	1 Liter	0.84
7	Kobata& Ginsberg Method	1 Liter	0.80
8	Kobata& Ginsberg Method	1 Liter	0.79
9	Kobata& Ginsberg Method	1 Liter	0.78
10.	Kobata& Ginsberg Method	1 Liter	0.77
	Maximum Value		**0.84**
	Minimum Value		**0.77**
	Average Value		**0.80**

Table 2.8.4

2.8.5 SEPHADEX G-25 GEL FILTRATION OF MARE MILK OLIGOSACCHARIDE MIXTURE

The lyophilized material of Mare milk was further purified on Sephadex G-25 column chromatography for separation of macromolecules from oligosaccharide by using glass distilled water as eluant at a flow rate of 5 ml/min. Presences of neutral sugars were monitored in all eluted fractions by phenol-sulphuric acid test. The U.V. monitored Sephadex G-25 chromatography of milk oligosaccharide mixture showed seven peaks. A substantial amount of macromolecules were eluted in void volume confirmed by positive colouration with p-dimethyl aminobenzaldehyde reagent. Fractions under peaks II and III gave a positive phenol-sulphuric acid test for sugars, which showed the presence of oligosaccharide mixture in Mare milk. These fractions were polled and lyophilized together.



Fraction No.	SOLVENT	Fraction No.	PHENOL H_2SO_4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-27	Glass triple distilled H2O		+ve	-
28-46	’’	I	-ve	
47-67	’’	II	+++ve	HPLC monitoring
68-79	’’	III	++ve	
80-98	’’	IV	-ve	

Table 2.8.5

CONFIRMATION OF HOMOGENEITY OF MARE'S MILK OLIGOSACCHARIDE BY REVERSE PHASE HPLC

Pooled fractions (peaks II, III, IV and V) obtained from Sephadex G-25 column, containing oligosaccharide mixture were qualitatively analysed by reverse phase HPLC. The column used for this purpose was C18 Purosphere 25 cm x 0.4 cm x 5-µ m (from E. Merck). A binary gradient system of acetonitrile : 0.5% trifluro-acetic acid (5:95) in triple distilled water (TDW) to CH3CN : 0.5% TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 220 nm. Ten peaks were noticed in the sample (pooled fraction II and III) at the varied retention times from 1.092 min to 20.675 in, for convenience the peaks were numbered in their increasing order of retention time i.e. 1.092 (R_1), 2.025 (R_2), 3.275 (R_3), 4.750 (R_4), 7.517 (R_5), 11.500 (R_6), 13.800 (R_7), 19.000 (R_8), 19.875 (R_9), 20.675 (R_{10}).

2.8.6 HPLC TABLE OF CRUDE MARE'S MILK OLIGOSACCHARIDE

Detector A-1 (220 nm			
Pk#	**Retention Time**	**Area %**	**Height**
1	1.092	1.664	7032
2	2.025	4.765	17430
3	3.275	16.707	104875
4	4.750	14.284	33992
5	75517	55.265	61327
6	11.500	5.583	18020
7	13.800	0.418	2549
8	19.000	0.322	6356
9	19.875	0.756	6152
10	20.675	0.235	3160

Table 2.8.6

The aim of this study was to investigate the antioxidant properties of different ruminant's milk. The LPO, SOD and catalase assay; antioxidative enzyme activities were used to determine the antioxidant properties of various milks of different

ruminants as each ruminant has its own unique property and the antioxidative agents present can give rise to a new horizon of natural antioxidants in the field of neutriceuticals

2.9 Reagents and Consumables

All the specified chemicals and reagents were purchased from Sigma (Sigma St Louis, MO, USA) unless otherwise stated. Culture wares and other plastic wares used in the study were procured commercially from Nunc, Denmark. Milli Q water (double distilled deionized water) was used in all the experiments.

Isolation of milk oligosaccharides

Milk oligosaccharides have been isolated by using the protocol of Kobata and Ginsberg[107]. In brief, 1000 ml of milk was collected and stored at -20^0 C and centrifuged for 15 min at 5000 rpm at 4^0 C. The solidified lipid layer was removed by filtration through glass wool column in cold. Ethanol was added to the clear filtrate to a final concentration of 68% and the resulting solution was left overnight at 0^0 C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^0 C. The supernatant and washings were combined and filtered through a micro filter to remove remaining lactose and lyophilized affording crude oligosaccharide mixture. The lyophilized material (mixture of oligosaccharides) was further purified by fractioning it on sephadex G-25 column using glass double distilled water as eluant at a flow rate of 3 ml/min. Each fraction was analyzed for sugars by phenol sulphuric acid reagent for presence of sugars.

2.9.1 Estimation of lipid peroxidation (LPO) levels

Lipid peroxidation (LPO) was performed using the thiobarbituric acid reactive substances (TBARS). In brief, 0.2 ml of saliva was taken and added with 2 ml of thiobarbituric acid reagent (15% TCA, 0.7% TBA, and 0.25 N HCl) and heated at 100^0 C for 15 min in a water bath. The sample were then placed in cold and centrifuged at 1000xg for 10 min. Absorbance of the supernatant was measured at 535 nm using microplate reader (Synergy HT, BioTek, USA). The data are expressed as nmol MDA/ml.

2.9.2 Superoxide dismutase (SOD) activity

The activity was measured following the protocol described earlier by Mc Cord and Fridovich. Im brief, the experiment was carried out in two setups. In one setup, 1.1 ml pyrophosphate buffer, 0.2 ml NBT, 0.2 ml PMS and 20 µl enzyme source were taken. Second setup received all the above reagents minus the enzyme source. The reaction was started simultaneously in two sets by the addition of 0.2 ml NADH. After a interval of 90 seconds, 0.5 ml glacial acetic acid was taken to each tube for checking the reaction, after this same amount of enzyme source was added in reference tubes. The absorbance was read at 560 nm using microplate reader (Synergy HT, BioTek, USA) against reagent blank. Difference between reference and experimental optical density (OD) gives the inhibition of NBT reduction by an enzyme source. Protein was also estimated in enzyme source. The unit of SOD enzyme activity was defined as the amount of enzyme required to inhibit the OD at 560 nm of NBT reduction by 50% in ine minute under the assay conditions.

2.9.3 Estimation of catalase levels

The experimental setup for catalase activity was quite similar to the LPO. The activity was measured using commercially available kit for catalase activity (Catalog no. 707002; Cayman Chemicals, USA) following the protocol provide by manufacturer. In brief, 100 µl of assay buffer (supplied in the kit), 30 µl of methanol, and 20 µl of sample were mixed in the 96-well plate. Reaction was initiated by adding 20 µl of hydrogen peroxide (0.882 M) and incubated in shaker for 20 min at room temperature. Reaction was stopped by adding 30 µl of potassium hydroxide. Then chromogen (30 µl) was added and incubated for 10 min followed by addition of potassium periodate (10 µl). The plates were kept at room temperature for 5 min and read at 540 nm using multiwell micro plate reader (Synergy HT, BioTek, USA).

2.9.4 Invitro antioxidant assay by DPPH method[90-95]

DPPH (2, 2-Diphenyl-1-picrylhydrazyl)is a stable free radical which is blue coloured in the presence of an antioxidant, this radical is reduced and the purple colour of DPPH is bleached to yellow. The absorbance measured at 517nm is the extent of bleaching recorded as the antioxidant activity of the coumpound.

Method:

100 micro litre of sample is taken in this assay was performed by gulluce et al (2006).The hydrogen atom or neutron donation ability of the samples was measured from the bleaching of the purple colored methanol solution of DPPH this

spectrophotometric assay uses stable DPPH as a reagent 100 micro litre of the sample was added to 5 ml of 0.004% methanol solution DPPH after 30 mins of incubation time at room temperature and the absorbance was read against the blank at 570nm. The control does not contain any sample. BHT was taken as the control. Butylated hydroxyl toluene a natural antioxidant . The antioxidant activity of the sample was calculated in percentage from comparing with BHT,s absorbance .

S. No.	Sample	Amt	OD	Absorbance%
1	BHT	100 mg	0.401	100 %
2	COW	100 mg	1.090	Negative
3	MARE	100 mg	1.080	Negative
4	CHAURI	100 mg	0.751	87.50
5	SHEEP	100 mg	1.029	Negative

Table 2.9.4

BHT ——————Chauri
0.4 0.75

$$\frac{100}{0.4} \times 0.75 = 87.5\ \%$$

Hence results showed that Chauri milk had the potential property anti oxidants which helped to reduce oxidation as it was compared with a natural antioxidant BHT and showed approximate 87% of oxidation reducing capacity of Milk oligosacchords.

General Procedures

The evaporation of alcohol from crude extract of milk oligosaccharides was done on Buchi Rotatry evaporator. The freeze drying of the compounds was done with the help of CT 60e (HETO) lyophilizer and centrifuged with the help of cooling centrifuge Remi instruments C-23 JJRCI 763. The HPLC system was equipped with Shimadzu CLASS-VP V6.13 solvent delivering System, 235-diode array detector and G.P. 100 printer plotter. The column used for this purpose was C18 Purosphere 25 cm x 0.4 cm x 5-µm (from E. Merck). A binary gradient system of acetonitrile: 0.5% trifluro-acetic acid (5:.95) in triple distilled water (TDW) to CH_3CN: 0.5 % TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 220 nm. Authentic samples of glucosamine, galactosamine, galactose, glucose, fucose and sialic acid were purchased from Aldrich Chemicals.

CHROMATOGRAPHY

The chromatographic techniques that have been used for the isolation and identification of the compounds are given below.

THIN LAYER CHROMATOGRAPHY

In the thin layer chromatography glass plates coated with slurry of silica gel G (SRI,) in water were used which were dried at room temperature for about 24 h and activated at

100-110^{o}C. For TLC;Chloroform and methanol in different proportions were used as solvent system.

PAPER CHROMATOGRAPHY (PC)

Paper chromatography was performed on Whatman No. I papers by using a three solvent system of ethyl acetate, pyridine and H_20 and spots were detected by appropriate reagent for specific moieties.

GEL PERMEATION CHROMATOGRAPHY

In this chromatography Sephadex G-25 (PHARMACIA) was used for the separation of protein, glycoprotein and oligosaccharides etc. from crude milk oligosaccharide extract. In the Column chromatography silica gel (SRL, 60-120 mesh) measured as adsorbent and solvent system used was Hexane: Chloroform, Chloroform and varied proportions of Chloroform: Methanol were used. The solvent of each fraction was reduced off on Buchi rotary evaporator on 50-60^{0}C which gave syrupy compound it was further detected by TLC.

TEST FOR SUGARS

Many types of tests were performed for the identification of normal, deoxy, amino or sialylated sugar residue present in oligosaccharide moiety. They were described as under-

PHENOL SULPHURIC ACID TEST FOR SUGARS

For the phenol-sulphuric acid test for sugars, in a carbohydrate containing fraction (0.1 ml), distilled water (1ml), 5% phenol (1 ml) and conc. H_2SO_4 (2.5 ml) was added and the mixture was vigorously shaken. After 30 min the carbohydrate-rich fractions gave yellow colour for presence of carbohydrate.

FIEGL TEST FOR SUGARS

In the Fiegl test for sugars, the test sample (0.1 mg)was placed in a microcrucible and then one drop of syrupy phosphoric acid was added and the crucible was covered with filter paper moistened with a 10% solution of aniline in AcOH (10%). A small watch glass was used as a paper weight. The bottom of the crucible was cautiously heated for 30-60 min with micro-burner, avoiding superheating. The development of pink to red colour was imparted on filter paper by normal sugars and 6-deoxy sugars exhibited brown coloration.

PARTRIDGE REAGENT FOR SUGARS

Freshly distilled aniline (0.93 g) in water saturated with butanol (100 ml) was mixed with phthalic acid (1.66 g) and was shaken to dissolve. The reagent was sprayed on the paper on which a spot of test sample was applied and then heated at 100-118°c for 3-5 min. A pink-brown colour indicated the, presence of normal hexoses.

MORGON-ELSON TEST FOR SUGARS

Acetyl-acetone reagent I: Solution A, 0.5 ml of acetyl-acetone was dissolved in butanol (50 ml). Solution B, 50% (w/v) aq. KOH (5 ml) was dissolved in ethanol (20 ml). 0.5 ml of solution B was added to 10 ml of Solution A to get reagent I.

p-Dimethylaminobenzaldehyde reagent II: 1g of p-dimethylaminobenzaldehyde was dissolved in 30 ml of ethanol and 30 ml of conc. HCl and. the solution was then diluted with 180 ml of distilled butanol to get reagent II.

Chromatograms were sprayed with reagent I and heated in the oven for 5 min. at 105°C. The dry paper strips were then sprayed with reagent II and returned to oven for a further short hot treatment of 5 min at 90°C. The appearance of purple-violet colour indicated the presence of amino sugars.

