CHAPTER 2

REVIEW OF LITERATURE

“The characteristic of scientific progress is our knowing that we did not know”

-GASTON BACHELARD

French philosopher
CHAPTER -2

REVIEW OF LITERATURE

The review of literature pertaining to the study “Evaluating Beneficial Effect of Breast Milk through Biochemical, Microbiological and Molecular Methods” is presented under the following headings.

2.1. Lactation

2.1.1. Milk as a Fluid

2.1.2. Human Milk

2.1.3. Functional Anatomy of the Human Breast

2.1.4. Overview of the Mammary Alveolus

2.1.5. Regulation of Breast Development and Lactation

2.1.6. Physiology and Secretion of Human Milk

2.1.7. Composition of Human Milk

2.1.8. Nutritive Value of Human Milk

2.1.9. Biochemical Parameters and Their Changes In Lactating Women

2.12. The History and the Definition of Probiotics

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2.15. Immune System and Probiotics

2.15.1 Diarrhoea

2.15.2 Cancer

2.15.3 Cholesterol Reduction

2.16. Mechanism of Probiotics
2.17. Selection Criteria for Probiotics

2.18. Human Milk – A Source of Potential Probiotic Strain

2.2 Mastitis

2.1 **LACTATION**

Changes in body weight and composition in response to the metabolic load imposed by lactation are highly variable among and within diverse populations. In most reports, rates of weight loss did not differ between lactating and non-lactating women. Despite differences in the hormonal milieu between lactating and non-lactating women only subtle short-term differences were observed in postpartum changes in body composition. Regional patterns of fat deposition and mobilization did not differ between lactating and non-lactating women in most studies. Changes in body composition during lactation are responses to a sequence of complex neuroendocrine and biochemical stimuli that may be significantly modified by environmental factors. Gestational weight gain was the strongest determinant of postpartum weight and fat mass change, which supports the premise those biological mechanisms. (Butte, F, Judty, Hopkinson, M, 1988)

Changes in the maternal body during reproduction are characterized by their diversity and this holds true for comparisons within as well as between species. An anticipatory role for the maternal body during reproduction occurs in a number of species, including humans that tend to deposit fat in their bodies during pregnancy and lose it during lactation. (Robinson, 1986).

In this review we will examine evidence that supports or refutes the application of these basic tenets in humans. It is generally recognized that the metabolic demand imposed by lactation in humans is relatively low, compared with other species. (Prentice and Prentice 1988).

Lactation requires both an increased supply of nutrients and development of mechanisms that ensure the preferential use of nutrients by the Mammary gland lactation is characterized by enhanced episodic secretion of prolactin and oxytocin, suppression of hypothalamic pituitary gonadal axis and hypoinsulinemia (Vernon, 1989)
Subcutaneous fat changes in low income lactating mothers and growth of breastfed infants we studied changes in body fat of lactating mothers and its relationship to milk fat and growth of exclusively breastfed infants during the first 3 months of life. Changes in body fat measured by body mass index (BMI) and skinfold thickness of 39 low socio economic status women were measured every 15 days during the first 3 months post partum. There was a decrease in maternal skinfold thickness from the 15th day postpartum. Milk fat concentrations decreased significantly only at the second month of lactation. (Fornes NS, DoreaJG, 1995)

Breast feeding reduces maternal lower body fat. Our findings indicate that a woman’s choice of infant feeding practice influences post partum anthropometric changes but these effects may be temporary. (Forsum E, Sadurskis A, 1989).

Energy cost of lactation, and energy balances of well nourished Dutch lactating women reappraisal of the extra energy requirements of lactation. The present study helps in the understanding of how well nourished women with an adequate lactational performance may cope in everyday life with the energy stress of lactation, and suggests that current recommendation of energy needs during lactation are too high. (Van Raaij, JM, Schonk, CM, Peek, ME, 1991)

Based on cross sectional measurements in lactating and non lactating women, (Efficiency was found to be 94.2+ 3.5%). It must be stressed that this value is not directly comparable with the livestock definition of efficiency which incorporates all components of daily energy expenditure in the denominator. It should also be noted that this approach assumes that milk synthesis is equally active during the measurement of BMR. BMR was actually lower in lactating women than prior to conception. The need for physiological down-regulation seems to be caused by an in adequate diet and might have detrimental consequences to the mother. Such higher apparent. Efficiencies should not therefore be used as the basis of recommended requirements. If BMR is not suppressed in lactating women. (Lawrence et. al., 1986)

Diet induced thermo genesis (DIT) in lactating and non-lactating women has been evaluated in two cross sectional studies. In both, the energy content of the test meal was larger in the lactating than in the non lactating women, and therefore the results cannot be compared. (Motil et. al., 1990)
The B vitamin folate and its co enzymatic forms are essential for one-carbon transfer reactions which are underlying events for protein, DNA and RNA biosynthesis. Folate requirements are greatest during periods of growth development, and reproduction but may not always be met because folate deficiency occurs frequently during these nutritionally vulnerable stages of life. It is estimated that globally up to one third of lactating women some degree of folate under nutrition. (Senete FR, Pilch SM, 1985)

There are several potential mechanisms of energy conservation during lactation in addition to the mobilization and utilization of fat. Three such “energy sparing adaptations” that may permit lactation to proceed normally when energy intake is limited, including decreases in basal metabolic rate (BMR), thermogenesis, and physical activity. (Prentice, 1998)

Long chain polyunsaturated fatty acids and perinatal development for lactating women we consider it premature to recommend specific LcPUFA intakes. However, it seems prudent for pregnant and lactating women to include some food sources of DHA in their diet in view of their assumed increase in Lc PUFA demand and the relationship between maternal and fetal DHA status (Koletzko B., Agostioni, et al., 2001)

Apoptosis in normal and neoplastic mammary gland development. Apoptosis plays important roles in mammary development from early embryonic formation of the mammary gland to the regression that follows cessation of cycling. The most dramatic occurrence of apoptosis is found during mammary involution. Most of the secretory epithelium in the lactating breast undergoes apoptosis as the mammary gland regresses and is reorganized for another cycle of lactation (Strange R, 2001)

Body compositions changes during lactation are highly variable among women changes in body weight composition in response to the metabolic load imposed by lactation are highly variable among and within diverse populations. In most reports, rates of weight loss did not differ between lactating and non lactating women. Despite differences in the hormonal milieu between lactating and non lactating women. Only subtle short-term differences were observed in post partum changes in body composition. Regional patterns of fat deposition and studies. Changes in body composition during lactation are responses to a sequence of complex
neuroendocrine and biochemical stimuli that may be significantly modified by environmental factors. (Butte NF, Hopkinson JM, 1998)

2.1.2 MILK AS A FLUID

Milk is a complex fluid composed of several phases that can be separated by centrifugation (Neville, 1995a) into a cream layer, an aqueous phase, and a two-phase pellet. The upper phase consists of milk cells and membranous debris, the lower of casein micelles. Casein can also be precipitated by micelle-destroying treatments such as the enzyme rennin or low pH leaving an aqueous phase that is often termed whey. If the whey is made from skim milk it is a true solution that contains all the milk sugar as well as the major milk proteins lactoferrin and secretory immunoglobulin A (sIgA), the monovalent ions sodium, potassium and chloride, citrate, calcium, free phosphate and most of the water-soluble minor components of milk. Depending on the species, varying proportions of the lactoferrin, lysozyme, citrate and calcium may be found associated with the casein pellet. The casein fraction is a small proportion of human milk, about 0.2% by weight. However, casein makes up 4% of cow's milk and as much as 12% of rodent milks. The casein fraction from cow's milk, usually obtained by rennin precipitation, is used in cheese-making while the whey finds a multiplicity of uses, most notably as the base for infant formula.

2.1.3 HUMAN MILK

During the first two or three days, the fluid that comes out from the breast is small in quantity, yellowish in color and has a high protein content and is called colostrum. It contains fat globules, which appear under the microscope as colostrums corpuscles. They are probably mononuclear phagocytes containing fat droplets. However the total fat content is less than in milk. The protein which is mainly globulin and some albumin is more in colostrums there is no caseinogens. It also contains more minerals. (Miranda, P, Saravia, NG, 1983)

Breast milk is the ideal food for babies as it contains all the constituents in suitable concentrations and is easily digestible. The proteins are lactalbumin, lactoglobulin and casein. The chief carbohydrate is those minerals and all vitamins except vitamin K are present in varying concentrations. Iron content is poor. Immunoglobulin (IgA) is present. The amount of proteins,
fats, carbohydrates present in human milk. Only if breast milk is scanty or there contraindication to breast feeding should products be given. (Leneno, KJ, 1993)

During lactation blood flow to the breast considerably increased. Milk secretion lasts for 9 months and is prolonged for a year is suckling is continued when lactation is well established about 850ml of milk is secreted. It is estimated that the average daily milk secretion during the year, in Indian women is 400ml. During the period of breast feeding ovulation and menstruation are suppressed, and lactation is regarded as a natural method of conception. However, ovulation may occur be the return of the first menstrual period. (Taggart N., Holliday R, 1967)

Milk is synthesized within the cells lining the mammary gland alveoli. Milk is a complex nutrient mainly of milk lipids, the milk as monovalent cations, and interesting, antibodies. converted to lactose by the lactose which is two individual catalytic enzyme galactosyltransferase and Lactalbumin is the regulatory subunit. In addition a number of major and minor milk proteins are synthesized. (Mcnilly AS, 1979)

A review of the literature indicates that apparently healthy women can become folate depleted in the early postpartum period. Milk folate content can be maintained at a level that prevents the development of folate inadequacy in exclusively breast-fed infants,