THIOBARBITURIC ACID ASSAY (WARREN ASSAY) FOR SIALIC ACID

Reagent A: Dissolved 1.07 g of sodium metaperiodate in 1.0 ml of water was added to 14.5 ml of concentrated orthophosphoric acid and made up to 25 ml with water.

Reagent B: Dissolved 1.0 g of sodium arsenite and 0.71 g of sodium sulphate in 0.1 M sulphuric acid (made by diluting 0.57 ml concentrated sulphuric acid with 100 ml of water) to a total volume of 10 ml.

Reagent C: Dissolved 0.12 g of thiobarbituric acid and 1.42 g of sodium sulphate in water to a total volume of20 ml.

In the sample (0.05 μ moles in a volume of 0.2 ml) 0.1 ml of reagent A is added. The tube was shaken and allowed to stand at room temperature for 20 minutes. 1 ml of reagent B was then added and the tube was shaken until a yellow-brown colour fades3 ml of reagent C was added in the tube with shaking; it was capped with a glass bead land then heated in a vigorously boiling water bath for 15 minutes. The tube was then removed and placed in cold water for 5 minutes. During cooling the colour faded and the solution becomes cloudy. From this solution, 1 ml was transferred to another tube which contained 1 ml of cyclohexanone. The tube was shaken and then centrifuged for 3 minutes. The clear upper cyclohexanone phase was red and the colour was more intense than it was when in water.

BROMOCRESOL-GREEN TEST

Spraying reagent: Bromocresol green (0.04 g) was dissolved in ethanol (96%, 100 ml). Drops of 0.1 N NaOH was added until a blue colouration just appeared. A spot of the test sample when applied on a paper moistened with this reagent (blue), if giving a yellow colouration indicated the presence ofa carboxylic group.

Kiliani Hydrolysis of Cow milk oligosaccharides

5 mg mixture obtained from Kobata Ginsberg method was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100°C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH to it and was evaporated under reduced pressure to afford glucose, galactose, fucose, and N-acetyl glucosamine which were identified by comparison with authentic sample (TLC, PC)

Kiliani Hydrolysis of Buffalo milk oligosaccharides

5 mg mixture obtained from Kobata Ginsberg method was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7: 11 :2) and heated at 100°C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH to it and was evaporated under reduced pressure to afford glucose,

galactose, fucose,N-acetyl glucose and N-acetyl Neuraminic acid whichwere identified by comparison with authentic sample (TLC, PC).

Kiliani Hydrolysis of Sheep milk oligosaccharides

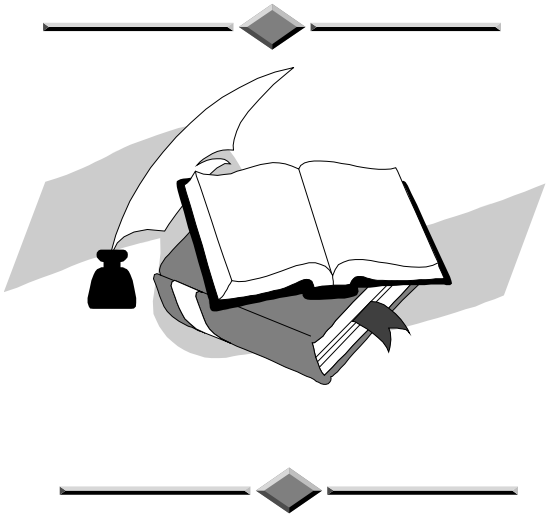
5 mg mixture obtained from Kobata Ginsberg method was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7: 11 :2) and heated at 100°C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH to it and was evaporated under reduced pressure to afford glucose, galactose, fucose,N-acetyl glucose and N-acetyl Neuraminic acid whichwere identified by comparison with authentic sample (TLC, PC).

Kiliani Hydrolysis of Camel milk oligosaccharides

5 mg mixture obtained from Kobata Ginsberg method was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7: 11 :2) and heated at 100°C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH to it and was evaporated under reduced pressure to afford glucose, galactose, fucoseand N-acetyl glucosamine which were identified by comparison with authentic sample (TLC, PC).

CHAPTER III

RESULTS AND DISCUSSION



CHAPTER III

3.1 RESULTS AND DISCUSSION

The results of this study have reaffirmed the fact that milk can be most beneficial and nutritionally complete food for all age groups. Milk not only has excellent nutritional properties. It also has immunological properties. In this study, milks of seven animals i.e. Cow, buffalo, sheep, goat, Chauri-cow, camel and mare were tested and studied for their nutritional and Bioactive properties. The macronutrients i.e. the fat, protein and carbohydrate contents of the milks mainly contributed to their nutritional properties whereas oligosaccharides mainly contribute to their immunological and other biological characteristics.

NUTRITIVE TABLE OF MACRO NUTRIENTS PRESENT IN VARIOUS MILKS

SL. NO.	SPECIES / NUTRIENTS %	COW	BUFFALO	SHEEP	CAMEL	CHOWRI	MARE	GOAT
1.	PROTEIN	4.1	5.9	5.7	4.0	1.9	2.8	3.9
2.	FAT	5.58	10.44	5.40	5.0	1.41	1.77	3.53
3.	TOTAL SOLUBLE SUGAR	7.42	6.58	7.10	7.23	8.81	6.30	6.57
4.	LACTOSE	4.91	4.15	5.0	5.10	7.01	8.06	4.65
5.	OLIGOSACC-HARIDES	0.85	0.88	0.81	0.86	0.91	0.80	0.79

Table 3.1

The Table shows the comparative amount of various nutrients in Milks of different animals. The protein content of buffalo's milk is 5.9% which is the highest followed by

sheep's milk which has 5.5% protein. The cow, camel, goat, mare and Chauri have got 4.1, 4.0, 3.9, 2.8 and 1.9 % protein respectively. The high percentage of protein in buffalo's milk makes it an ideal drink for the vulnerable sections of the Society, especially for pregnant women, lactating women, growing children and adolescents. However, because of its high fat content which is 10.44% buffalo's milk should not be recommended for adult people and old aged. Buffalo's and sheep's milk can be of great therapeutic importance in patients suffering from severe catabolic disorders like cancer, tuberculosis, typhoid, anaemia, post-surgical conditions, burns and trauma. These milks can supply the much needed high protein and calories for tissue repair and energy. Cow milk has 3.9% protein which can be easily digested and absorbed by the growing infant. Cow milk also has a high lactose content, this combination makes it most suitable for infantile digestion and absorption. Since buffalo's milk has a high fat content (10.44%), it cannot be given to people suffering from metabolic disorders such as diabetes, hypertension, cardiovascular diseases, obesity and certain hepatic and kidney diseases. In these conditions, milk of Chauri-cow and marecan provide the patient with non-fat calories and proteins.

The fat content of buffalo's milk was the highest 10.44% followed by cow milk 5.58, sheep 5.40%, camel 5.05%, goat 3.53%, mare 1.77% and Chauri 1.41%. Buffalo's milk because of its high fat content would be suitable for underweight persons and people suffering from catabolic diseases. However, it cannot be given to people who are either obese of suffer from cardiac diseases. Chauri-cow milk and mare's milk can be given in weight reducing diets also.

The lactose content of Chauri's milk was the highest which is 6.9% followed by mare 6.1%, camel 5.1%, cow 4.91%, sheep 4.90%, goat4.35% and buffalo 4.02%.

Lactose a disaccharide composed of glucose and galactose is the principle sugar of mammalian milk and the main carbohydrate energy source for infants and children. Thus lactose plays a central metabolic role in human nutrition. Lactose is hydrolysed in the intestine by the enzyme lactase-phlorizin hydrolase to glucose and galactose. However, in some conditions small intestine does not make enough enzyme lactase. In the absence of this enzyme lactose does not split into glucose and galactose which is essential for its absorption by the cell lining of small intestine. This condition known as **lactose intolerance** has symptoms of gastrointestinal origin mainly abdominal pain, cramping, diarrhoea, flatulence, stomach bloating, abdominal distension and nausea Symptoms occur because unabsorbed lactose passes through the small intestine into the colon. It can also have long term effects like calcium and vitamin "D" deficiency leading to osteoporosis. Another disease galactosemia which is charactersized by high blood levels of galactose is caused by one or more of the enzyme necessary for metabolizing galactose. A metabolic build up is made which is toxic to the lever and kidneys and also damage the lens of the eyes causing cataract. Galactose is an important constituent of the complex polysaccharide which is the part of the cell glycoconjugates, key elements of immunologic determinants, hormones, cell membranes, structures and it is also incorporated in galacto lipids which are important structure elements of the central nervous system. Neonates suffering from galactosemia have symptoms like failure to thrive, diarrhea, jaundice, hepatomegaly cataracts, galactoseuria, gonadal dysfunction, developmental delay and neurological symptoms etc. In cases of secondary lactose intolerance which occurs because of a disease which causes harm to the intestinal lining milks of reduced lactose content by cow, buffalo and sheep milk can be given. In cases of galactosemia a strict galactose free diet is the only permanent

treatment. Commercially prepared milk in which the lactose content has been reduced, are prepared at processing plants by adding the liquid enzyme lactase to pasteurized milk and storing it for 24 hours. Milk that has 99.9% of this lactose hydrolysed is leveled lactose free.

In all the milks Chauri's milk had the highest total soluble sugars content 8.81% followed by cow 7.42%, camel 7.23, sheep 7.10%, buffalo 6.58%, mare 6.30% and goat 6.57% because of their high soluble sugar content Chauri's and mare's milk can serve as reservoirs of ready energy to infants as well as adults. Apart from supplying energy the various sugars change to acid in the gastro intestinal tract thus making environment acidic and healthy for pro biotic bacteria sugar helps in absorption of calcium and phosphorus. The sugar content of milk makes it nutritionally and therapeutically very important. Milk is a perfect blend a non-reactive buffers, high quality protein sugars and lipids. The main sugars present in milk are lactose, Sucrose and Glucose in small amounts. This nutritional make up makes milk a healthy diet for all age groups and nearly all clinical conditions.

The oligosaccharide contents of various milks was as follows ; Chauri-cow (0.91%), buffalo (0.88%), cow (0.85%), camel (0.83%), mare (0.80%), sheep (0.79%) and goat (0.79%). Oligosaccharides provide the milk unique immunological properties they inhibit adherence of many pathological microorganisms to human epithelial cells, thus protecting the infants as well as adults from various life threatening infections. Apart from this they also inhibit binding a toxin realest by these microorganisms to the mucosal cells. Milk because of its excellent nutritional profile can saved the consumer from many deficiency diseases. In the growing years deficiency of the protein and calories can cause growth retardation, show mental development and diseases like

kwashiorkor and marasmus. Growing children can be victim of vitamin A deficiency showing symptoms or night blindness. Deficiency of vitamin D and Calcium can cause rickets. Consumption of milk can be helpful in these entire situations.

3.2 Analysis of Oligosaccharides of various Milks

The chemical degradation of cow's milk oligosaccharides have shown the presence of glucose, galactose, fucose and N-acetyl glucose. They are all biologically and immunologically bio active compounds and they also provide unique immune resistance to the neonates.

The chemical degradation of buffalo milk oligosaccharides have shown the presence of glucose, galactose, fucose and N-acetyl glucose in it. Due to presence of these monosaccharide units in branched form have shown immunostimulant activity.

When the chemical degradation of sheep's milk oligosaccharides was done, they have shown the presence of glucose, galactose, fucose and N-acetyl glucose in them. With the presence of these compounds sheep milk has got health promoting properties.

The chemical degradation of camel milk oligosaccharides have shown the presence of glucose, galactose, fucose and N-acetyl glucose in it. Because of the presence of these oligosaccharides camel milk can restrict the adherence of many pathogenic bacteria to the human epithelial cells and also has immune enhancing properties.

The chemical degradation of Chauri's milk oligosaccharides shownthe presence of glucose, galactose, fucose and N-acetyl glucose in it. It has also shown the presence of N-acetyl Neuraminic acid (sialic acid). Due to the presence of Neuraminic acid varied biological activity like immunostimulant and cosmetic properties have been

shown by Chauri's milk.After the chemical degradation, goat milk oligosaccharides have shown the presence of glucose, galactose, fucose and N-acetyl glucose. The goat milk have shown health promoting and immune enhancing properties.The chemical degradation of mare's milk oligosaccharides have shown the presence of glucose, galactose, fucose, N-acetyl glucose and N-acetyl neuraminic acid. It has shown anti epilepsy and anti tubercular properties.Due to the presence of these oligosaccharides moieties in milk various biological activities have been shown by various milks. The biological activities depend upon the glycosidic linkages, and position of monosaccharides in the straight or branched chains of the milk sugars. Milk naturally contains an array of bioactive compounds which exert a regulatory activity in the human organism beyond basic nutrition. These bioactivities are due to lysozymes, lactoferrins, immunoglobulin's, growth factors and hormones which are secreted in their active form by the mammary gland "bidifus-factor", that gives immunity against infections and also promote "good" intestinal health. The bifidus bacteria produce lactic acid and thus help in maintaining acidic environment in the intestine.

3.2.1 Core series of milk oligosaccharides detected in samples

Structure	Name
Gal(β1-4)Glc	Lactose
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-N-tetraose
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-N-neotetraose
Gal(β1-4)GlcNAc(β1-6) Gal(β1-3)GlcNAc(β1-3)	Lacto-N-hexaose
Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)GlcNAc(β1-3)	
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-3)Glc(β1-4)Glc	Lacto-N-neohexaose
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-3)Glc(β1-4)Glc	
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc	para-Lacto-N-hexaose
Gal(β1-4)GlcNAc(β1-3) Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)GlcNAc(β1-3) Gal(β1-4)Glc	para-Lacto-N-neohexaose
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc	Lacto-N-octaose
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	
Gal(β1-4)GlcNAc(β1-6) Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)GlcNAc(β1-6) Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc	Lacto-N-neooctose
	iso-Lacto-N-octaose
Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)GlcNAc(β1-3) Gal(β1-4)GlcNAc(β1-6) Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc	para-Lacto-N-octaose
	Lacto-N-decaose
	Lacto-N-neodecaose

Table 3.2.1

3.2.2 Acidic milk oligosaccharides

1. Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
2. Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
3. Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-6)
   GlcNAc(β1-3) } Gal(β1-4)Glc
4. Neu5Ac(α2-3) { Gal(β1-4)GlcNAc(β1-6)
   GlcNAc(β1-3) } Gal(β1-4)Glc
5. Neu5Ac(α2-6Gal(β1-4)GlcNAc(β1-6)
   GlcNAc(β1-3) } Gal(β1-4)Glc
6. Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)
   Gal(β1-3) } Gal(β1-4Glc)
7. Neu5Ac(α2-3) { Gal(β1-4)GlcNAc(β1-
   Gal(β1-4)GlcNAc(β1- } Gal(β1-4)Glc
8. Neu5Ac(α2-6) { Gal(β1-4)GlcNAc(β1-
   Gal(β1-4)GlcNAc(β1- } Gal(β1-4)Glc
9. Neu5Ac(α2-6)GalNAc(β1-4)GlcNAc
10. Neu5Ac(α2-3)Gal(β1-4)Glc
11. Neu5Gc(α2-3)Gal(β1-4)Glc
12. Neu5Ac(α2-6)Gal(β1-4)GlcNAc
13. Neu5Gc(α2-6)Gal(β1-4)GlcNAc
14. Neu5Ac(α2-6)Gal(β1-4)Glc
15. Neu5Gc(α2-6)Gal(β1-4)Glc
16. Neu5Ac(α2-6)Gal(β1-4)Glc
    |
    GlcNAc(β1-3)
17. Neu5Ac(α2-3)Gal(β1-4)Gal(β1-4)Glc
18. Neu5Ac(α2-6)Gal(β1-4)Glc
    |
    Gal(β1-3)

19. Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc
20. Neu5Gc(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc
21. Neu5Ac(α2-8)Neu5Gc(α2-3)Gal(β1-4)Glc
22. Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)GlcNAc

3.2.3 Neutral milk oligosaccharides

1. GalNAc(β1-4)GlcNAc
2. GalNAc(β1-4)Glc
3. GalNAc(β1-4)GlcNAc
4. Fuc(α1-2)Gal(β1-4)Glc
5. GalNAc(α1-3)Gal(β1-4)Glc
6. GalNAc(β1-3)Gal(β1-4)Glc
7. Gal(β1-6)Gal(β1-4)Glc
8. Gal(α1-3)Gal(β1-4)Glc
9. Gal(β1-3)Gal(β1-4)Glc
10. Gal(β1-4)Gal(β1-4)Glc
11. Fuc(α1-2)Gal(β1-4)Glc
    |
    GalNAc(α1-3)
12. Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc

13. Gal(β1-4)GlcNAc(β1-6) \
    GlcNAc(β1-3) — Gal(β1-4)Glc
14. Gal(β1-4)GalNAc(β1-6) \
    Gal(β1-3) — Gal(β1-4)Glc
15. Gal(β1-4)GlcNAc(β1-6) \
    Gal(β1-4)GlcNAc(β1-) — Gal(β1-4)Glc

BPC of both Chauri-cow and Camel milk oligosaccharides

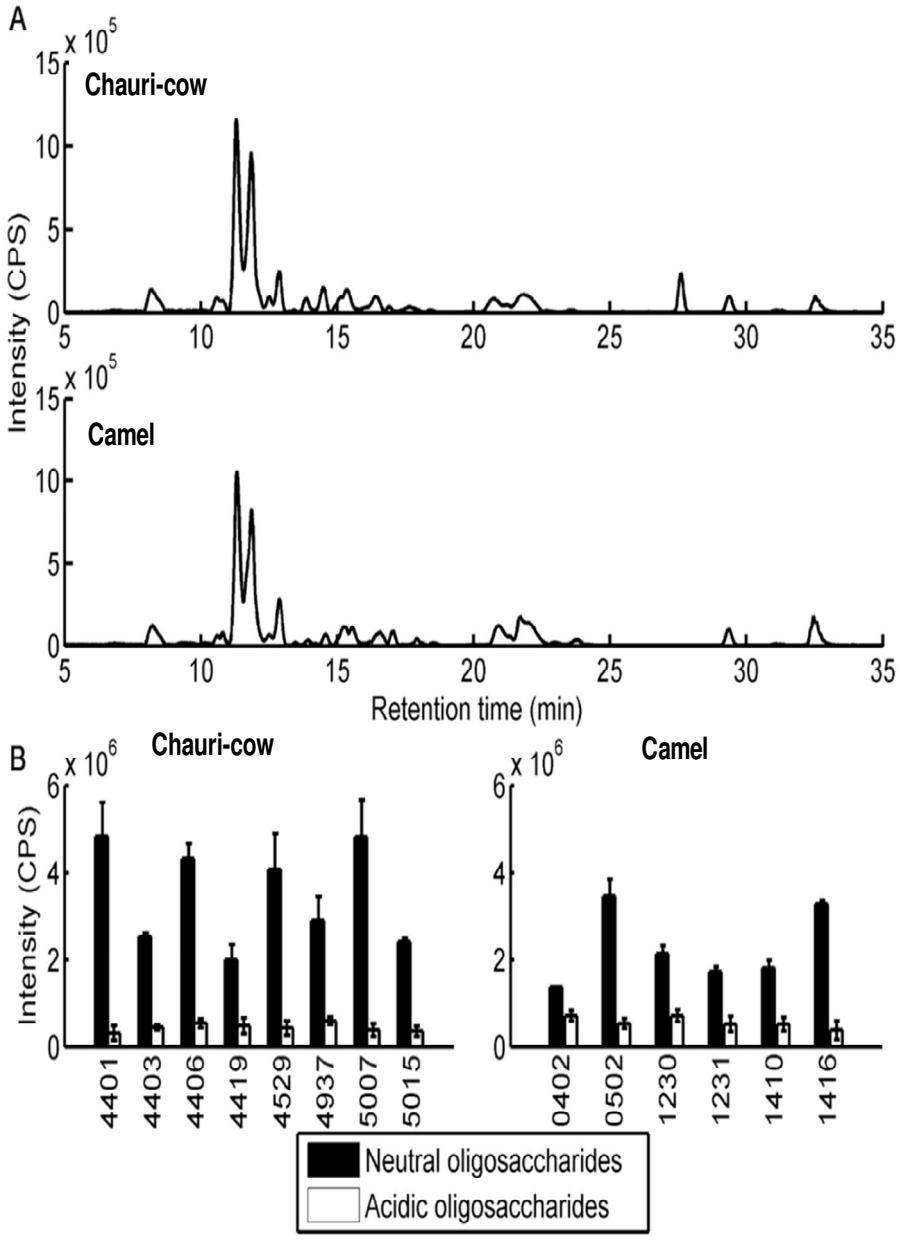


Figure-3.2.1

[Neutral and acidic oligosaccharide abundances in 14 samples measured in duplicates]

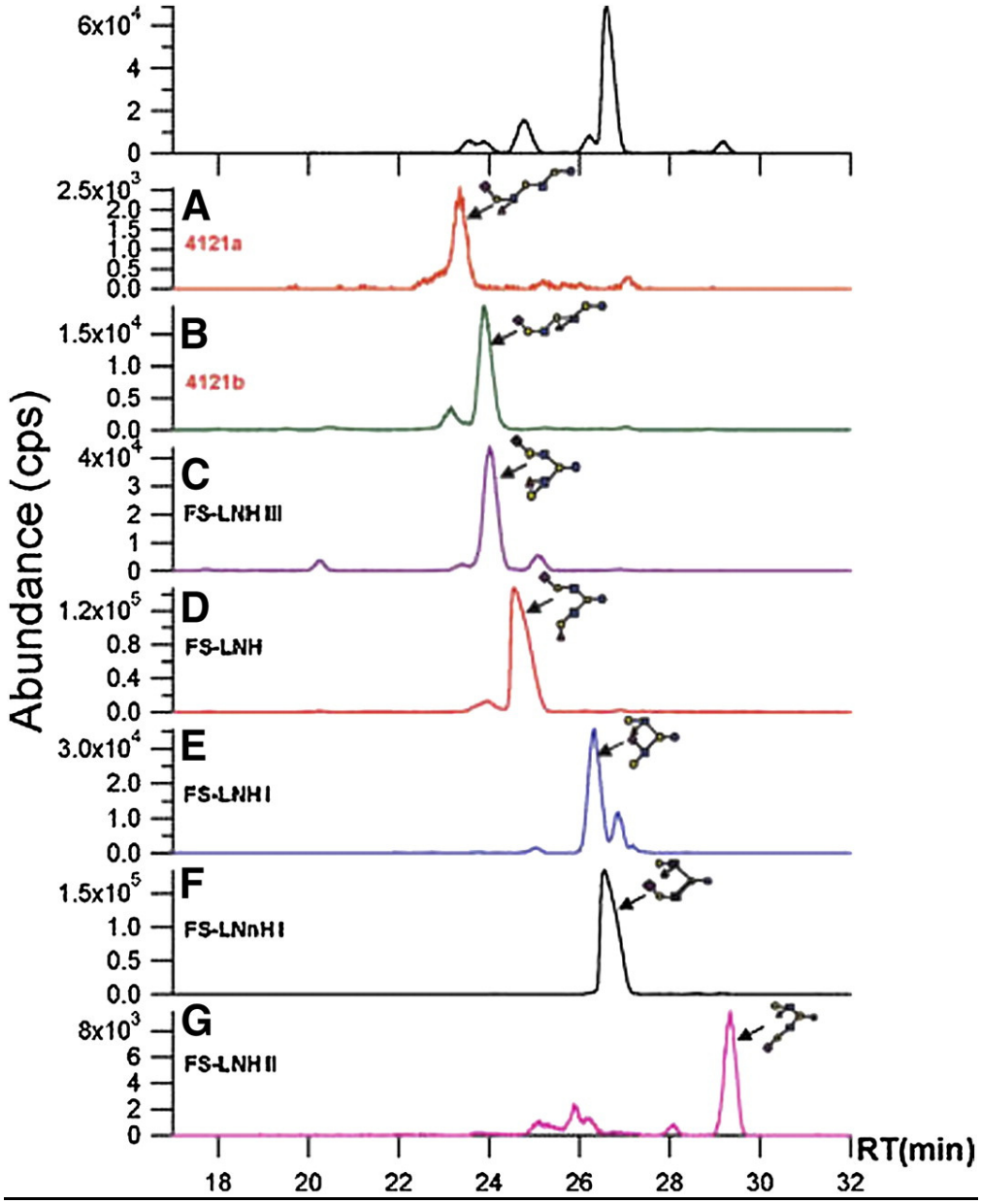


Figure-3.2.2

[Separation of isomers of reduced FS-LNH using LC-MS-MS]