<table>
<thead>
<tr>
<th>Showing concentrations in 100ml</th>
<th>Human milk</th>
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<tbody>
<tr>
<td>1. Water in ml</td>
<td>8.8</td>
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<tr>
<td>2. Protein in g</td>
<td>1.2</td>
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<tr>
<td>3. Casein g</td>
<td>1.2</td>
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<tr>
<td>4. Carbohydrate in g</td>
<td>7.0</td>
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<tr>
<td>5. Fat in g</td>
<td>3.8</td>
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<tr>
<td>6. Calcium in mg</td>
<td>3.3</td>
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<tr>
<td>7. Calories</td>
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Consisting of sugar, lactose, proteins as well and divalent most immune Glucose is the milk sugar, enzyme, synthetase, composed of subunits. The subunit of the
but often at the expense of maternal folate stores. During lactation folate is preferentially taken up by actively secreting mammary glands. And only in the case of frank maternal folate deficiency is milk folate reported to decline to critically low concentrations. (Metz J, Zelusky R, 1968)

Folate in human milk is reported to remain stable or to increase as lactation progresses and there is considerable variability in reported mean values for folate in human milk. It is now well established that methodological difficulties rather than differences among individuals are responsible for the wide range in values reported for milk folate previously, our laboratory reported that in the analysis of human milk folate. (Lirn HS, Mackey AD, 1998)

The folate content of human milk is likely to be underestimated unless an antioxidant is used to prevent it from being oxidized, conjugase pretreatment is performed to cleave the long-chain forms of the vitamin, heat treatment is applied to release the folate from its binding proteins before microbiological analysis and test organisms are selected that are able to use all the forms of folate in the samples. (Connor et al., 1990a)

Bioactive constituents’ enzymes and other bioactive constituents of human milk may alter the composition of expressed milk (Greenberg and Graves 1984). The hydrolysis of fats in human milk appears to generate fatty acids and monoglycerides with antiviral properties (Isaacs et al., 1986).

Many other host resistance factors in human milk, lysozyme is relatively resistant to proteolysis and to denaturation resulting from the high acidity within the stomach (Goldman et al., 1982, 1983 a, b).

The morphology of human milk macrophages suggests that they are activated, indeed, that is born out by the fact that they are more motile than their precursors in blood. The macrophages in human milk are involved in antigen processing and presentation to T lymphocytes and thus may serve in the recognition of foreign materials. Further more, these macrophages display class II major histocompatibility antigens, which suggests that they may participate in the process of Immunogenesis in the Infant (Leyva cobian and clemente, 1984)
Thymic-dependent lymphocytes (T-cells) account for the majority of lymphocytes in milk, the relative proportions of the major sup populations of these cells may be similar to those in blood. (Keller et al., 1986)

The kinds of fatty acids present in human milk are strongly influenced by maternal diet; the type and amount of fat in the diet and the adequacy of energy intake. However, maternal stores or tissues include the macronutrients, most minerals and folate. (Zimecki and Coworkers, 1987)

Modifications to infant formulas are continually being made as the components of human milk are characterized and as the nutrient needs of diverse groups of infants are identified. Long chain polyunsaturated fatty acids added in amounts similar to those in human milk have recently become available in the United States; infants fed these formulas or human milk have higher tissue concentrations of long chain polyunsaturated fatty acids and reportedly have better visual acuity than do infants fed non-supplemented formulas. Selenium, an important antioxidant, is present in higher concentrations in human milk than in non-fortified cow milk based formula, and the selenium intakes of infants fed non-fortified formulas are reported to be at or below recommended levels. Blood selenium concentrations and plasma glutathione peroxidase activity are higher in infants fed selenium supplemented formulas or human milk in infants fed non-fortified formulas. Nucleotides and their related products play key roles in many biological processes. Although nucleotides can be synthesized endogenously, they are considered “conditionally essential” Nucleotide concentrations in human milk are higher than in un-supplemented cow milk based formulas, and studies in animals and human infants suggest that dietary nucleotides play a role in the development of the gastrointestinal and immune systems. (Carver, JD, 2003)

Docosahexaenoic acid (DHA) and arachidonic acid (ARA) are important structural components of the central nervous system. These fatty acids are transferred across the placenta, are present in human milk, and are accumulated in the brain and retina during fetal and infant development. The high concentration of DHA in the retina and of DHA and ARA in brain gray matter suggests that these fatty acids have important role in retinal and neural function. Animal studies have shown that depletion of DHA from the retina and brain results in reduced visual
functions and learning deficits. The latter effects may be explained by changes in the membrane bilayer that alter membrane associated receptors and signal transduction systems, ion channel activity, or direct effects on gene expression. DHA can be formed in the liver from alpha linolenic acid but it is unclear if the rate of DHA synthesis in human is sufficient to support optimal brain and retinal development. (Innis. SM, 2003)

The parenchyma of the breast consists of approximately 10 to 15 ducts extending from the nipple and coursing through the mammary fat pad to terminate in grape-like clusters of alveoli (Fig. 1). Each duct serves a specific lobule. The lobules are separated and supported by thick connective tissue septa and, in the non-pregnant, non-lactating breast, by large amounts of adipose tissue. Blood vessels, nerves and lymphatic run in the septa which merge imperceptibly with the fascia at the anterior thoracic wall. The nipple, which serves as the termination point for the lactiferous ducts, is surrounded by an area of pigmented skin, the areola, containing sebaceous glands and sweat glands.

2.1.4 FUNCTIONAL ANATOMY OF THE HUMAN BREAST

Figure-2

BREAST ANATOMY

The areola serves as the termination point for the fourth intercostal nerve which carries sensory information about suckling to the spinal cord and brain. This is extremely important in the regulation of oxytocin secretion from the posterior pituitary and prolactin from the anterior pituitary. The mammary ducts expand slightly to form sinuses beneath the areola.

Figure-3
During pregnancy the alveolar complexes increase in number and complexity and the cells lining the alveoli and small ducts mature, acquiring the capability to secrete milk. However, milk secretion is kept in check by high concentrations of circulating sex steroids, primarily progesterone. At parturition a series of programmed changes transforms the cells into the fully secretory state. This transformation is termed lactogenesis. Thereafter milk is secreted more or less continuously into the alveolar lumens and stored there until the let-down reflex brings about contraction of the myoepithelial cells, forcing milk through the ducts to the sinuses beneath the areola where it becomes available to the suckling infant.

2.1.5 OVERVIEW OF THE MAMMARY ALVEOLUS
**Figure 4.** Model alveolus (a) with subtending duct (d) showing blood supply, adipocyte stroma, myoepithelial cells, and plasma cells (PC).

Although the location and external form of the mammary glands differ from one species to another, the mechanisms of milk production are remarkably similar. Milk is produced and stored in alveolar units like that diagramed in Figure 2. Removal of milk from the alveoli is accomplished by contraction of the myoepithelial cells surrounding the alveoli (a) and ducts (d). This process is called milk ejection. Milk exits through ductules into ducts draining several clusters of alveoli. In the human the small ducts coalesce into 15 to 25 main ducts that drain sectors of the gland. The main ducts dilate into small sinuses as they near the areola where they open directly on the nipple. In many species including both ruminants and rodents the ducts empty into a single primary duct or a cistern which in turn is drained by a single teat canal. In dairy animals the cistern provides additional milk storage.

In comparison to related dermal glands such as the salivary and sweat glands, the rate of milk secretion is slow, about 1.5 ml of milk per gram of tissue per day (Peaker, 1977). Histologically, the cells lining the smaller ducts resemble the alveolar cells, even reacting with anticasein antibodies (Smith & Vonderhaar, 1981). The larger ducts play a passive role in milk secretion, merely transferring the milk from the alveolar stores to the sub-areolar sinuses where it becomes available to the suckling infant. Because the composition of the aqueous phase of milk changes very little during a feed or milking (Neville et al., 1984), it is unlikely that reabsorptive
processes, like those important in the formation of saliva or sweat, play a significant role in determining milk composition.

Although the mammary epithelial cells are ultimately responsible for converting most precursors into milk constituents and transporting them to the mammary lumen, as illustrated in Figure 2 other cell types are also intimately involved in milk production. We have already mentioned the myoepithelial cells responsible for milk ejection from the breast. The mammary ducts and alveoli are embedded in a stroma that contains fibroblasts, adipocytes, plasma cells and blood vessels. Blood flow is greatly expanded during lactation to make available the large amounts of substrate required for milk synthesis. Interactions with stromal cells are intimately involved in mammary development and milk secretion. Stromal fibroblasts and/or adipocytes are known to be the source of growth factors such as hepatic growth factor/scatter factor and IGF-1 and are probably responsible for production of the enzyme lipoprotein lipase, important in milk lipid synthesis. During lactation B lymphocytes "home" to the mammary gland where they become plasma cells and settle in the interstitial space producing the immunoglobulins that ultimately find their way into milk (Hayward, 1983). The mammary epithelium should, therefore, be viewed as an integrator of activities in many cells and tissues that contribute in a coordinated fashion to the synthesis of milk.

Five distinct processes are utilized by the mammary epithelium in the secretion of milk (Figure 3). These pathways operate in parallel to transform precursors derived from the blood or interstitial fluid into milk constituents. Although the biochemical processes involved are fundamentally the same in all mammals, differences in their relative rates and, in some cases, in the nature of the products synthesized result in milks whose composition differs widely from species to species. Some of the milk secretion pathways, e.g., exocytosis of protein-containing vesicles and transcytosis of immunoglobulins, are similar to processes in many exocrine organs. In contrast, the mechanism for fat secretion is unique to the mammary gland. Because the cell biology of the five pathways is well-defined, the nineteenth century terms apocrine, merocrine and holocrine secretion, occasionally still applied to milk secretion, no longer adds to our understanding and should be abandoned.
2.1.6 CELLULAR MECHANISMS FOR MILK SYNTHESIS AND SECRETION

Figure 5. Alveolar Cell from lactating mammary gland. N, nucleus; TJ, tight junction; GJ, gap junction; D, desmosome; SV, secretory vesicle; FDA, fat-depleted adipocyte; PC, Plasma Cell; BM, basement membrane; ME, cross section through process of myoepithelial cell; RER, rough endoplasmic reticulum. See text for explanation of secretory pathways I (exocytosis), II (lipid), III (apical transport), IV (transcytosis) and V (paracellular pathway).

Four secretory processes are synchronized in the mammary epithelial cell of the lactating mammary gland: exocytosis, lipid synthesis and secretion, transmembrane secretion of ions and water and transcytosis of extra-alveolar proteins such as immunoglobulins, hormones and albumin from the interstitial space. A fifth pathway, the paracellular pathway, allows the direct transfer of materials between the milk space and the interstitial space. This pathway is open in the pregnant gland and allows the transfer of molecules at least as large as intact immunoglobulin. It is closed in the fully lactating gland providing a tight barrier between the milk and interstitial spaces. This barrier opens again in the presence of mastitis and during involution (Neville, 1995b).

2.1.6.1 Exocytosis

Most of the components of the aqueous phase of milk are secreted by the exocytotic pathway (Pathway I, Figure 3). Proteins synthesized on ribosomes are transferred to the lumen of the rough endoplasmic reticulum where their signal sequences are cleaved and the protein molecules folded. Vesicles transfer the proteins to the Golgi stack where they are further processed by the addition of carbohydrate, phosphate or other groups and packaged into secretory vesicles.
In addition to processing of milk proteins \textit{per se}, the Golgi vesicles in the lactating mammary cell synthesize lactose from precursor UDP-galactose and glucose that enter from the cytoplasm. Because the Golgi membrane is impermeable to lactose, the sugar is osmotically active and water is drawn into the terminal Golgi vesicles. The swollen appearance of the \textit{trans}-Golgi and the secretory vesicles which arise from it are specific characteristics of the lactating mammary cell. Casein micelle formation begins in the \textit{cis}-Golgi with condensation of casein molecules; addition of calcium, possibly in the secretory vesicle, leads to maturation of the casein micelles into particles sufficiently dense to be seen in the electron microscope. Secretory vesicles are thought to be the source of most of the constituents of the aqueous phase of milk including citrate, nucleotides, calcium, phosphate and probably monovalent ions and glucose. However, the apical membrane of the mammary alveolar cell has transporters for monovalent ions and glucose and the concentration of these substances may be adjusted by direct membrane transport (see below).

Secretory vesicles move to the plasma membrane where they fuse and release their contents into the milk space by exocytosis. The exocytotic pathway involved in milk secretion appears to be largely constitutive. This means that, once secretion begins after parturition, exocytosis is continuous and secretory products are not stored within the epithelial cell. However, Burgoyne and his colleagues (Turner et al., 1992) have observed that a portion of the mammary secretion could be stimulated by increased cell calcium in an in vitro system suggesting the presence of a regulated secretion pathway in the lactating mammary epithelial cell.

2.1.6.2 Lipid Synthesis and Secretion.

Triglycerides, synthesized in the smooth endoplasmic reticulum of the mammary alveolar cell from precursor fatty acids and glycerol, coalesce into large droplets that are drawn to the apex of the cell (Pathway II, Figure 3). The lipid droplets bulge against and gradually become enveloped in apical plasma membrane, finally separating from the cell as the milk fat globule. The occasional inclusion of a crescent of cytoplasm within the membrane-bound globule enables any substance contained in the cytoplasm to enter milk. The membrane surrounding the milk fat globule has two functions: it is the primary dietary source of phospholipids and cholesterol for the breast-fed infant and it prevents the fat globules from coalescing into large fat droplets that
might prove difficult to secrete. Butter results when a suspension of separated milk fat droplets is beaten or churned to remove the membranes allowing the fat droplets to condense.

2.1.6.3 Transport across the Apical Membrane.

In contrast to the other pathways for milk secretion the pathways for the direct transport of substances across the apical membrane of the mammary alveolar cell are poorly understood (Pathway 3, Figure 3). Linzell and Peaker (Linzell & Peaker, 1971) devised a clever technique to determine what molecules could utilize this pathway, infusing isotopes of small molecules up the teat of a goat and calculating how much of the substance left the milk and entered the blood. They found that sodium, potassium, chloride and certain monosaccharides as well as water directly permeated this membrane (Linzell & Peaker, 1971) but calcium, phosphate and citrate did not (Neville & Peaker, 1981). Studies of bicarbonate secretion led these investigators to postulate the presence of chloride-bicarbonate exchange at the apical membrane (Linzell & Peaker, 1975). Stable isotope studies in women confirmed the presence of a glucose pathway across the apical membrane in the human mammary gland as well (Neville et al., 1990). The function of the apical pathways is not clear, since the composition of the aqueous phase of milk is thought to be determined in the Golgi and secretory vesicles. What is clear, however, is that apical pathways are limited to a modest number of small molecules. Although often overlooked, many drugs enter milk by direct transfer across both basolateral and apical membranes of the mammary alveolar cell. For this reason most therapeutic drugs are transferred efficiently into milk (Fleishaker & McNamara, 1988)

2.1.6.3.1 Transcytosis of Interstitial Molecules.

Intact proteins can cross the mammary epithelium from the interstitial fluid either by transcytosis or through the paracellular pathway. During lactation only the transcytotic pathway is available (Pathway IV, Figure 3). Immunoglobulins are the best studied of the molecules that enter milk via transcytosis. In most-non-ruminants IgA is synthesized by plasma cells in the interstitial spaces of the mammary gland or elsewhere in the body (Hayward, 1983). The protein binds to receptors, the polymeric immunoglobulin receptor, on the basal surface of the mammary alveolar cell; the entire IgA-receptor complex is endocytosed and transferred across the cell. At the apical membrane the extracellular portion of the receptor is cleaved and secreted together with the IgA. The cleaved receptor portion is known as secretory component and the secreted
product is thus secretory IgA or sIgA. The many proteins, hormones and growth factors that find their way into milk from the plasma are also thought to be secreted by similar, but much less well-studied, mechanisms.

**2.1.6.4. The Paracellular Pathway**

The paracellular pathway (Pathway V, Figure 3) allows passage of substances between epithelial cells, rather than through them. During full lactation the passage of even small molecular weight substances between alveolar cells is impeded by a gasket-like structure called the tight junctions (*Zonula occludens*) that joins the epithelial cells tightly, one to another. Although immune cells apparently can diapedese between epithelial cells to reach the milk (Lin et al., 1995; Seelig, Jr. & Beer, 1981), the junctions seal tightly behind them leaving no permanent gap. During pregnancy, with mastitis and after involution the tight junctions become leaky and allow components of the interstitial space to pass unimpeded into the milk. At the same time milk components can enter the plasma. This leakiness is useful during these periods since secretion products are allowed to leave the gland, inflammatory cells and protective molecules can enter the milk space and products of the dissolution of the mammary cells during involution can be cleared from the breast. When the junctions are open the mammary secretion has high sodium and chloride concentrations, a fact that is sometimes useful in diagnosing breastfeeding problems (Morton, 1994).

**2.1.7 LACTOGENESIS: THE TRANSITION FROM PREGNANCY TO LACTATION**

Formally lactogenesis is defined as the onset of milk secretion. Hartmann (1973) and Linzell and colleagues (Fleet et al, 1975), based on work in ruminants, divided lactogenesis into two stages. Lactogenesis Stage 1 occurs during pregnancy when the gland becomes sufficiently differentiated to secrete small quantities of specific milk components such as casein and lactose. Lactogenesis Stage 2 is defined as the onset of copious milk secretion associated with parturition. It is brought about by a decline in progesterone around the time of parturition in the presence of maintained prolactin concentrations. A differentiated mammary epithelium is necessary for stage 2 lactogenesis to occur. In humans the epithelium reaches this stage of differentiation about mid-pregnancy.

In the early post-partum period the secretion product of the mammary gland is called colostrum. This fluid contains high concentrations of immunoglobulins and the protective
protein, lactoferrin. In species such as ruminants that lack transplacental transport of immunoglobulins, feeding of colostrum is necessary to provide passive immune protection to the young until their immune systems become mature. In other species, such as humans, where transplacental transport of immunoglobulins provides humoral immunity in the early post-partum period, the presence of secretory IgA, lactoferrin and high concentrations of oligosaccharides is important in protection of mucosal surfaces from infection, particularly under conditions where optimal sanitation cannot be maintained.

Lactogenesis represents a profound and rapid series of changes in the activity of differentiated mammary epithelial cells from a quiescent state to a fully active secretory state. As can be illustrated by examining the composition of human milk during the first week post-partum, these changes occur as an orderly progression of events that starts with closure of the tight junctions between the epithelial cells, followed by a transient increase in the secretion of the protective proteins sIgA and lactoferrin. After about 36 hours a rapid increase in the synthesis of all the components of mature milk begins that is complete by about day 5 postpartum.

**Figure 6.** Changes in human milk composition and volume in the early postpartum period. The concentration scale for lactose sodium and chloride, whose concentrations begin to change immediately post-partum, is on the left. Volume increases from 50 ml/day on day 1 to 600 ml/day on day 5 with the greatest increase taking place between days two and four.

The first change to occur is a fall in the sodium and chloride concentrations in the milk and an increase in the lactose concentration (Figure 4). These modifications commence immediately after birth and are largely complete by 72 hours postpartum (Neville et al., 1991). They precede the onset of the increase in milk volume by at least 36 hours and can be explained
by closure of the tight junctions. With blockage of the paracellular pathway lactose, made by the epithelial cells, can no longer pass into the plasma. Sodium and chloride can no longer pass from the interstitial space into the lumina of the mammary alveoli. Thus this phase is marked by a rapid fall in the sodium and chloride concentrations of milk and a rapid increase in the lactose concentration.