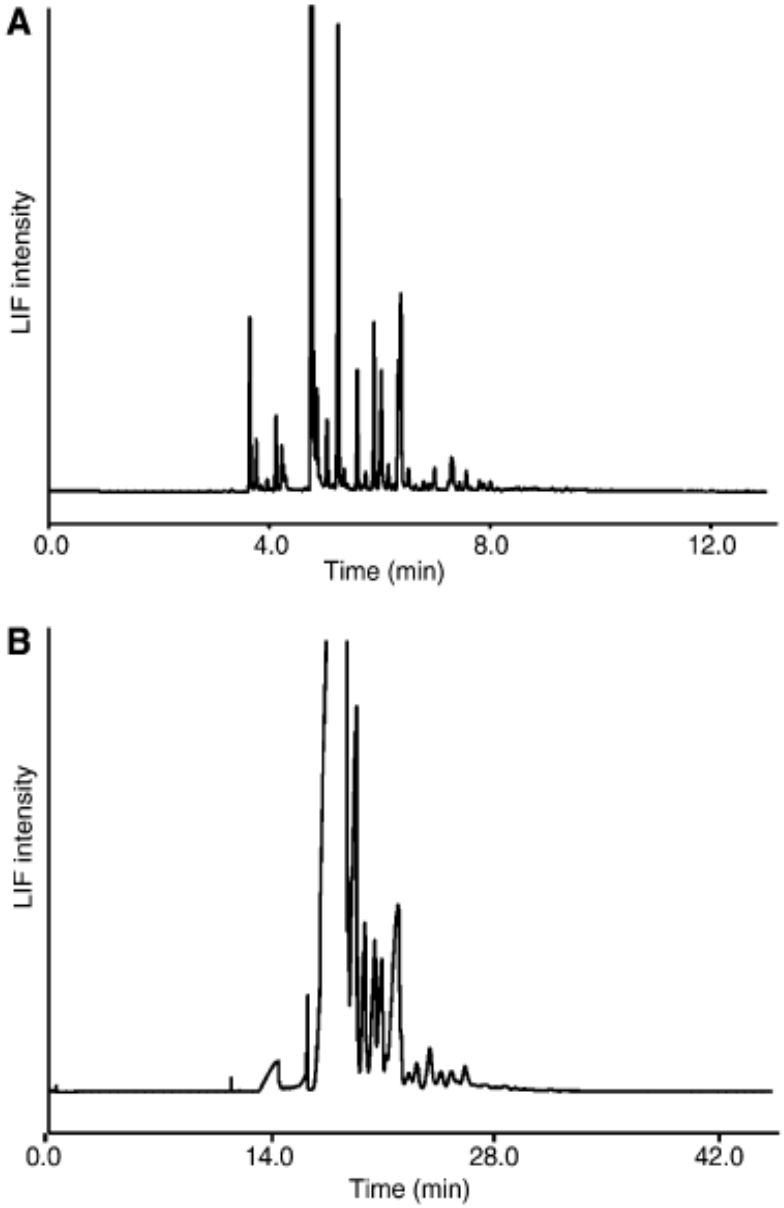


Figure-3.2.3

[Separation of 8-aminopyrene-1,3,6-trisulfonic acid–labeled milk oligosaccharides using capillary electrophoresis with laser-induced fluorescence (LIF) (A) and capillary electrophoresis with LIF MS (B). The adjustments needed for hyphenation with MS result in reduced resolving power and longer analysis times. MS, however, facilitates direct identification.]

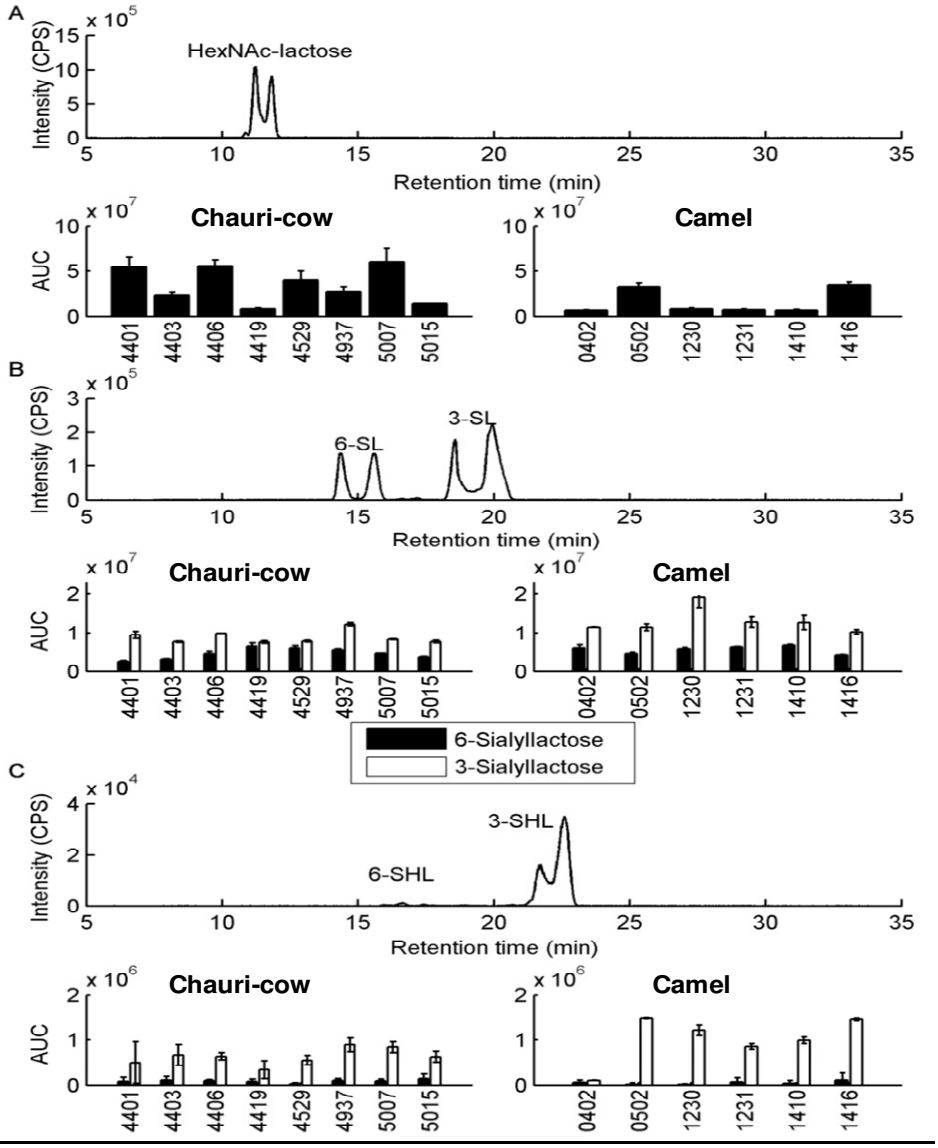


Figure-3.2.4

[Extracted ion chromatograms and calculated AUC in 14 samples measured in duplicates (A) HexNAc-lactose. (B) the two isomers of sialyllactose (3-SL and 6-SL). (C) sialyl-hexosyl-lactose.]

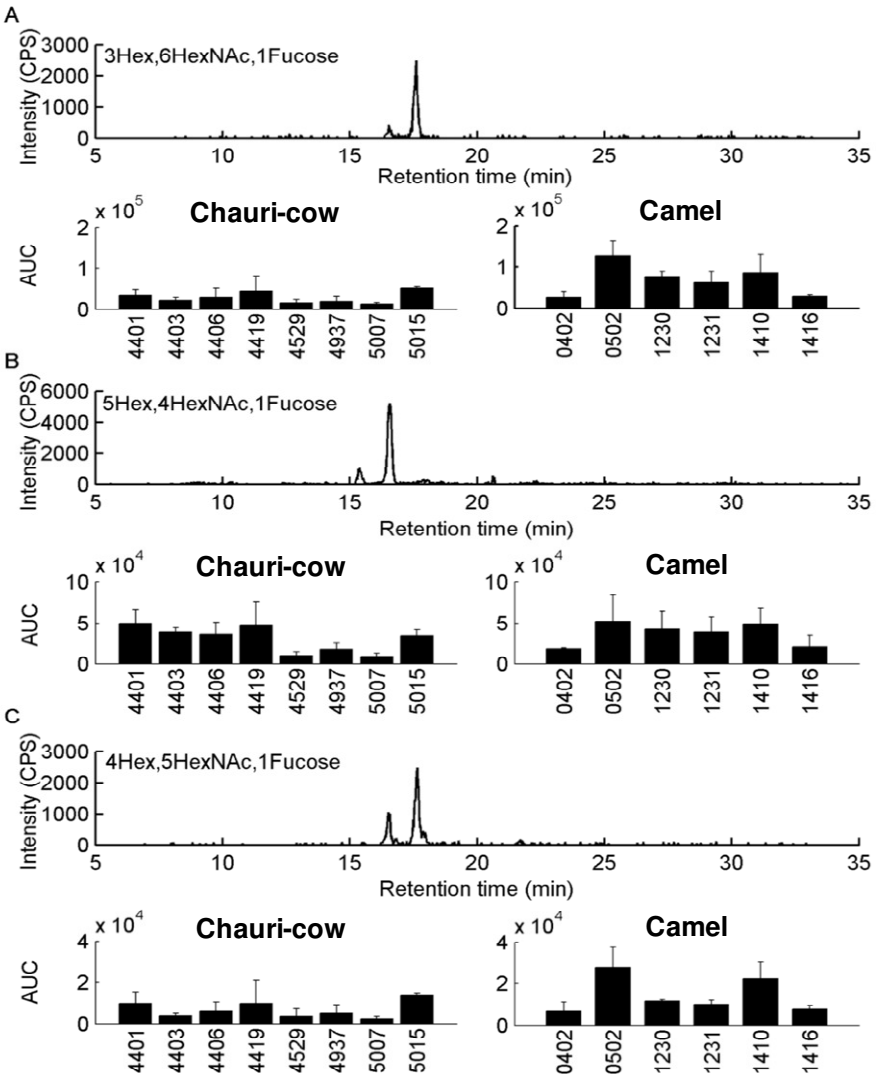


Figure-3.2.5

[Extracted ion chromatogram and calculated AUC in 14 samples measured in duplicates (A) 3Hex, 6HexNAc. (B) 5Hex, 4HexNAc, 1Fucose. (C) Hex, 5HexNAc,1Fucose.]

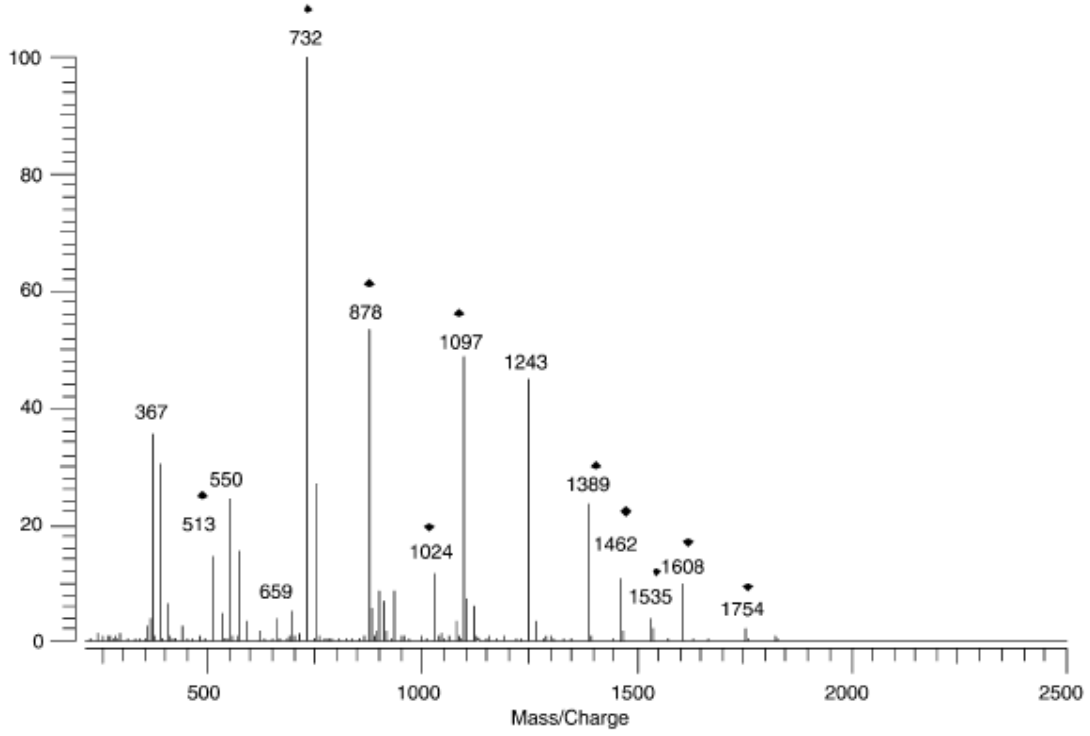


Figure-3.2.6

[Matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance MS spectrum of reduced milk oligosaccharides (MO) using 2,5-dihydroxybenzoic acid matrix in the positive ionization mode. Signals originating from MO are marked with a diamond]

Oligosaccharides analysis[80-88]

Investigationof the absolute quantification of oligosaccharides from milksamples, and identified a possible co-crystallizing effect of lactose and oligosaccharides by ethanolprecipitation. The oligosaccharides were extracted from milk samples as previouslyestablished earlier (Lebrilla CB, et al., 2008), and care was taken in order to maximize reproducibility of the extractionprocedure. The present study compares relative abundancesofindividual oligosaccharides of different milk samples and the composition of the identified oligosaccharides arepresented in Table-1.The variable oligosaccharides were found to have different retention times (RT), indicating different positional isomers,e.g. oligosaccharides ID 1, consisting of 3 hexoses, which elute at 4 different RT corresponding to 4different isomers. Additional isomers were found for several other oligosaccharides including v ID 3, 7-9, 14-15, and 21. Figure-A displays the representative BPC of Chauri cow and Camel oligosaccharides. Figure-B shows the distribution of neutral versus acidic oligosaccharides species in the milk samplesstudied measured as peak height intensities (counts per second, CPS) of all neutral andacidic oligosaccharides species pooled together. Neutral oligosaccharides species are significantly higher than acidic oligosaccharides species ($p < 0.05$).