**Figure 7.** Changes in the concentrations of IgA and lactoferrin in human milk in the post-partum period.

The next change is an increase in the rates of secretion of sIgA and lactoferrin (Figure 5). The concentrations of these two important protective proteins remain high for the first 48 hours after birth, together comprising as much as 10% by weight of the milk. Colostrum also contains high concentrations of cells including lymphocytes, macrophages, neutrophils and sloughed secretory epithelium (Ho et al., 1979). The concentrations of lactoferrin, sIgA and cellular elements fall rapidly after day 2, a consequence of both dilution as milk volume secretion increases and a decrease in their absolute rate of secretion. Although both lactoferrin and IgA are found at high concentrations in colostrum, their secretion is likely under separate control since lactoferrin is secreted by the exocytotic pathway (I in Figure 3) while sIgA is secreted by the transcytotic pathway (IV in Figure 3). Further the secretion rate of lactoferrin peaks about one day after the peak of sIgA secretion.

Finally, starting about 36 hours postpartum there is a 10-fold increase in milk volume from about 50 ml/day to 500 ml/day (Figure 4). This volume increase is perceived by the parturient woman as the "coming in" of the milk and is brought about by a massive increase in the rates of synthesis and/or secretion of almost all the components of mature milk (Neville et
al.1991), most notably lactose, casein (Patton et al., 1986), alpha-lactalbumin, lipid, calcium, potassium, etc. Three milk components, citrate, glucose and free phosphate, are quite interesting because their concentrations increase in proportion to the increase in milk volume. In the case of glucose the change in concentration has been shown to be due to an increase in glucose transport from the interstitial space into the mammary alveolar cell (Neville et al., 1990) whereas the increase in phosphate possibly results from dephosphorylation of the UDP generated during lactose synthesis. The increase in citrate may be related to fat synthesis; however, it does not occur in all species since rodents do not have citrate in their milks.

2.1.8 HORMONAL REGULATION OF BREAST DEVELOPMENT AND LACTATION

Optimum development of the breast requires the coordinated action of many hormones, PRL, estrogen, progesterone, adrenal steroids, Insulin GH and thyroid hormone. The estrogen promotes primarily ducts growth, whereas PRL and progesterone are necessary for lobulo-alveolar development, and PRL alone governs lactation.

2.1.8.1 Prolactin

Of all hormones, PRL appears to be the dominant one governing the breast. It is important in all phases of breast development was clearly shown in the careful studies of Lyons, Li and Johnson Talwalker and meites, though it would seem that ordinarily PRL requires estrogen to function as a stimulator of epithelial cell proliferation. With the addition of progesterone, PRL particularly fosters lobulo-alveolar development. Its growth promoting properties in various animal preparations have been substantiated by DNA measurement in addition to microscopic observation.

PRL is also the controlling hormone regulating many steps of the secretory process, including the formation of milk proteins casein and α-lactalbumin.

The chorionic hormone placental lactogen (HPL) also circulates in large amounts in maternal blood during human pregnancy. It appears to have essentially the same action as PRL. Although of slightly lesser potency than PRL on a weight basis, it is present in considerably greater quantities and therefore must be regarded along with PRL as being a major contributor to breast growth during gestation. (Chatterjee. CC, 1985)
2.1.8.2 Estrogen

The role of estrogen is complex. Although a highly potent mammogen. It is ineffective by itself in the absence of anterior pituitary hormones. In humans it also increases GH secretion. In the presence of these two hormones estrogen acts on breast tissue to promote primarily ductal development.

Although helping to prepare the breast for eventual milk formation, estrogen inhibits actual lactation, and in this respect appears to act as antagonist to PRL. Estrogen receptors, both cytoplasmic and nuclear, have been demonstrated in normal as well as in tumorous breast tissue. Concentrations of cytoplasmic receptors rise during later pregnancy and the first part of lactation. The significance of this rise and the factors that regulate estrogen receptor synthesis are still largely unknown. (Sempulingarn, 1988).

2.1.8.3 Progesterone

Like estrogen, progesterone has no effect on the breast in the absence of anterior pituitary hormones. Even in the presence of PRL Progesterone has little or no effect unless there is concomitant or preceding estrogen stimulation under those conditions progesterone acts principally to synergize with PRL in promoting lobulo alveolar development like estrogen, Progesterone inhibits actual lactation. This begins after the decline of progesterone that follows parturition. Exogenously administered progesterone is considerably less effective.

2.1.8.4 Growth hormone

Growth hormone appears to synergize with PRL, and may be able to substituted for it in promoting certain phases of breast growth, such as ductal development. Different animal GH’S possess different degrees of prolactin. Like activity in homologous and heterologous species. Although GH seems to improve the degree of breast growth obtainable with combinations of other hormones hypophysectomized animals. Its essentiality for breast growth is questionable, at least in humans. Despite the fact that human and primate GH’S have strong intrinsic PRL like activity the fact that ateriotic dwarfs who are
essentially completerly lacking in GH develop breasts and lactate normally post partum, suggests that GH is not important for lactation in the human.(Barba C.V.C.,Vanaai. M.A., 1992).

1.8.5 Insulin

Insulin is necessary for PRL and other hormones to exert their effects on breast tissue invitro and insulin serum factors resembling it are probably necessary invivo as well. Insulin receptors have been demonstrated in breast tissues. Although necessary for breast growth and lactation insulin probably does not play a regulatory role in any of these processes by virtue of changes in its concentration. (Hussey JR. Sharpe PA, 1995).

2.1.8.6 Adrenal steroids

Like insulin, corticosteroids appear to be necessary for most phases of breast growth and secretion both invitro and invivo. The requirement is probably for a glucocorticoid or steroid having glucocorticoid activity (e.g. aldosterone). Rather than for a mineralocorticoid. Cytoplasmic glucocorticoid receptors have been demonstrated in lactating mammary tissue. As with insulin, corticosteroids probably exert a permissive rather than a regulatory role. (Lapidus L, Bengtsson, C, 1996)

2.1.8.7 Thyroid hormone

Thyroid hormone does not appear to be essential for their breast development or lacted although both processes may be adversely affected in states of thyroid hormones, deficiency or excess. (Andrew G, Frantz, 1989)

Suppression of hypothalamic pituitary-adrenal axis responses to stress in lactating women. The final 5 mm of exercise was set to elicit 90% of the maximal oxygen up take of each subject. Plasma ACTH, cortisol and glucose responses to exercise were significantly attenuated in lactating women. Basal nor epinephrine levels were also reduced in lactating women. These results indicate that stress responsive neuro hormonal systems are restrained in lactating women. (Altemus. M, ecuste. PA, 1995)

Insulin, cortisol and thyroid hormones modulate maternal protein status and milk production and composition in humans. The partitioning of dietary and endogenous nutrients
during lactation is not well understood. To examine association between plasma hormone and substrate profiles and indices of either maternal body protein metabolism or lactational performance, we measured plasma insulin, cortisol, prolactin, thyroxine, triiodothyronine, individual amino acid, blood urea nitrogen and prealbumin concentrations in lactating women in the post absorptive state. (Motil. KJ, Thotathu Chery. M, 1994)

In adult life, adaptation to acute and sub acute alterations in calcium homeostasis is largely accomplished through the actions of parathyroid hormone (PTH) and 1, 25 dinydroxy vitamin D. During the pregnancy and lactation other “non classical” hormones, such as parathyroid hormone related Protein (PTHrP) appear to contribute to the alterations in calcium and skeletal horneostasis which occur in this setting maternal calcium homeostasis is geared to provide sufficient calcium flux across the placenta during pregnancy and into breast milk during lactation to ensure normal fetal, and neonatal skeletal mineralization. These requirements are. Substantial and cannot met solely by augmented intestinal calcium absorption. (Urszulas, Masiukiewie M.D, 2003).

2.1.9 COMPOSITION OF HUMAN MILK

2.1.9 .1 Differences between milks

Mature Human milk looks watery and bluisn compared with cows’ buffalo’s or goats milk. It does not look as ‘nutritious’ as these animal milks. Certainly all animal milks have high nutritional value but each has a different chemical composition and they are all different from human milk. Recent scientific advances have enabled researchers to show how human milk is exactly what a human infant needs. And human milk is a better food for a baby in every way in which it is different from animal milks (Benshaul, 1962)

The overall dilution of milk-ie. Low solute content of protein, sugar, salt. Probably some computation of all these factors operates but in any case, human milk has a very low energy concentration and is dilute in respect of all solutes, so according to either theory it should be natural, for humans to feed very often. (Lezoff et. al., 1977)
2.1.9.2 The First Milk- Colostrum

The bright yellowish colostrums secreted during the first 3-6 days after birth differs from mature milk. It has less fat and more protein, especially more of the immunoglobulin fraction of the protein especially more of the immune globulin fraction of the protein. These Ig are almost certainly valuable to the child. One particular kind of immunoglobulin IgA -may be important because the new born baby cannot produce its own colostrums contains more IgA than mature milk. It seems that IgA from milk lines the surface of the intestine, protecting the baby until it can make its own IgA. The protection is certainly against infections and may also help to prevent the development of allergies. (Jelliffe and Jellife, 1978)

2.1.9.3 Fat

Fat makes up almost half the energy content of human milk. Of an average of 70 kcal /l00ml, almost 30 kcal are from fat. (Hytten, 1954)

The fat content of milk not only varies between individuals, but it varies from month to month and from day to day in the same individual. There are even important difference between the beginning and end of a single feed. (Gunther and Stanier, 1949)

The fatty acids in milk follow the pattern in the women’s blood. This is turn partly follows the composition of the fat in her diet. (Ahrens, 1959)

There are two main ways in which the fats of human milk differ from those of other milks 1) Human milk contains more linoleic acid a special fatty acid which is essential for human. 2) The fats of human milk are easier for a baby to digest and absorb than are those of cow’s milk. (Widdowson, 1969, Gyorgy, 1971)

The cholesterol level in human milk is higher than in cow’s milk. The significance of this is not yet clear although many theories have been advanced (Tsang and Glueck, 1975)

2.1.9.4 Carbohydrates
The main carbohydrates in milk lactose, a sugar which is found only in milk. Lactose gives milk its sweetness and much of its special taste. Human milk contains more lactose than most other milks, which accounts for its particular sweetness.

Lactose is useful in several ways. It may be especially useful for the growing brain. Because when it is digested, galactose another sugar is formed which may be needed for brain tissue. (Abdullah, 1970)

2.1.9.5 Protein

The protein content of human milk may be even lower than previously believed. (Hambraeus, 1977)

Unlike fats the protein in human milk may be does not vary from day to day or during a feed. Further more the protein does not change much with the mother’s diet. Even moderately undernourished mother seem to have a normal protein level in their milk. (Lindblad and Rahimatoola, 1974)

The nutrient protein in milk but there are also other proteins whose main function are not nutrient, but anti-infective. Those are lysozyme (an enzyme with anti-infective properties); Ig; and proteins which bind, or carry vitamins and minerals such as vitamin Bl2, iron and zinc. The iron binding protein such as lactoferrin. These proteins mostly come from cells in the milk identical to white blood cells. Some cells are phagocytic, and other cells produce the IgA, and other anti-infective substance. So milk is a living fluid (white blood according to the Koran). A recent review of the anti infective properties of breast milk (Welsh and May, 1979)

2.1.9.6 Vitamins

Human milk contains sufficient, and most deficiencies in infants have been due to deficient formulas. Although we have known about the need for vitamins in general for some time, the exact quantities that a person need are still uncertain for most vitamins. (Allan Walker W, 1980)

2.1.9 .7 Water and Salt
Human milk is water rich that is, it is a rather dilute fluid in a normal infant, most nutrients remain in the body. As a result, there is little waste for the kidneys to excrete- a part from the excess water. Hence infant’s urine is much more dilute than adult urine. Infact an immature infant’s kidney cannot concentrate urine so well as an adult kidney. So an dilute milk is necessary.

It tropical climates or during febrile illness, or with diarrhea a child may lose water in other ways, for example by sweating or in the stool, so the high water content of breast milk is definite advantage,(Almroth,1978).

Cow’s milk has much more salt than human milk, and it can be difficult for an infant’s kidney to excrete it all. If formula is made up in the way suggested by the makers. There is often too much salt, and too much of some other substances. (Morley, 1978).

2.1.9.8 Other Minerals and Trace Elements

Human milk contains very little iron and cow’s milk even less but such iron as there is breast milk is very well absorbed. (Saarinen and Simes, 1997)

A baby is born with an iron store. As red blood cells are destroyed the iron in them is used again. The store and breast milk normally provide sufficient iron to last a child well over six months. Only a few infants actually become iron deficient (i.e. with a reduced serum ferritin). Exclusively breast-fed babies is Peru were no more iron deficient than healthy babies on a mixed diet, even at nine months of age (Paster, Howanitz, and Oski, 1981)

These are minerals which are present in the body in very small amounts but which are none the less essential for health. Zinc for instance, is needed for satisfactory growth, maturation, and immunity, zinc is also needed to prevent a disease called acrodermatitis enteropathica which affects the skin and intestines, and used to be fetal. A wide range values is given for the level of each substances in human milk. (Belavady, 1978)

2.10 NUTRITIVE VALUE OF HUMAN MILK

Longitudinal studies of the nutrition status of 60 lactating Bangladeshi mothers from an under privileged, periurban community and f the quantity and, composition of their milk were
completed to determine the relationships between maternal nutritional status and lactating capacity. Daily milk production was estimated by 24 hour test weighing the nitrogen, fat, lactose and total energy concentrations of extracted milk samples were analyzed at various stages of lactation to estimate total milk nutrient production. (Brown KH, Akhtar NA, 1986)

The nutritional status among lactating women. Reasons for assessing nutritional status among lactating women in general, assessments of the nutritional status of lactating women and other groups have many applications in research, in patient management, in public policy development and in program planning and evolution. The selection of the indicator if nutritional status to be used should consider its intended application. (Habicht and Pelletier, 1990)

The biochemical indicators have been established for nutritional status among lactating women the usefulness of values obtained from non pregnant, non lactating women as a reference standard for lactating women requires evolution. There are few indicators of risk of undesirable outcomes for lactating women. An example of such an indicator is an abnormally low concentration of riboflavin in milk. This is associated with the likelihood of nutritional deficiency in the nursing infant. (Bates et al., 1982)

Another is the classic association of low thiamin concentrations in the milk of mothers in rice eating population with a high incidence of infantile beriberi among breast fed babies. (Kinney and follis, 1958)

The poor nutritional status with respect to certain micronutrients iron) are well understood in lactating women and can be used for targeting of nutrient specific interventions. (Rothe, 1988).

The interpretation of levels of vitamins, minerals, hormones, and metabolites is unaffected by lactation. For this to be true, plasma volume in lactating women must be the same as that in non lactating women and in stable over the course for lactation. These ideas about plasma volume are difficult to evaluate with the currently available data. (Brown et. al., 1947)

The assumption that blood values of vitamins, minerals, hormones, and metabolites are unaffected by lactation is known not to be correct. For example insulin and glucose levels in
lactating women respond quite differently to a test meal than they do in the same women after
cessation of lactation. (Illingworth, et. al., 1986)

Protein metabolism also appears to change during lactation (Motif, et. al., 1989, 1990)
Nitrogen balance among lactating women is lower than that among non lactating post
partum and nulliparous women studied at similar.

Urinary-3 methyl histidine excretion (A measure of muscle protein breakdown) also is
lower in lactating women. Some changes in plasma volume for example, serum zinc
concentration increases while serum copper decreases between weeks 1 to 2 and 19 to 21 of
lactation (Vander et, elst, et. al., 1986).

Inferences about maternal nutritional status also can be made from the nutritional status
of the infant for example; infants with evidence of vitamin B, E deficiency (That is those with
increased concentration of methylmalonic acid in the urine) may have mothers with poor vitamin
B, E status (Speaker et. al., 1988).

The reverse is not necessarily true; however, for example the nutritional status of breast
fed infants of mothers with inadequate foracin (Salmenpera et. al., 1986) or vitamin C
(Salmenpera, 1984)

The effects of a dietary zinc supplement during lactation on longitudinal changes in
maternal zinc status and milk zinc concentrations. (Krebs NF, hambidge KM, 1985).

Storage beyond three hours at ambient temperature alters the Biochemical and nutritional
qualities of breast milk. A positive correlation between, lactose level and pH were obtained.
These results suggest that breast milk is stable for 3 hours, beyond which significant changes
occur in its Biochemical composition and nutritional quality. (Eteng MU, Ebong PE, 2001)

Mature milk is produced from approximately ten days after delivery up until the termination
of the breastfeeding. Mature milk contains on average:

Energy (750 kcal / liter)

Lipids (38 g / liter) - The main lipids found in human breast milk are the triacyl-
glycerols, phospholipids, and fatty acids including essential fatty acids. Maternal diet does not
affect the amount of fat in milk but does affect the types of fat. Cholesterol is present in breast milk.

Casein (2.5 g / liter) - protein - Casein or curds are proteins with low solubility which complex with calcium. These are present in breast milk in much lower concentration than in cow's milk.

Whey (6.4 g / liter) - protein - the whey proteins are located in the clear liquid left behind when clotted milk stands. The largest components are alpha-lactalbumen, lactoferrin, lyzozyme, albumen and immunoglobulins.

Nonprotein Nitrogen is used in amino acid synthesis and includes the nitrogen in urea, creatine, creatinine, uric acid and ammonia. Peptides, such as epidermal growth factor, somatomedin - C and insulin are also present in this fraction. Nucleotides such as cytidine monophosphate are derived from nucleic acids and play an important role in the immune system and protein synthesis.