We also used LC-MS-MS for the analysisof oligosaccharides. The advantage of such separation was segregation of good number of isomers Figure-2. Both neutral and sialylatedcompounds could be separated in one go and, using a librarycontaining retention time, mass, and fragmentation information,immediate identification became possible. Reductionof the reducing end of the oligosaccharides is necessarybecause the α- and β-anomers are separatedon thePGC stationary phase.The separationsobtained using both offline and online CE with LIFis depicted in Figure-3-6.

Conclusion of oligosaccharides analysis:

- Chauri cow's milk contained higher relative amounts of both sialylated and the more complex neutral fucosylated oligosaccharides
- Abundance of smaller and simpler neutral oligosaccharides was observed in Camel and buffalo's milk
- Chauri cow's milk contains levels of fucosylated oligosaccharides significantly higher than that of Indian Cow, Gaddi sheep, Camel, Mare and buffalo's milk.
- Chauri cow's milk also possesses oligosaccharides with a higher degree of complexity and functional residues (fucose and sialic acid), suggesting it may therefore offer advantages in term of a wider array of bioactivities.

3.3 Biological activities due to various milk oligosaccharides present:

Milk oligosaccharides are constituted of various commonly known monosaccharides such as glucose, galactose, fucose, N-acetyl glucose, N-acetyl galactose, N-acetyl nueraminic acid etc. Moreover, some of the core group units are also present in milk oligosaccharides. Due to presence of these monosaccharides and core group units, oligosaccharides perform varied biological activities which are as follows;

3.3.1 Biological activities due to Galactose

Galactose is widely distributed throughout body tissues and apart from being a source of energy, it is also a major part of biologically active glycoconjugates. Galactose is incorporated into glycoproteins and glycolipids in brain. Galactose is found in immune system glycoconjugates found in immunoglobulins and macrophages and appear to play a functional role in the etiology of immune diseases such as rheumatoid arthritis. Galactose is markedly decreased in upper airway epithelial cells in severely ill patients, which could be important in the development of opportunistic respiratory infections in these patients, since cell-surface carbohydrates mediate the adherence of many pathogenic bacteria to epithelial cells. Galactose content of red blood cells is also decreased in alcoholics, suggesting either increased hydrolysis of galactose or inhibition of the synthesis of galactose containing glycoproteins and glycolipids. There are galactose enzyme deficiency disorders, which result in inability to utilize galactose with subsequent build up of unmetabolized galactose, a condition known as galactosemia. Although these inborn errors of metabolism are infrequent, they have serious medical consequences. Deficiency in galactokinase enzyme mainly causes cataracts, which regress without complications if a galactose-free diet is started early enough. Deficiency in the epirnerase enzyme, which interconverts glucose and galactose, although extremely rare, causes nerve deafness. In transferase enzyme deficiency galactosemia, there are severe neonatal symptoms, such as failure to thrive, hepatomegaly and bacterial sepsis; these symptoms probably result from inability to synthesize necessary glycoconjugates due to reduced availability of UDP-galactose. Galactosyltransferase (GalTase) appears to play an important role in the etiology of rheumatoid arthritis (RA).

Abnormalities of immunoglobulin (IgG) glycosylation are recognized in RA individuals, where there is a lower content of galactose in glycoconjugates in both serum and synovial fluid.Galactose demonstrates a variety of biological activities. In addition to a role for galactose glycoconjugates in IgG, galactose polysaccharides are potent immune system modulators. Arabinogalactan, which is comprised of a galactose backbone with side-chains of galactose and arabinose sugars, activates phagocytosis, potentiates reticulendothelial system action, and demonstrates anti-complement activity. Galactose inhibits tumor growth and tumor cell metastasis, In vitro, galactose depresses the rate of growth of transplantable rat mammary tumors in cell cultures. In in vivo studies in mice, treatment with galactose significantly decreases the number of liver tumor colonies produced by lymphosarcoma. In humans, intravenous galactose (1.5 g/kg body weight) inhibits liver metastases in prospective, randomized, clinical trials in patients with colon and stomach cancer. The galactose glycoproteins on surfaces of tumor and host cells, which are involved in tumor cell spread, are blocked by competitive galactose glycoconjugates and autoantibodies, resulting in reduced tumor cell attachment. The galactose polysaccharide, arabinogalactan, has been shown to stimulate natural killer cell cytotoxicity and inhibit metastasis of tumor cells to the liver. In experimental animal studies, galactose has shown enhanced wound healing in rats and has inhibited cataract formation in mice fed with galactose-supplemented diet. In humans, galactose stimulates GIP release from the stomach, which results in increased insulin release and glucose utilization.

3.3.2 Biological activities due to Fucose

Fucose is widely distributed throughout the body in glycoproteins and glycolipids. Fucose is also incorporated into human skin epidermal cells where it is involved in synthesis of membrane of cells involved in maintaining skin hydration. Fucose glycoconjugates have been identified in various other tissues as well. Fucose glycoproteins are found in animal and human brain cells. They are present in synaptic junction areas where nerve cells meet, implying a role in synaptic membrane involvement in nerve impulse transmission. Fucose is also distributed in macrophages, which are critically important cells in the immune system. Fucose is found in glycoprotein and glycolipid red blood cell antigens, which are involved in determining blood type. Fucose metabolism is important for formation of glycoproteins and glycolipids.

Fucose is an important immune modulator which is active in inflammatory diseases. Fucose suppressed the skin reaction of allergic contact dermatitis induced by dinitrochlorobenzene in guinea pigs. Fucose stimulates macrophage migration which is an essential component of the immune system. Moreover, fucose inhibits macrophage-chemotactic and neutrophil chemotactic factors, which are also important in the immune system cascade. Fucose inhibits cancer growth and metastasis. Fucose inhibited rat mammary tumor cell growth in vitro. Different concentrations of fucose uniformly produced suppression in the growth rate and a change in the morphology of cells grown in tissue culture. Fucose also inhibited mouse tumor ceIl-induced platelet aggregation, a process important in cancer cell metastasis. A natural product derivative of fucose (2-deoxy-L-fucose) inhibited leukemia and mammary tumor cell growth in cell culture

systems in vitro. Fucose also has therapeutic implications in treating or preventing respiratory tract infections. Fucose also reduces collagen production in cultured cerebral microvessel endothelial cells.

3.3.3 Biological activities due to various core units

Biological functions of oligosaccharides are closely related to their conformation. The monomers of milk oligosaccharides are D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc) and sialic acid [N-acetylneuraminic acid(NeuAc)].The predominance of *Bifidobacterium bifidium*in the intestinal flora of breast-fed infants led Moro in 1900 to the conclusion that HM contains a growth factor for these microorganisms using a "bifidum mutant" called*Bifidobacterium bifidium* subspecies *pennsylvanicum* which was isolated from feces of breast-fed infants, found that gynolactose, a mixture of 10 oligosaccharides containing GlcNAc, is the growth-promoting bifidus factor.

3.3.4 Oligosaccharides As Nonspecific Defense Mechanisms

There is increasing eyidence that oligosaccharides and glycoconjugates in HM have a direct inhibitory effect on certain virulence-related abilities of pathogenic microorganisms. Although the exact pathophysiological mechanism of the genesis of diarrhea is not fully known, these ability_of.microorganisms adhere to mucosal surface and inhibit spreading of these bacteria – (e.g. Escherichia coli,Helicobactor jejuni etc.) in the duodenum.

Oligosaccharides, naturally occurring in human milk, as receptors for microbes

Receptors	Microorganisms
Mannose-containing glycoproteins	Escherichia coli (type 1 fimbriae)
Fucosylated oligosaccharides	E. coli (heat-stable enterotoxin)
Fucosylated tetra and pentasaccharides	E. coli
Sialyl (a 2-3) lactose and glycoproteins	E. coli (S-fimbriae)
Sialyl (a 2-3) galactosides in mucins	E. coli (S-fimbriae)
Neutral oligosaccharides (LNT, neo-LNT)	Streptococcus pneumoniae
Gal (b1-4)GlcNAc or Gal (b1-3)GlcNAc	Pseudomonas aeruginosa
Fuca 1 -2Gal epitopes	Candida albicans
Sialyl-lactose	Helicobactor pylori
Sialyl-lactose	Streptococcus sanguis
Sialyl-lactose and sialylated glycoproteins	H. pylori
Sialylated glycoproteins (a2-3-linked)	Mycoplasma pneumoniae
Sialylated poly-N-acetyllactosamine	M. pneumoniae
Sialylated (a2-3)poly-N-acetylactosaminoglycans	Streptococcus suis
Sialyl(a 2-6) lactose	Influenza virus A
Sialyl(a2-3)lactose	Influenza virus B
9-O-Ac of NeuAc(a2-3)R	Influenza virus B

Table 3.3.1

3.4 Antioxidant Properties Of Various Milk Oligosaccharides[89-110]

Milks were collected from various ruminant species such as buffalo, cow, gaddi sheep, camel, Chauri-cow, and mare which were processed by kobata and Ginsburg method as described earlier Chapter 2 Methodology, crude mixture of oligosaccharides of milk along with other essential milk components like vitamins, minerals, enzymes, were analysed for various bioactivities mainly antioxidant. Antioxidant bioactivity was

determined as many reactive oxygen species (ROS), including the superoxide radical, hydroxyl radical, hydrogen peroxide, and the peroxide radical, are known to cause oxidative damage to all living systems. Minute changes in the level of ROS inside cells, affect the rate of metabolism, gene expression, post-translation protein alterations and participate in regulating the cycle of cell division and the programming of apoptosis. Due to presence of reactive oxygen species oxidation of DNA occurs resulting in possible mutations. Recent studies and evidences suggests that reactive oxygen species and their subsequent effect on cellular macromolecules play a significant pathological role in some dreadful human diseases such as cancer, atherosclerosis, hypertension, and arthritis. Although the human body naturally has an inherently antioxidative system (i.e., superoxide dismutase, glutathione peroxidase, and uric acid) to protect and prevent itself from the damage caused by peroxidants, but after a certain extent our systems are not sufficiently equipped to totally prevent such damages. Hence, the quench of finding natural antioxidants from food sources is increasing remarkably, because it is believed that antioxidants can protect the living system not only from the attack of free radicals but also retard the progress of many chronic diseases, slow down the process of biological oxidation in the human body as well as retarding the lipid oxidative rancidity in foods. Antioxidants from natural sources are lmore desirable than those chemically produced, because some synthetic antioxidants have been reported to be carcinogenic. Antioxidants from natural sources would be easier to digest, health promoting and can be taken as supplements for a longer period of time to fight oxidative stress caused by prolonged illness minus the side effects of the synthetically created.Mother,s milk is the ideal and a complete planned food during infancy. Breast feeding is the best mode of nutrition for infants whether premature or term. In certain cases when the neonate is

devoid of the mother,s milk then formula milk,s can be the only life saving mode of nutrition. Both mothers' milk and formulas contain macronutrients, vitamins and minerals that support healthy growth and development of the infants however, strong evidences suggests mother,s milk specially the first milk known as the colostrum is best for the infants which provides better protection for premature infants and normal infants against various oxidative stress. But in special cases when the neonate is devoid then mother,s milk id replaced by formula milk,s which are formulated from certain ruminant species which matches the nutritional value of human milk to remarkable extents. Human milk contains antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and non-enzymatic antioxidants lactoferrin, ascorbic acid, and vitamin E that helps the premature infant to cope with ROS-mediated diseases. Cu/Zn-SOD is found in human milk. Cu/Zn-SOD is very resistant to various types of denaturing stress including heating and commercial pasteurized milk retains its SOD activity at a similar level to unpasteurized milk. CAT is one the most heat-labile enzymes of human milk with most of its activity being destroyed by treatment at 72^0 C for 15 sec. Glutathione peroxidase (GSHPx) removes H_2O_2 and other peroxides. GSHPx activity is lost by heating at 80^0C for 10 min. Lactoferrin (Lf) is a member of iron-binding transferrin protein family that inhibits the formation of ROS by binding iron and thus attenuating the conversion of hydrogen peroxide into hydroxyl radical via the Fenton type reaction. Lf is abundant in human milk. Vitamin C is a water-soluble anti-oxidant and a free radical scavenger, able to moderate the oxidative stress effects of various diseases. Vitamin E functions primarily as an antioxidant. It consists of eight vitamers (related chemical substances that fulfill the same specific vitamin function) four tocopherols and four tocotrienols. A-Toco-pherol, the form of vitamin E in human

milk is an important fat-soluble antioxidant that acts as a radical scavenger. Both human milk and formulas (F) contain vitamin C and E.In the human body, lipid peroxides are toxic and capable of damaging most of the body cells. The process of lipid peroxidation is initiated by an attack upon a fatty acid or fatty acyl side chain by any chemical species that features sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain. The resulting lipid radicals then undergo molecular rearrangement, followed by reacting with oxygen to produce peroxyl radicals, which are capable of abstracting hydrogen from adjacent fatty acid side chains and thus propagating a chain reaction of lipid peroxidation.The in vitro trials results demonstrated the highest total antioxidant capacity (TAC) in goats' milk known for its great therapeutic value and easy digestion, especially from Prisca breed. Ex vivo trials showed that Prisca goats' milk inhibits platelet aggregation at lower amounts than milk from other species. Milk fat contains a number of individual components that can be described as having anticarcinogenic properties. In particular, conjugated linoleic acid (CLA) and sphingomyelin have been suggested to have important anticancer properties. McIntosh et aldemonstrated a protective role for dietary dairy proteins against tumour development, showing that dietary whey protein and casein were more protective against the development of intestinal cancers in rats than was red meat or soy bean protein. They concluded that dietary proteins differ in their ability to protect against cancer development and that the proteins in dairy foods, particularly the whey proteins, appear to play a significant role in cancer prevention. Daily ingestion of foods containing peptides with potent ACE-inhibitory activities may be effective at keeping the human blood pressure low. Praveesh et al. have shown that an angiotensin I-

converting enzyme-inhibitory cow milk hydrolysate and its *in vitro* antioxidant and anticancer activity.