Lactose (70 g / liter) carbohydrate - Lactose is the major carbohydrate in breast milk. It is composed of galactose and glucose. Lactose concentration in breast milk increases over the duration of breastfeeding

Changes in the nutritional status of the lactating women during exclusive lactation. In order to evaluate the influence of breast feeding upon selected nutritional parameters of lactating women. Only one significant change was detected in measurements done for nutritional assessment: loss of body weight. No changes were detected in hemoglobin levels or in serum protein and albumin. (Arteaga A, et. al., 1981)

2.11 BIOCHEMICAL PARAMETERS AND THEIR CHANGES IN

Retinol derived from circulating RBP- retinol complex is transferred from blood to milk most of it is re-esterified in the mammary glands and occurs as retinayl esters in milk. Some is provided by carotene, β-Carotene is stored in the mammary glands during pregnancy and is rapidly secreted into milk during the first few days of lactation thus carotene provides almost 20% of the retinol equivalents during the first day. But this drops to less than 5% by the end of the first week. Unlike retinol — o- carotene is very effective antioxidant and thus provides the
infant a defense against oxygen toxicity. Thus may be particularly important during the first several days of life, as the infant adjutant to its new oxygen rich environment. (Shirarigs, 1989)

The vitamin A content of human milk is significantly affected by maternal nutrition during pregnancy and lactation. The fat content of the milk, time after birth, gestational age at birth, parity of the mother, and individual variation also have an influence. The use of oral contraceptives is reported to affect the amount of retinol in human milk. Thus it is important to consider these factors when evaluating milk vitamin A levels. (ConenN. Meashame, 1983)

Bound forms several of the vitamins (such as vitamin D, folate, and pantothenic acid are secreted bound to other compounds, and they must be released before they can be completely extracted or detected for example accurate measurement of the total content of pentothenic acid in human milk requires double enzyme hydrolysis. (Song et al., 1984) Distribution in aqueous and lipid fractions, vitamin D and its metabolites are secreted in the aqueous fraction of human milk and are attached to binding proteins (Hollis et al., 1982)

Certain constituents, such as secretory IgA exist in a different physical form than they do in other tissues, such as blood, and therefore require discrete detection procedures. (Holmgren et.al., 1981)

The titer of specific antibodies in human milk depends on whether the women has recently been exposed to the relevant immunogen via the intestinal or respiratory tract (Svennerholm, 1982)

Many of the whey proteins in human milk have directive protective effects against infection, lactoferrin. One of the dominant whey proteins in human milk through lactation. (Butte et al., 1984: Goldman et al., 1982, 1983)

Fibronectin, a protein that enhances phagocytosis, has recently been found in human milk. (Friss et. al., 1988). Compared the concentrations and daily output of secretory IgA and secretory IgA antibodies to somatic antigen to serotypes of E. coli. (Cruz et. al., 1982).

No differences in the levels of IgA, IgM, IgG, Lactoferrin, or lysozyme in the colostrums from well nourished and poorly nourished women.
(Reddy et al., 1977). The lower levels of IgA but similar levels of IgA in colostrum from well nourished and poorly nourished women. (Narul et al., 1982)

Malnutrition was characterized by lower weigh to height rations by lower creatinine height indices and serum concentrations of total protein albumin, IgG and IgA. (Miranda et al., 1983)

The effects of maternal nutrition upon the avidity of secretory IgA antibodies to \textit{E.coli} polysaccharides and diphtheria toxin in human milk. Decreased avidity was found in antibodies from the malnourished group. (Robertson et al., 1988). In addition to the soluble immunologic agents mentioned above human milk contains living white blood cells (leukocytes). (Crago et al., 1979, Smith and Goldman 1968)

Neutrophils and macrophage account for approximately 90\% of white blood cells in human milk. Remaining white blood cells are lymphocytes. The neutrophils have phagocytic activity and intracellular killing power similar to those of neutrophils in human blood. (Suda et al., 1984)

The certain protein fractions in human milk may aid in generating helper cell responses and in performing other immuno regulatory functions. Finally the presence of antidiotyptic antibodies in milk may act as immunizing agents these antibodies mimic other antibodies in the infant that in turn are directed against the original microbial antigens on the mother thus, they may be natural, safe immunizing agents. (Okarnoto and Ogra, 1989)

The nutrients in human milk most likely to be present in lower than normal concentrations in response to chronically low maternal intakes are the vitamins, especially vitamins B6, B12, A, and D. Those maintained at the expense of maternal stores or tissues include the macronutrients, most minerals and folate. (Zimecki et al., 1987)

Diets of adolescent typically contain less iron (an average of 5.7mg/1.000 kcal) than recommend during lactation. (6.8 mg/ 1.000 kcal)

Diets of low in come adult women are characterized by lower densities of calcium and vitamin A than are typical of diets of women above the poverty level (NHS, 1983)
Serum prolactin levels in lactating women using progestasert system. (Bararaovi MH et al., 1981).

Bone mineral density changes during lactation. The objectives of this study were to characterize the effects of lactation and weaning on maternal bone mineral density (BMD). The results also suggest that the bone loss may be attenuated by a generous dietary ratio of calcium to protein. (.Krebs NF, et. al., 1997)

The effect of oral contraceptives in protein metabolism. Overall there was a trend towards increased hepatic protein synthesis with a resultant reduction in concentrations of plasma amino acids and albumin, multivitamin supplementation did not alter any of these patterns. (Amatayakulk et al., 1994)

Vitamin B12 metabolism and status during lactation and infancy. The overview of vitamin B12 metabolism and requirements during the continuum of lactation has identified several gaps in our knowledge more information is needed concerning the roles of the different transcobalaminis during pregnancy and lactation. Including their impact on placental and mammary transfer of cobalmine and their effect on intestinal absorption in the infant. Knowledge is needed about the relative importance of maternal stores and current dietary intake on fetal storage of the vitamin and on it’s concentrated in breast milk. (Allen LH, 1994)

Acute effect of an oral calcium load in pregnancy and lactation. An oral calcium supplement may benefit breast-feeding women by reducing lactation. Related elevated rate of bone resorption and consequent loss of trabecular bone. (Kentlan et al., 1991)

2.12. THE HISTORY AND THE DEFINITION OF PROBIOTICS

The word ‘probiotic’ comes from Greek language ‘pro bios’ which means ‘for life’ opposed to ‘antibiotics’ which means ‘against life’. The history of probiotics began with the history of man by consuming fermented foods that is well known Greek and Romans consume very much (Gismondo, et al. 1999, Guarner, et al. 2005). In 1908 a Russian researcher Ellie Metchnikoff, who has a nobel prize, firstly proposed the beneficial effects of probiotic microorganisms on human health. Metchnikoff hypothesized that Bulgarians are healthy and long lived people because of the consumption of fermented milk products which consists of rod
shaped bacteria (*Lactobacillus* spp.). Therefore, these bacteria affect the gut microflora positively and decrease the microbial toxic activity (Gismondo, et al. 1999, Çakır 2003, Chuayana, et al. 2003).

The term ‘probiotic’ firstly used in 1965 by Lilly and Stillwell to describe substances which stimulate the growth of other microorganisms. After this year the word ‘probiotic’ was used in different meaning according to its mechanism and the affects on human health. The meaning was improved to the closest one we use today by Parker in 1974. Parker defined ‘probiotic’ as ‘substances and organisms which contribute to intestinal microbial balance’. In 1989, the meaning use today was improved by Fuller. Thus, probiotic is a live microbial supplement which affects host’s health positively by improving its intestinal microbial balance. Then this definition was broadened by Havenaar and Huis in’t Veld in 1992 including mono or mixed culture of live microorganisms which applied for animal and man (Çakır 2003, Guarner, et al.2005, Sanders 2003).

In the following years lots of researchers studied on probiotics and made so much definition. They are listed below.

1- ‘Living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition’ by Shaafasma, 1996.

2- ‘A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract’ by Naidu et al., 1999.

3- ‘A live microbial food ingredient that is beneficial to health’ by Salminen et al. 1998.

4- ‘A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the micro flora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host’ by Schrezenmeir and de Vrese, 2001.


Probiotics are also challenging for the industrial applications. The probiotic concept is open to lots of different applications in a large variety of fields relevant for human and animal health. Probiotic products consist of different enzymes, vitamins, capsules or tablets and some fermented foods contain microorganisms which have beneficial effects on the health of host.
They can contain one or several species of probiotic bacteria. Most of products which destine human consumption are produced in fermented milk or given in powders or tablets. These capsules and tablets do not used for medicinal applications. They are just used as health supporting products. The oral consumption of probiotic microorganisms produces a protective effect on the gut flora. Lots of studies suggest that probiotics have beneficial effects on microbial disorders of the gut, but it is really difficult to show the clinical effects of such products. The probiotic preparations use for traveller’s diarrhea, antibiotic associated diarrhoea and acute diarrhea which is showned that they have positive therapeutic effect (Gismondo, et al. 1999, Çakır 2003, Quwehand 1999). More than 400 bacterial species exit in human intestinal tract. It is an enormously complex ecosystem that includes both facultatively anaerobic and anaerobic microorganisms (Naidu, et al. 1999). The numbers of genera is nearly steady, because they each have their own growth niches (Fooks, et al.1999). The composition of the gut microflora is constant but can be affected by some factors such as; age, diet, environment, stress and medication (Albertcllasic 2007). To have a healthy intestine the balance of the bacteria must be maintained but this is difficult as the lifestyles change. Lots of factors may change the balance away from potenially beneficial or health promoting bacteria like lactobacilli and bifidobacteria to potentially harmfull or pathogenic microorganisms like clostridia, sulphate reducers and Bacteroides species. It makes the host more susceptible to the illnesses.

In this case the prevalence of the beneficial bacteria must be supported. Using of probiotics help to protect the host from various intestinal diseases and disorders while increasing the number of beneficial bacteria and make the balance steady again (Fooks, et al. 1999). Probiotics are suggested as food to provide for the balance of intestinal flora (Holzapfel, et al. 1998). Probiotics are used for long times in food ingredients for human and also to feed the animals without any side effects. Also probiotics are acceptable because of being naturaly in intestinal tract of healthy human and in foods (Çakır 2003, Albertcllasic 2007).

2.13. THE EFFECTS OF PROBIOTICS ON HEALTH

There are lots of studies on searching the health benefits of fermented foods and probiotics. However, in most of these studies researchers did not use sufficient test subjects or they use microorganisms were not identified definitely (Çakır 2003). So, while a number of reported effects have been only partially established, some can be regarded as well established
and clinically well documented for specific strains. These health-related effects can be considered as in the below (Çakır 2003, Scherezenmeir and De Vrese 2001, Dunne, et al. 2001, Dugas, et al. 1999).

- Managing lactose intolerance.
- Improving immune system.
- Prevention of colon cancer.
- Reduction of cholesterol and triacylglycerol plasma concentrations (weak evidence).
- Lowering blood pressure.
- Reducing inflammation.
- Reduction of allergic symptoms.
- Beneficial effects on mineral metabolism, particularly bone density and Stability.
- Reduction of Helicobacter pylori infection.
- Suppression of pathogenic microorganisms (antimicrobial effect).
- Prevention of osteoporosis.
- Prevention of urogenital infections.