Statistical analysis: The results are expressed as mean and standard error of means (Mean±SE) for at least three experiments. One way ANOVA followed by post hoc Tukey's test was employed to detect differences between the groups of treated and control. $P < 0.05$ was taken to indicate significant differences

Results:

3.4.1 Estimation of lipid peroxidation (LPO) levels

A significant decrease in the levels of LPO was found in sheep and yak i.e. 5.60±0.11 and 5.082±0.16; 1 mg/500 µl saliva group respectively as compared to controls.No significant changes were observed in the levels of LPO in camel and mare. No significant changes were found in the levels of LPO in 2 mg/500 µl saliva groups.

LPO Assay (Antioxidant assay)

S.No.	Sample	1 mg /500 µl saliva sample	2 mg /500 µl saliva sample
1	Control	8.43	8.98
2	Camel	7.98	8.09
3	Sheep	5.60	8.22
4	Mare	7.46	9.23
5	Chauri	5.082	8.50

Table 3.4.1

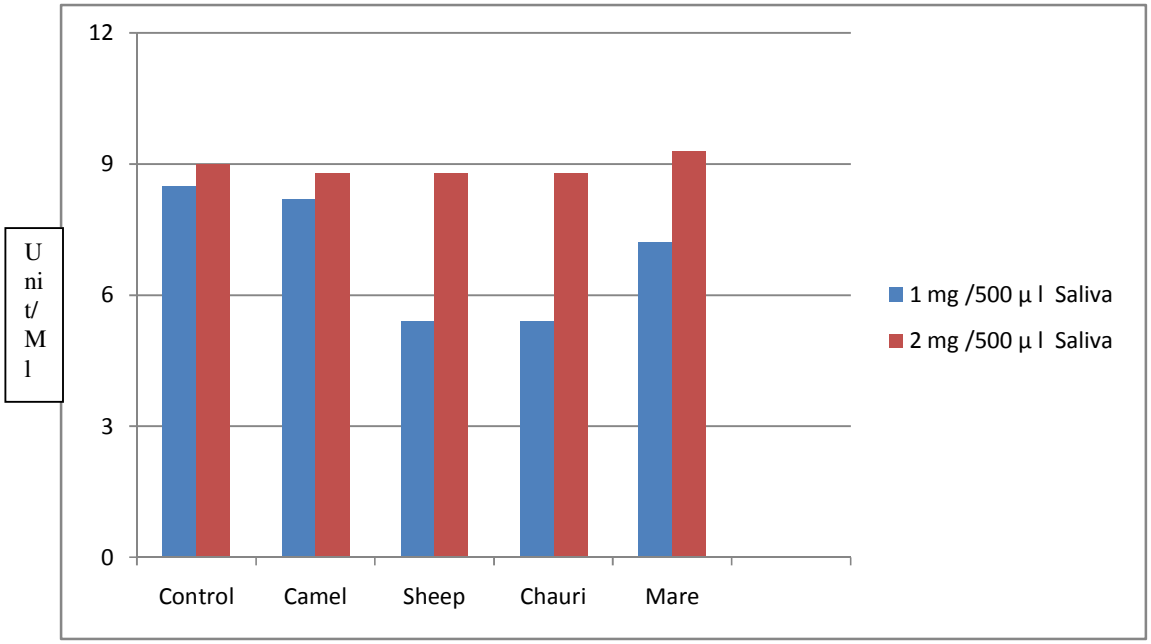


Figure 3.4.1

Figure 3.4.1 Levels of lipid peroxidation in different ruminants. Values are given as mean ± SE of the data obtained from three independent experiments. *= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$.

3.4.2 Superoxide dismutase (SOD) activity

A significant increase in the activity of superoxide dismutase in Mare (15.82±0.51; 1 mg/500 µl saliva group) was observed as compared to controls.However, a significant decrease in the activity of SOD was observed in camel (7.54±0.14; 1 mg/500 µl saliva group) and yalk (6.10±0.21; 1 mg/500 µl saliva group) as compared to control but no significant changes were observed in the activity of SOD in sheep. No significant changes in the activity of SOD were found in 2 mg/500 µl saliva group.

SOD Assay (Antioxidant Property)

S.No.	Sample	1 mg /500 µl saliva sample	2 mg /500 µl saliva sample
1	Control	9.38	10.01
2	Camel	8.04	9.58
3	Sheep	5.90	9.55
4	Chauri	5.95	9.54
5	Mare	7.10	9.80

Table 3.4.2

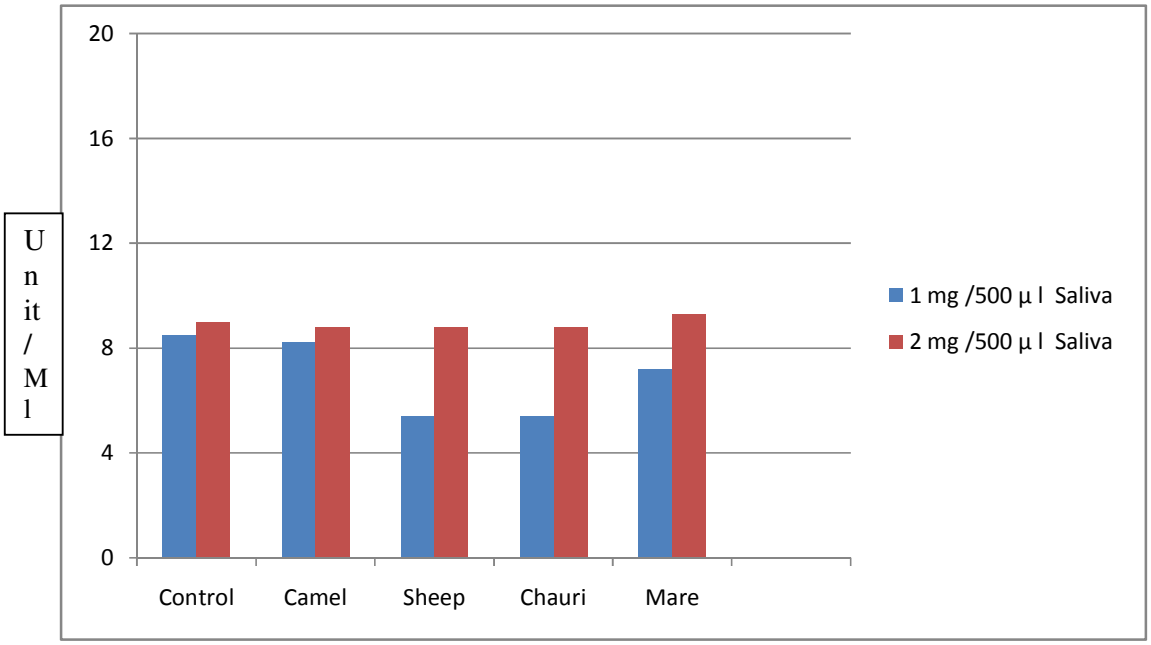


Figure 3.4.2

Figure 3.4.2: Levels of superoxide dismutase in different ruminants. Values are given as mean ± SE of the data obtained from three independent experiments. *= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$.

3.4.3 Estimation of catalase levels

A significant increase in the levels of catalase was also found in Mare (0.87±0.08; 2 mg/500 µl saliva group) as compared to controls.However, a significant decrease in the levels of catalase was observed in camel (0.15±0.01; 2 mg/500 µl saliva group) and yalk (0.33±0.03; 2 mg/500 µl saliva group) as compared to control but no significant changes were observed in the levels of catalase in sheep. No significant changes were found in the levels of catalase in 1 mg/500 µl saliva groups.

CATALASE Assay (Antioxidant Property)

S.No.	Sample	1 mg /500 µl saliva sample	2 mg /500 µl saliva sample
1	Control	0.48	0.60
2	Camel	0.50	0.35
3	Sheep	0.62	0.75
4	Mare	0.69	0.87
5	Chauri	0.62	0.23

Table 3.4.3

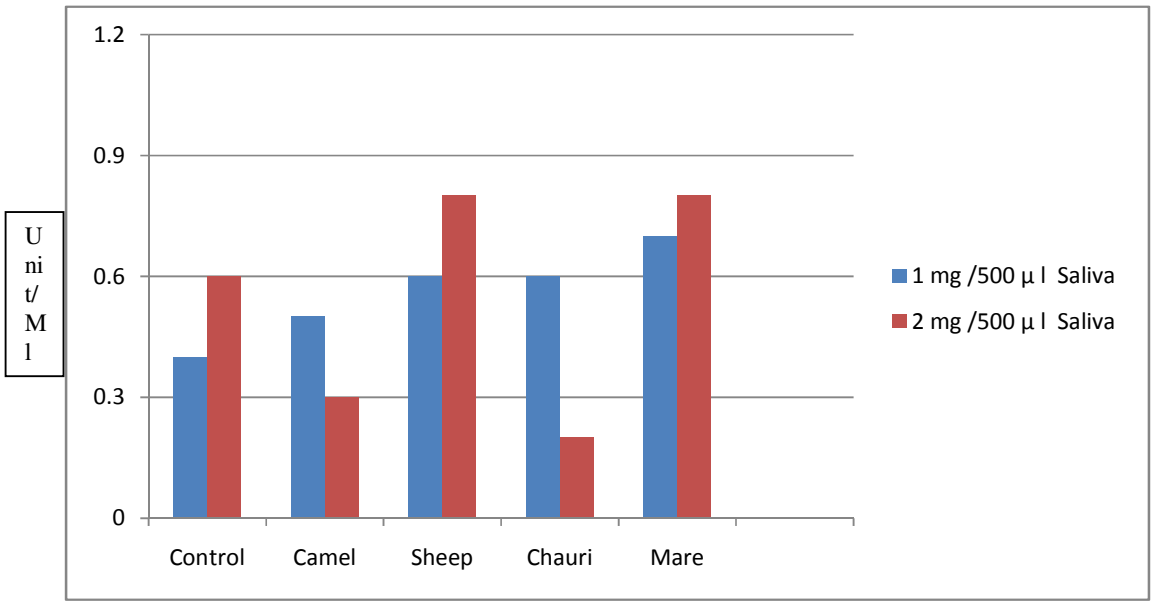


Figure 3.4.3

Figure 3.4.3: Levels of catalase in different ruminants. Values are given as mean ± SE of the data obtained from three independent experiments. *= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$.

Intake of milk oligosaccharides was found positively associated with health promoting factors and lower chances of many diseases analyzed by epidemiological studies and clinical trails. Antioxidant capacity was believed to be the antidote of various dreadful and incurable diseases like Cancer, AIDS and tumors. Antioxidant capacity of various milk oligosaccharides are demonstrated be collection of milk samples from various ruminants like cow, Chauri-cow, buffalo, camel, mare, gaddi sheep and was processed by Kobata and Ginsburg method and then the crude oligosaccharides was assessed for their bioactivities. When an excess of free radicals is formed they can analyze protective enzymes like superoxide dismutase, catalase and peroxidase cause destructive and lethal cellular effects for examples apoptosis. It has been known that oxidation also plays a significant role at the cellular level, in addition oxidative stress is known to cause age related diseases. The factors involved in these diseases are the lipid peroxides and it has been observed increased oxidative damage leads to incurable diseases like Alzheimer's in human. Cancer is probably a consequence of DNA damage. Various antioxidative agents are artificially induced to lower the lipid oxidation rate but natural antioxidants like milk oligosaccharides could also be used and to inhibit the oxidation rate.