2.14. LACTOSE INTOLERANCE

Most of human commonly non-Caucasians become lactose intolerant after weaning. These lactose intolerant people can not metabolize lactose due to the lack of essential enzyme β-galactosidase. When they consume milk or lactose-containing products, symptoms including abdominal pain, bloating, flatulence, cramping and diarrhoea ensue. If lactose passes through from the small intestine, it is converted to gas and acid in the large intestine by the colonic microflora. Also the presence of breath hydrogen is a signal for lactose maldigestion. The studies provide that the addition of certain starter cultures to milk products, allows the lactose intolerant people to consume those products without the usual rise of breath hydrogen or associated symptoms (Fooks, et al. 1999, Scheinbach 1998, Quewand and Salminen 1998, Lin, et al. 1991).

The beneficial effects of probiotics on lactose intolerance are explained by two ways. One of them is lower lactose concentration in the fermented foods due to the high lactase activity of bacterial preparations used in the production. The other one is; increased lactase active lactase enzyme enters the small intestine with the fermented product or with the viable probiotic bacteria (Salminen, et al. 2004). When the yogurt is compared with milk, cause the lactose is converted to lactic acid and the yogurt consist of bacterial β-galactosidase enzyme; it is suitable end beneficial.
to consume by lactose intolerants. Furthermore, the LAB which is used to produce yogurt, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, are not resistant to gastric acidity. Hence, the products with probiotic bacteria are more efficient for lactose intolerant human. It is thought that the major factor improves the digestibility by the hydrolyses of lactose is the bacterial enzyme β-galactosidase. Another factor is the slower gastric emptying of semi-solid milk products such as yogurt. So the β-galactosidase activity of probiotic strains and other lactic acid bacteria used in dairy products is really important. B-galactosidase activity within probiotics varies in a huge range. It has to be considered both the enzyme activity of probiotic strain and the activity left in the final product for their use in lactose intolerant subjects (Salminen, et al. 2004).

### 2.15. IMMUNE SYSTEM AND PROBIOTICS

The effects of immune system are promising. However, the mechanism is not well understood. Human studies have shown that probiotic bacteria can have positive effects on the immune system of their hosts (Mombelli and Gismondo 2000). Several researchers have studied on the effects of probiotics on immune system stimulation. Some in vitro and in vivo searches have been carried out in mice and some with human. Data indicate that oral bacteriotherapy and living bacteria feeding in fermented milks supported the immune system against some pathogens (Scheinbach 1998, Dugas, et al. 1999). Probiotics affect the immune system in different ways such as; producing cytokines, stimulating macrophages, increasing secretory IgA concentrations (Çakır 2003, Scheinbach 1998, Dugas, et al. 1999). Some of these effects are related to adhesion while some of them are not (Quwehand, et al. 1999).

Link-Amster et al. (1994) examined whether eating fermented milk containing *Lactobacillus acidophilus* La1 and bifidobacteria could modulate the immune response in human. They give volunteers the test fermented milk over a period of three weeks during which attenuated *Salmonella typhi* Ty21a was administered to mimic anenteropathogenic infection. After three weeks, the specific serum IgA titre rise to *S. typhi* Ty21a in the test group was >4-fold and significantly higher (p=0.04) than in the control group which did not ate fermented foods but received *S. typhi* Ty21a. The total serum IgA increased. These results showed that LAB which Cn survive in the gastrointestinal tract can act as adjuvants to the humoral immune response (Lime- Amster, et al. 1994, Quwehand, et al. 1999).
Perdigon et al. (1986) feed the mice with lactobacilli or yogurt and it stimulated macrophages and increased secretory IgA concentrations (Scheinbach 1998). Also in a human trial Halpern et al. (1991) feed human with 450 g of yogurt per day for 4 months and at the end a significant increase is observed in the production of γ-interferon (Fooks, et al. 1999). Mattilla-Sandholm and Kauppila (1998) showed that *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 derived extracts suppress lymphocyte proliferation in vitro. Further evidence for immunomodulation by these two strains a children trial with severe atopic eczema resulting from food allergy. Children fed with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 showed improvement in Clinical symptoms compared to the placebo group (Saarela, et al. 2000).

2.15.1 Diarrhoea

Diarrhoea is many causes and many types so it is difficult to evaluate the effects of probiotics on diarrhoea. But there are lots of searches and evidence that probiotics have beneficial effects on some types of diarrhoea. Diarrhoea is a severe reason of children death in the worldwide and rotavirus is its common cause (Scheinbach 1998). In the treatment of rotavirus diarrhea, *Lactobacillus* GG is reported really effective. The best documented probiotic effect is shortened duration of rotavirus diarrhea using *Lactobacillus* GG. It has been given proof in several studies around the world by some researchers like Guandalini *et al.* (2000), Pant *et al.* (1996). Also *Lactobacillus acidophilus* LB1, *Bifidobacterium lactis* and *Lactobacillus reuterii* are reported to have beneficial effects on shortening the diarrhea (Salminen, et al. 2004).

One of types of diarrhoea is traveller’s diarrhea (TD) which affects the healthy travellers not only in developing countries but also in Europe. Probiotics have beneficial effects in preventing some forms of TD. Oksanen *et al.* (1990) evaluated the efficacy of *Lactobacillus* GG in preventing diarrhea in 820 people travelling from Finland to Turkey. In a double-blind study by Black *et al.* (1989) lyophilised bacteria (*L.acidophilus, B.bifidum, L.bulgaricus, S.thermophilus*) were given to 56 Danish tourists on a 2-week trip to Egypt. The occurrence of diarrhea in the group receiving the lactic acid bacteria was 43% while it was 71% in the placebo group (Gismondo, et al. 1999).

Antibiotic therapy causes mild and severe outbreaks of diarrhoea The normal microflora may be suppressed during the microbial therapy and resulting with filling with pathogenic strains. The changes of microflora may also encourage the resistant strains at least *Clostridium difficile*
which is the reason of antibiotic associated diarrhea (ADD). Several clinical trials (Surewicz, et al., Adam, et al., Mcfarland, et al., etc.) have used *Saccharomyces boulardii*, *Lactobacillus* spp. and *Bifidobacterium* spp. in ADD. Probiotics which are able to restore and replace the normal flora should be used. Also they should be used in high risk patients such as old, hospitalised or immunocompromised. Studies with *Saccharomyces boulardii* proved that *Clostridium difficile* concentration is decreased in the presence of *Saccharomyces boulardii* (Gismondo, et al. 1999).

### 2.15.2 CANCER

Epidemiological studies point out that if the consumption of saturated fats increases in the diet, the occurrence of colon cancer increases in Western World. Bacterial enzymes ($\beta$-glucoronidase, nitroreductase and azoreductase) convert precarcinogens to active carcinogens in the colon. It is thought that probiotics could reduce the risk of cancer by decreasing the bacterial enzymes activity. Although the exact mechanism for the anti tumor action is not known, some suggestions have been proposed by McIntosh as follows (Fooks, et al. 1999, Scheinbach 1998):

1. Carcinogen/procarcinogen are suppressed by binding, blocking or removal.
2. Suppressing the growth of bacteria with enzyme activities that may convert the procarcinogens to carcinogens.
3. Changing the intestinal pH thus altering microflora activity and bile solubility.
4. Altering colonic transit time to remove fecal mutagens more efficiently.
5. Stimulating the immune system.

There are in vitro and in vivo evidences not only from animal studies but also from human studies that probiotics have beneficial effects on suppression of cancer. Oral administration of lactic acid bacteria has been shown to reduce DNA damage caused by chemical carcinogens, in gastric and colonic mucosa in rats. The consumption of lactobacilli by healthy volunteers has been demonstrated to reduce the mutagenicity of urine and faeces associated with the ingestion of carcinogens in cooked meat. When it comes to epidemiological studies, they show an association between fermented dairy products and colorectal cancer. The consumption of a large quantity of dairy products especially fermented foods like yogurt and fermented milk with containing *Lactobacillus* or *Bifidobacterium* may be related to a lower occurrence of colon cancer (Rafter 2003, Hirayama and Rafter 2000). A number of studies have shown that predisposing factors (increases in enzyme activity that activate carcinogens, increase
procarcinogenic chemicals within the colon or alter population of certain bacterial genera and species) are altered positively by consumption of certain probiotics (Brady, et al. 2000).

2.15.3 CHOLESTEROL REDUCTION

Lots of researchers proposed that probiotics have cholesterol reduction effects. However, the mechanism of this effect could not been explained definitely. There are two hypotheses trying to explain the mechanism. One of them is that bacteria may bind or incorporate cholesterol directly into the cell membrane. The other one is, bile salt hydrolysis enzymes deconjugate the bile salts which are more likely to be exerted resulting in increased cholesterol breakdown (Çakır 2003, Scheinbach 1998, Prakash and Jones 2004).

A study on the reduction of cholesterol was showed that *Lactobacillus reuteri* CRL 1098 decreased total cholesterol by 38% when it is given to mice for 7 days in the rate of $10^4$ cells/day. This dose of *Lactobacillus reuteri* caused a 40% reduction in triglycerides and a 20% increase in the ratio of high density lipoprotein to low density lipoprotein without bacterial translocation of the native micro flora into the spleen and liver (Kaur, et al. 2002).

2.16. MECHANISM OF PROBIOTICS

Probiotic microorganisms are considered to support the host health. However, the support mechanisms have not been explained (Holzapfel, et al. 1998). There are studies on how probiotics work. So, many mechanisms from these studies are trying to explain how probiotics could protect the host from the intestinal disorders. These mechanisms listed below briefly (Rolfe 2000, Çakır 2003, Salminen, et al. 1999, Castagliuola, et al. 1999).