The effect of various ruminant milks such as cow, camel, buffalo, mare sheep show oxidant/antioxidant properties against markers such as MDA, SOD and catalase in Assimilated saliva sample which was assessed spectrophotometrically. The results indicated a significant decrease in the concentration of saliva in both the groups (1 mg/500 µl saliva and 2 mg/500 µl saliva) as compared to the control group of the samples. More significant decrease in MDA level was found in sheep and chauri cow crude oligosaccharide mixture as compared to the control in 1 mg/500 µl saliva. No

significant change was observed in camel and mare MDA activity.In group 2 mg/500 µl saliva, there was difference found. All compounds (camel, sheep, mare, chauri cow) showed a variation, it could be assessed that the protective effect of milk against oxidative stress in all groups was due to its antioxidant properties. Camel milk in group 2 mg/500 µl saliva and sheep & chauri cow milk in group 1 mg/500 µl saliva was found to contain high concentration of antioxidant property. Vitamins and enzymes found in milk act as antioxidants and have been found to be useful in decrease of oxidative stress. It has been previously reported that proteins deriving from dairy products reveal some antioxidant potential. Pena-Ramos and Xiong found that peptides deriving from milk protein hydro lysates inhibited lipid oxidation, suggesting that the specific amino acid residue side-chain groups or the specific peptide structure of the antioxidative peptides may be attributable to chelation of pro-oxidative metal ions and termination of the radical chain reactions.The antioxidant assays like SOD and Catalase in groups 1 mg/500 µl and 2 mg/500 µl saliva was found antioxidant specially the mare milk showed more antioxidant property in both groups 1 mg/500 µl and 2 mg/500 µl saliva in different concentrations. The catalase assay camel, sheep, mare, chauri cow showed less significant changes when compared to the control in group 1 mg/500 µl and 2 mg/500 µl saliva.

SOD scavenging capacity of saliva is considered very important in reducing the oxidative stress caused by day to day oxidation .The elevation of antioxidant assay like SOD and Catalase in milk of ruminants may be due to the association with a loss of balance between pro oxidation. It has been found that oligosaccharides present in milk have positive health promoting factors which not only help against oxidative stress but

also have remarkable immune stimulant properties.It has been reported previously that some milk-derived proteins and peptides demonstrate some level of antioxidative activity. Wong and Kitts found that the reducing activity of certain buttermilk solids was mainly attributed to the sulfhydryl content of the group and that free hydroxyl groups could have also contributed, in part, to the observed reducing activity. Further, it has also been previously reported that some lactic acid bacteria may exhibit excellent reducing power. Highly reactive free radicals formed by exogenous chemicals or endogenous metabolic processes in the human body or in food systems are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Almost all organisms are well-protected against free-radical damage by antioxidative enzymes such as SOD, catalase, peroxidase, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, GSHPx, and GR. Among a number of different antioxidative enzymes, catalase, GSHPx, and SOD have been demonstrated to be present in milk[93]. Catalase is one of the most heat-labile enzymes known, with most of the activity of the enzyme being destroyed by even modest heat treatment.In vitro antioxidant activities of cow milk were studied using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging and total reducing power assay. The observed result showed that cow milk fermented with the combination of Lactobacillus plantar.

3.4.4 In vitro antioxidant assay by DPPH method

DPPH (2, 2-Diphenyl-1-picrylhydrazyl)is a stable free radical which is blue coloured in the presence of an antioxidant, this radical is reduced and the purple colour of DPPH is bleached to yellow. The absorbance measured at 517nm is the extent of bleaching recorded as the antioxidant activity of the coumpound.

Method: 100 micro litre of sample is taken in this assay was performed by gulluce et al (2006).The hydrogen atom or neutron donation ability of the samples was measured from the bleaching of the purple colored methanol solution of DPPH this spectrophotometric assay uses stable DPPH as a reagent 100 micro litre of the sample was added to 5 ml of 0.004% methanol solution DPPH after 30 mins of incubation time at room temperature and the absorbance was read against the blank at 570nm. The control does not contain any sample. BHT was taken as the control. Butylated hydroxyl toluene a natural antioxidant. The antioxidant activity of the sample was calculated in percentage from comparing with BHT,s absorbance .

S. No.	Sample	Amt	OD	Absorbance%
1	BHT	100 mg	0.401	100 %
2	COW	100 mg	1.090	Negative
3	MARE	100 mg	1.080	Negative
4	CHAURI	100 mg	0.751	87.50
5	SHEEP	100 mg	1.029	Negative

Table 3.4.4

BHT ——————— Chauri
0.4 0.75

$$\frac{100}{0.4} \times 0.75 = 87.5\ \%$$

Hence results showed that Oligosaccharides in milk had the potential anti-oxidant property with antioxidative assays suggesting that it can be used as an natural anti-oxidants which helps to reduce biological oxidation produced by stress or prolonged illness. Milk oligosaccharides was compared with a natural antioxidant BHT and suggested that **Chauri-Cow Milk Oligosaccharides as potent antioxidants.**

BIBLIOGRAPHY

1. The World Health Organisation(2014) Global strategy on infant and young child feeding,
2. World diary ruminant numbers(2014) Food outlook . A global analysis, United nations pages 51-54
3. Tadasu Washima, Tadasaita et al (2001) Review, Glycoconjugates, Jownai 18, 357-371.
4. T. Nakamura Kowese H. (2003) J. Dairy Science, 86, 1315-1320.
5. ARVANITAKIS, C.: Diet Therapy in Gastrointestinal Diseases: A Commentary," J. Am. Diet. Assoc., 75: 449-52, 1979.
6. Sumiyoshi W, Urashima T, Nakamura T, Arai I, Saito T, Tsumura. , Wang B, Brand-Miller J, Watanabe Y, Kimura K. (2003), Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. Br J Nutr. 2003 Jan; 89(1):61-9.
7. T.Nakamura T., Kawase H., Kimura K., Watanabe Y., Ohtani M., Arai I., Urashima T. (2003), Concentration of Sialyloligosaccharides in Bovine Colostrum and milk during the Prepartum and early Lactation. J.Dairy Sci. 86, 1315- 1320.
8. Lidestri M, Agosti M, Marini A, Boehm G.(2003), Oligosaccharides might stimulate calcium absorption in formula-fed preterm infants. Acta Paediatr Suppl. 91(441):91-2.
9. Boehm G, Stahl B, Jelinek J, Knol J, Miniello V, Moro GE.(2005), Prebiotic carbohydrates in human milk and formulas. Acta Paediatr Suppl. 94(449): 18-21.
10. Boehm, G., & Stahl, B. (2003-). Oligosaccharides, Functional dairy products (pp. 203-243). Cambridge, England: Woodhead Publishing Limited.
11. Newburg DS, Ruiz-Palacios GM, Morrow AL.(2005), Human milk glycans protect infants against enteric pathogens. Annu Rev Nutr. 25: 37-58.
12. Lara-Villoslada F, Debras E, Nieto A, Concha A, Galvez J, Lopez-Huertas E, Boza J, Obled C, Xaus , (2005), ,Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis. Clin Nutr.
13. Kobata A, (2003), Possible application of milk oligosaccharides for drug development. Chang Gung Med J. 2003 Sep; 26(9):621-36.
14. Erney R, Hilty M, Pickering L, Ruiz-Palacios G, Prieto P., (2001), Human milk oligosaccharides: a novel method provides insight into human genetics. Adv Exp Med BioI. 200 I; 50 I :285-97.
15. Rudloff S, Stefan C, Pohlentz G, Kunz C. (2002), Detection of ligands for selectins in the oligosaccharide fraction of human milk. Eur J Nutr. 2002 Apr; 41 (2):85-92.
16. Newburg DS, Ruiz-Palacios GM, Morrow AL.(2005), Human milk glycans protect infants against enteric pathogens. Annu Rev Nutr. 25: 37-58.
17. Rudloff S, Stefan C, Pohlentz G, Kunz C. (2002), Detection of ligands for selectins in the oligosaccharide fraction of human milk. Eur J Nutr. 2002 Apr; 41 (2):85-92.
18. Sharon N, Ofek I. (2000), safe as mother's milk: carbohydrates as future antiiadhesion drugs for bacterial diseases. Glycoconj J. 2000 Jul-Sep; 17(7-9):659-64.
19. Martin MJ, Martin-Sosa S, Hueso P. (2002), Binding of milk oligosaccharides by several enterotoxigenic Escherichia coli strains isolated from calves. Glycoconj J. 2002 Jan; 19(1):5-1 1.
20. Autar R, Khan AS, Schad M, Hacker J, Liskamp RM, Pieters RJ.(2003), Adhesion inhibition of F1 C-fimbriated Escherichia coli and Pseudomonas aeruginosa PAK and PAO by multivalent carbohydrate ligands. Chembiochem.4(12): 1317-25.

21. Morrow AL, Ruiz-Palacios GM, Altaye M, Jiang X, Guerrero ML, Meinzen-Derr JK, Farkas T, Chaturvedi P, Pickering LK, Newburg DS.(2004), Human milk oligosaccharide blood group epitopes and innate immune protection against campylobacter and calicivirus diarrhea in breastfed infants. Adv Exp Med BioI. ;554:443-6.
22. Huang P., Farkas T., Marionneau S., Zhong W., Ruvoen-Clouet D.V.M., Morrow A.L., Altaye M., Pickering L.K., Newburg D.S., LePendu .I., Jiang X. (2003), Noroviruses bind to human ABO, lewis and secretor histo-blood group antigens identification of four distinct strain -specific patterns. J.Infect. Dis. 188, 19-31.
23. Marionneau S., Ruvoen N., Le Moullac- vaidye 8., Clement M., Cailleau-Thomas A., Ruiz- Palacoisis, G., Huang P., Jiang X., Le Pendu J. (2002), Norwalk virus binds to histo-blood group antigens on gastroduodenal. epithelial cells of secretor individuals. Gastroentrology, 122, 1967-1977.
24. Hakkarainen J, Toivanen M, Leinonen A, Frangsmyr L, Stromberg N, Lapinjoki S, Nassif X, Tikkanen-Kaukanen C. (2005), Human and bovine milk oligosaccharides inhibit Neisseria meningitidis pili attachment in vitro. J Nutr.135(I 0):2445-8.
25. Johansson P, Nilsson J, Angstrom J, Miller-Podraza H.,(2005),Interaction of Helicobacter pylori with sialylated carbohydrates: the dependence on different parts of the binding trisaccharide Neu5Ac{alpha}3Gal {beta}4GlcNAc. Glycobiology. 2005 Jun; 15(6):625-36.
26. Martin-Sosa S, Martin MJ, Hueso P. (2002), The sialylated fraction of milk oligosaccharide is partially responsible for binding to enterotoxigenic and uropathogenic Escherichia coli human strains. J. Nutr. 132(10):3067-72.
27. Mark J. Gnoth, Clemens Kunz, Evamaria Kinne-Saffran and Silvia Rudloff, (2000), Human Milk Oligosaccharides Are Minimally Digested [n Vitro I . J. Nutr. 130: 3014~3020.
28. Buddington RK, Kelly-Quagliana K, Buddington KK, Kimura Y.(2002), Nonndigestible oligosaccharides and defense functions: lessons learned from animal models. Br J NutL87 Suppl 2:S231-9.
29. Agostoni C, Axelsson I, Goulet 0, Koletzko B, Michaelsen KF, Puntis JW, Rigo J, Shamir R, Szajewska H, Turck 0; ESPGHAN Committee on Nutrition, (2004), Prebiotic o[igosaccharides in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition. J Pediatr Gastroenterol Nutr. 39(5):465-73.
30. Sudarmo SM, Ranuh RG, Rochim A, Soeparto P. (2003), Management of infant diarrhea with high-lactose probiotic-containing formula. Southeast Asian J Trop Med Public Health.34(4):845-8.
31. Euler AR, Mitchell OK, K[ine R, Pickering LK, (2005), Prebiotic effect of fructoooligosaccharide supplemented term infant formula at two concentrations compared with unsupplemented formula and human milk. Pediatr Endocrinol Rev. 2(4):683-4.
32. Fanaro S, Boehm G, Garssen J, Knol J, Mosca F, Stahl B, Vigi V.(2005), Galactoooligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas: a review. Acta Paediatr Suppl. 94(449):22-6.
33. Ben XM, Zhou XY, Zhao WH, Yu WL, Pan W, Zhang WL. Wu SM, Van Beusekom CM, Schaafsma A.(2004), Supplementation of milk formula with galactoooligosaccharides improves intestinal micro-flora and fermentation in term infants.Chin Med J (Engl).117(6):927-31.
34. Boehm G, Lidestri M, Casetta P, Jelinek J, Negretti F, Stahl B, Marini A.(2002), Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. Arch Dis Child Fetal Neonatal Ed. 86(3):F178-81.

35. Natal'ya A. Karataeva, Dmitry Gorbunov, Ivan V. Prokudin, Valentina N. Buneva, Anna A. Kulminskaya, Kirill N. Neustroev and Georgy A. Nevinsky, (2006), Human milk antibodies with polysaccharide kinase activity, Immunology Letters, Volume 103, Issue I , Pages 58-67 .
36. Malandain H. (2005),lgE-reactive carbohydrate epitopes--classi fication, crosssreactivity, and clinical impact. Allerg Immunol (Paris). 37(4): 122-8.
37. Johnson MA, Eniade AA, Pinto BM.(2003),Rational design and synthesis of peptide ligands for an anti-carbohydrate antibody and their immunochemical characterization.: Bioorg Med Chern. 11(5):781-8.
38. Debre, R, Ramon, G., Levy-Solal, E., and Thiroloix, P.L. 1930. Sur le passage des antitixines par le lait et colostrums, en particulier dans I'espece humaine. Nourrisson. 18:235.
39. Lara-Villoslada F, Debras E, Nieto A, Concha A, Galvez J, Lopez-Huertas E, Boza J, Obled C, Xaus , (2005), ,Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis. Clin Nutr.
40. Milo LA, Correa-Matos NJ, Donovan SM, Tappenden KA.(2004), Neutrophil and small intestinal lymphocyte migration after Salmonella typhimurium infection: impact of fermentable fiber. J Pediatr Gastroenterol Nutr.39(I):73-9.
41. Gnoth MJ, Kunz C, Rudloff S. (2000), Endotoxin-reduced milk oligosaccharide fractions suitable for ce.ll biological studies. Eur J Med Res. 5(1 1):468-72.
42. Erney R, Hilty M, Pickering L, Ruiz-Palacios G, Prieto P., (2001), Human milk oligosaccharides: a novel method provides insight into human genetics. Adv Exp Med BioI. 200 I; 50 I :285-97.
43. Newburg DS, Ruiz-Palacios GM, Morrow AL.(2005), Human milk glycans protect infants against enteric pathogens. Annu Rev Nutr. 25: 37-58.
44. Tom Gardiner. (2000), Biological Activity of Eight Known Dietary Monosaccharides Required for Glycoprotein Synthesis and ·Cellular Recognition Processes, Glycoscience and Nutrition, Vol I, No. 13.
45. Tom Gardiner,(2000),Dietary Galactose:Absorption, Distribution, Metabolism, Excretion (ADME) and Biological Activity, Glycoscience and nutrition VOL I, N07.
46. Boehm, G., & Stahl, B. (2003-). Oligosaccharides, Functional dairy products (pp. 203-243). Cambridge, England: Woodhead Publishing Limited.
47. Tadasu Urashima I, Tadao Saito2, Tadashi Nakamura I and Michael Messer, 3., (2001), REVIEW, Oligosaccharides of milk and colostrums in non-human mammals. Glycoconjugate Journal 18,357-371.
48. T.Nakamura T., Kawase H., Kimura K., Watanabe Y., Ohtani M., Arai I., Urashima T. (2003), Concentration of Sialyloligosaccharides in Bovine Colostrum and milk during the Prepartum and early Lactation. J.Dairy Sci. 86, 1315- 1320.
49. Histo-blood group antigen and human milk oligosaccharides: genetic polymorphism and risk of infectious diseases. Adv Exp Med BioI. 2004; 554: 135543.
50. C. Kunz,l S. Rudloff,2 W. Baie r,3 N. Klein,4 and S. St robe, (2000), Oligosaccharjdes In Humanmjlk:, Structural, Functional, and Metabolic Aspects, Annu. Rev. Nutr. Vol-20:699-722.
51. Koscielak J. Transfus Med. 2001 Aug; ABH blood group active glycoconjugates from human red cells. Vol-1 1(4):267-79.
52. Thurl S, Henker J, Siegel M, Tovar K, Sawatzki G., (1997), Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. Glycoconj J. 1997 Nov; 14(7):795-9.
53. Metabolic oligosaccharide engineering as a tool for glycobiology, Current Opinion in Chemical Biology ,Volume 7, Issue 5 , October 2003, Pages -616-625.

54. Guillermo M. Ruiz-Palacios, Luz Elena Cervantes, Pilar Ramos, Bibiana ChavezzMunguia and David S. Newburg, (2003), Campylobacter jejuni Binds Intestinal H(O) Antigen (Fuc 1, 2Gall, 4GIcNAc), and Fucosyloligosaccharides of Human Milk Inhibit Its Binding and Infection*. 1. BioI. Chem., Vol. 278, Issue 16, 14112-14120.
55. Sialic acid-containing milk proteins show differential immunomodulatory activities independent of sialic acid. J Agric Food Chern. 2005 Oct 5; 53(20):7673-80.
56. Newberg D.S. Annual Review Nutr. 2005 75, 37-58..
57. J Agric Food Chern. '2005 Oct 5; 53(20):7673-80.sialic acid-containing milk proteins show differentialimmunomodulatory activities independent of sialic acid.
58. Sudramo S.M. Ranuth G.H. South east asian Jou. Of trop. Med. And public health 2003, 34(4), 845-848
59. Euler A.R. Pediat. Andocrio.rev. 2005, 2(4), 682-683
60. Kay F.O. Glycoconjugate Jou. 2001, 18, 373-390
61. Kins E.Y. Chem. Biochem. 2005, 6(2), 422-431
62. Boehm Arch. Dis. Child Fatal Neonatal Ed. 2002, 86(3), 178-81
63. Tadasu, urashima Glycoconjugate Jou. 2001,18, 357-371
64. Sharon N. Biochem Biophys. Acta 2006 1760(4), 527-537
65. Jain, R.; Sharma, J. Encyclopedia of Analytical Chemistry, R.A. Meyers (Ed.) , John Wiley & Sons Ltd. Chichester, 2000, pp. 1583-1603
66. Shen Z. Warren C. D. Jou. Of Chromatography 2001, 921, 315-321
67. Shen Z. Warren C. D. Jou. Of Chromatography 2001, 921, 315-321
68. Subramanian A. (2002),lmmunoaffinity Chromatography Molecular Biotechnology, Volume 20, Number I, January 2002, pp. 41-47(7).
69. NMR Spectroscopy of Glycoconjugates, Eds. J. Jimenez-Barbero and T. Peters WILEY-VCH, Weinheim, 2002.
70. BY E. F. HOUNSELL (2005), NMR of Carbohydrates, Lipids and Membranes , Nuclear Magnetic Resonance,Volume 34, The Royal Society ofChemistry,2005
71. Duus J. Q,Gotfredsen C.H., Bock K. (2000) , Carbohydrate structural determination by NMR spectroscopy: Modern methods and limitation , Chem.Rev., 100, 4589-4614.
72. Plock A, Beyer G, Hiller K, Grundemann E, Krause E, Nimtz M, Wray V.(2001), Application of MS and NMR to the structure elucidation of complex sugar moieties of natural products: exemplified by the steroidal saponin from Yucca filamentosa L. Phytochemistry. 2001 Jun; 57(3):489-96.
73. HIGH RESOLUTION I H -NMR SPECTROSCOPY IN THE STRUCTURE ANALYSIS OF CARBOHYDRATES DERIVED FROM GL YCOPROTIENS, Johannes F. G. Vliegenthart. Herman van Helbeek and Lambertlls Dorland
74. Zhang X, J. of American Soc. Mass spect. 2004, 15(10), 1471
75. Gasper L., (1984). General Laboratory Methods. In Methods of Protein Analysis (Kerese I. and Chalmers R.A., eds) pp 30-86, Ellis Horwood Ltd., Chichester
76. Pearson, Chemical Analysis of Food, 8th Edition, Churchill Livingstone, 1984.
77. Plummer D.T. (1971). An Introduction to Practical Biochemistry, Tata McGraw-Hill Publishing Company Ltd., Bombay.
78. ISI Handbook of Food Analysis, Part (I)-(XV), Indian Standards Institute, 1988.
79. Kobata, A. and Ginsburg V., J. Biol. Chem., 244, 5496 (1969)
80. Eugeino jose gracia, Tina luiza , Cadolin ordoni (2012) Department of dental materials, Dental school University of brazil
81. Alexdra rias servinomatias, (2013) Deparment of Agri food industry journal of food and nutrition voulume 9
82. Rosa Helena , Miranda grande (2013) Deparment of dentistry, dental school usp, university of brazil
83. Allias lucio, (2011) university of porta grossa, Brazil

84. Shuai wu, Naannan tao , Bruce german (2012) Development of neutral human milk oligosaccharides US national library of medicine
85. Thurman E. M Ferrer (2003) Analysis of emeriging contents, Columbus American chemical society
86. Andrey R.E Robert (2003) An introduction London , ISBN0471-49801-7
87. Mac Master, Marvin C (2005) A practical user,s guide New York, John wiley ISBN 0471-65531-7
88. Tao N, DePeters EJ, Freeman S, German JB, Grimm R, Lebrilla CB. Bovine milk glycome. J. Dairy Sci. 2008; 91:3768–3778. [PubMed: 18832198.
89. Sliwa-jóźwik A., Jóźwik A., Kołataj A., Influence of exogenous glutathione [GSH], as stress factor, on the activity of lysosome enzymes in some organs of mice. Archiv fur Tierzucht-Archives of Animal Breeding, 45 (3): 307-314, (2002)
90. Crujeiras AB., Parra D., Milagro FI., Goyenechea E., Larrarte E., Margareto J., Martínez JA., Differential expression of oxidative stress and inflammation related genes in peripheral blood mononuclear cells in response to a low-calorie diet: a nutrigenomics study. Omics: A Journal of Integrative Biology, 12: 251-61, (2008)
91. Poulsen HE., Oxidative DNA modifications. Experimental and Toxicologic Pathology, 57: 161-169, (2005)
92. Halliwell B., Gutteridge JMC., Oxygen toxicity, oxygen radicals, transition metals, and disease. Biochem J, 219: 1-4, (1984)
93. Frenkel K., Carcinogen-mediated oxidant formation and DNA damage: Pharmacol Ther, 53: 127-166, (1992)
94. Simic MG., Mechanisms of inhibition of free-radical processed in mutagenesis and carcinogensis. Mutat Res, 202: 377-386, (1988)
95. Pryor WA., The antioxidant nutrients and disease preventions what do we know and what do we need to find out? Am. J. Clin. Nutr, 53: S391-S393, (1991)
96. Imaida K., Fukushima T., Shivai T., Ohtani M., Nakanishi K., Ito N., Promoting activities of butylated hydroxyanisole and bultylated hydroxytoluene on 2-stage urinary bladder carcinogenesis and inhibition of ç-glutamyl transpeptidase-positive foci development in the liver of rats. Carcinogenesis, 4: 895-899, (1983)
97. Anderson GH., Human milk feeding. Pediatr Clin North Am, 32: 335-353, (1985)
98. Turoli D., Testolin G., Zanini R., Bellù R., Determination of oxidative status in breast and formula milk. Acta Paediatr, 93: 1569-74, (2004)
99. Friel JK., Martin SM., Langdon M., Herzberg G., Buettner GR., Human milk provides better antioxidant protection than does infant formula. Pediatr Res, 51: 612-8, (2002)
100. L'Abbe M., Friel JK., Glutathione peroxidase and superoxide dismutase concentrations in milk from mothers of premature and full-term infants. J Pediatr Gastr Nutr, 31: 270-4, (2000)
101. Lindmark-Mansson H., Akesson B., Antioxidative factors in milk. Br: J. Nutr, 84: S103-S110, (2000)
102. Asada K., Occurrence of superoxide dismutase in bovine milk. Agric Biol Chem, 40: 1659-60, (1976)
103. Hirvi Y., Griffiths MW., Milk catalase activity as an indicator of thermization treatments used in the manufacture of cheddar cheese. J Dairy Sci, 81: 338-45, (1998)

104. Hojo Y., Selenium concentration and glutathione peroxidase activity in cows milk. Biol Trace Elem Res, 4: 233-9, (1982)

105. Raghuveer TS., Mcguire EM., Martin SM., Lactoferrin in the preterm infants' diet attenuates iron-induced oxidation products. Pediatr Res, 52: 964-72, (2002)

106. Halliwell B., Gutteridge JM., Cross CE., Free radicals, antioxidants, and human disease: Where are we now? J Lab Clin Med, 119: 598-620, (1992)

107. Yannis S., Apostolos M., Ioannis V.,Angela-Gabriella D., Pasquale L., Emilio J., Pavlos C., Valantis K., Athina B.,Giovanni M.,Spyridon K., Antioxidant and anti-platelet properties of milk from goat, donkey and cow: An in vitro, ex vivo and in vivo study.International Dairy Journal, 21(11):901–906, (2011)

108. Ip C., Chin SF., Scimeca JA., Pariza MW., Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res, 51: 6118-6124, (1991)

109. Ip C., Singh M., Thompson HJ., Scimeca JA., Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res,124: 615-620, (1994)

110. Ip C., Briggs SP., Haegele AD., Thompson HJ., Storkson J., Scimeca JA., The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet. Carcin,17: 1045-1050, (1996).