1. Production of inhibitory substances: Production of some organic acids, hydrogen peroxide and bacteriocins which are inhibitory to both gram-positive and gram-negative bacteria.
2. Blocking of adhesion sites: Probiotics and pathogenic bacteria are in a competition. Probiotics inhibit the pathogens by adhering to the intestinal epithelial surfaces by blocking the adhesion sites.
3. Competition for nutrients: Despite of the lack of studies in vivo, probiotics inhibit the pathogens by consuming the nutrients which pathogens need.
4. Stimulating of immunity: Stimulating of specific and nonspecific immunity may be one possible mechanism of probiotics to protect the host from intestinal disease. This mechanism is not well documented, but it is thought that specific cell wall components or cell layers may act as adjuvant and increase humoral immune response.
5. Degradation of toxin receptor: Because of the degradation of toxin receptor on the intestinal mucosa, it was shown that *S. boulardii* protects the host against *C. difficile* intestinal disease. Some other offered mechanisms are suppression of toxin production, reduction of gut pH, attenuation of virulence (Fooks, et al. 1999).

### 2.17. SELECTION CRITERIA FOR PROBIOTICS

In order to be able to exert its beneficial effects, a successful potential probiotic strain is expected to have a number of desirable properties. The selection criteria are listed in Table 1.2 briefly. Some of them will be discussed in more details. A potential probiotic strains does not need to fulfill all such selection criteria (Quwehand, et al.1999).

The selection criteria can be categorized in four basic groups. Appropriateness, technological suitability, competitiveness, performance and functionality (Klaenhammer and Kullen 1999). Strains which have these criteria should be used in order to get effective on health and functional probiotic strains. Probiotics are chose by using the criteria in Table 1.2. Saarela et al. (2000) proposed the properties of probiotics in three basic groups as; safety aspects, aspects of functionality and technological aspects

Table 1.2. Selection criteria for probiotics.


<table>
<thead>
<tr>
<th>Probiotic Strain Properties</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human origin for human usage</td>
<td>Although the human probiotic <em>Saccharomyces boulardii</em> is not human origin, this criteria is important for species dependent health effects</td>
</tr>
<tr>
<td>Acid and bile tolerance</td>
<td>Important for oral consumption even if it may not be for other applications for survival through the intestine, maintaining adhesiveness and metabolic activity.</td>
</tr>
<tr>
<td>Adhesion to mucosal surface</td>
<td>Important to improve immune system, competition with Pathogens, maintain metabolic activity, prevent pathogens to adhesion and colonization. Safe for food and clinical use Identification and characterization of strains accurately, Documented safety. No invasion and no degradation of intestinal mucus.</td>
</tr>
<tr>
<td>Clinically validated and documented health effects</td>
<td>Minimum effective dosage has to be known for each particular strain and in different products. Placebo controlled, double-blinded and</td>
</tr>
</tbody>
</table>
randomized studies have to be run

| Good technological properties | Survival in products if viable organisms are required, Phage resistance, strain stability, culturable in large scales, oxygen resistance, has no negative effects on product flavour. |

2.18. HUMAN MILK – A SOURCE OF POTENTIAL PROBIOTIC STRAIN

After birth, breast milk is the best food for infants because it fulfills all the nutritional requirements for them during months. Also breast milk protects the newborn against infectious diseases. This effect seems a result of the action of some breast milk components, like different antimicrobial compounds, immunoglobulins, immunocomponent cells (Martín, et al. 2003) and also breast milk contains probiotic substances which stimulate the growth of the beneficial bacteria neonate gut (Martín, et al. 2004, Martín, et al. 2003). In a general view human milk contains fat, protein, carbohydrate, minerals and bacteria.

Table 1.3. Contents of human milk
(Source: Prentice 1996)

<table>
<thead>
<tr>
<th>Fat</th>
<th>Fatty acids, polyunsaturated fatty acids,</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Casein, α-lactalbumin, lactoferrin, IgA, IgG, lysozyme, serum albumin, ß-lactoglobulin</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Oligosaccharides, lactose</td>
</tr>
<tr>
<td>Minerals</td>
<td>Calcium, phosphorus, sodium, potassium, chlorine</td>
</tr>
</tbody>
</table>

When it comes to the microbiological point of breast milk, human milk is really an important factor in the initiation and development and of course composition of the neonatal gut micro flora since it constitutes source of microorganisms to the infant gut for several weeks after birth (Martín, et al. 2005). It is estimated that an infant ingests $19 \times 10^5$ – $19 \times 10^7$ commensal bacteria while suckling if the infant consumes approximately 800 ml breast milk per day (Martín, et al. 2004, Martín, et al. 2005, Heikilla and Saris 2003). From the studies the fact is that, the
bacterial composition of the infant fecal flora seems to reflect the bacterial composition of breast milk (Heikillä and Saris 2003).

The composition of the gut micro flora is thoroughly influenced by the diet of the infant. Thus, the presence of a few predominant Gram-positive species in breast milk may be a reason explaining why micro biota of breast-fed infants is composed of a narrow spectrum of species, and a more diverse micro biota develops after weaning (Martín, et al. 2004, Favier, et al. 2002).

The studies on the microbiology of human milk are restricted to the identification of potential pathogenic bacteria in clinical cases of mastitis or infant infections. However, it is clear that the prevention of infant from infectious diseases owing to the natural flora of human milk (Martín, et al. 2004). Although there are limited knowledge about the commensal or probiotic bacteria that breast milk contain, bacteria commonly isolated from this biological fluid include staphylococci, streptococci, micrococi, lactobacilli and enterococci (Martín, et al. 2004, Martín, et al. 2003, Martín, et al. 2005, Heikillä and Saris 2003). Bacteria from these genera can be easily isolated from fresh milk of healthy women. So, these groups of bacteria should be considered the natural microbiota of human milk rather than mere contaminant bacteria (Martín, et al. 2004, Martín, et al. 2005).

There are surprisingly not so much studies on the isolation and analysis of commensal or potential probiotic bacteria from breast milk (Martín, et al., 2003). However, if the bacteria with the ability to provide health benefits such as protection the host from pathogenic bacteria were isolated from human milk, they would be considered attractive probiotic organisms (Martín, et al. 2004). These isolated bacteria would fulfill some of the main criteria like being human origin, adaptation to dairy substrates and a history of long duration and safe intake by infants (Martín, et al. 2004, Klaenhammer and Kullen 1999). From the bacteria isolated from breast milk, Lactobacillus gasseri, Lactobacillus rhamnosus, Lactobacillus fermentum, or Enterococcus faecium are founded and they can be regarded as potential probiotic bacteria (Martín, et al. 2004, Holzapfel, et al. 1998). Hence, breast milk, a natural source of potentially probiotic or biotherapeutic LAB, protects mother and infants against infectious diseases (Martín, et al. 2004).

There are lots of studies on the effect of human milk on the health of infants and the infant diseases but surprisingly lack of studies on the microbiology of breast milk. From the few studies, it is found that human milk is an attractive source for potential probiotic strains. As, the
bacteria implement some of the main criteria for being probiotic strains such as, human origin, survival in the gastrointestinal conditions and particularly low pH and bile, production antimicrobial compounds, adhesion to the intestinal mucosa (Martín, et al. 2005, Olivares 2005).

Martin et al. (2003) aimed to investigate whether human breast milk contains potentially probiotic lactic acid bacteria, and therefore, whether it can be considered a synbiotic food. For this purpose; they isolated lactic acid bacteria from milk, mammary areola, and breast skin of eight healthy mothers and oral swabs and feces of their respective breast-fed infants. They identified the lactic acid bacteria by RAPD-PCR analysis and 16S rDNA sequencing. From the results they identified the bacteria isolated from human milk as *Lactobacillus gasseri*, *Lactobacillus fermentum* and *Enterococcus faecium*. These species are considered among the probiotic bacteria (Holzapfel, et al. 1998, Collins, et al. 1998) and contain strains that are used in commercial probiotic products. They concluded as their work indicates that breast milk contains lactic acid bacteria is a natural source of LAB for the newborns and may be considered a symbiotic food.

Martin et al. (2004) studied on three lactobacilli strains isolated from breast milk. Whether they were potential probiotic bacteria. They performed some assays to investigate some criteria need to be used as probiotic bacteria such as; survival to conditions simulating in the gastrointestinal tract, production of antimicrobial compounds, adherence to intestinal cells, production of biogenic amines, degradation of Mucin, enzymatic profile and pattern of antibiotic resistance. 2 *Lactobacillus gasseri* and 1 *Lactobacillus fermentum* strains were evaluated and the results showed that the probiotic potential of lactobacilli isolated from human milk is similar to strains commonly used in commercial probiotic products.

Heikkilä and Saris (2003) were focused on the antimicrobial activity against *Staphylococcus aureus* of bacteria isolated from human milk. They identified the bacteria by different molecular characterization techniques and named the bacteria as staphylococci, streptococci, and LAB as *Lactobacillus crispatus, Lactobacillus rhamnosus, Lactococcus lactis* and *Leuconostoc mesenteroides* and also *Enterococcus faecalis*. Then they examined the antimicrobial activity of these bacteria against Staphylococcus aureus. They concluded that the commensal bacteria in human milk may have a role in protecting the infant and mother against *Staphylococcus aureus*). Also the results supported that the commensal staphylococci and
streptococci are predominant bacterial species in breast milk. The other isolated bacteria *Lactobacillus rhamnosus* had RAPD profile identical to the commercial strain *Lactobacillus rhamnosus* GG, which is a commonly used probiotic strain in milk products in Finland. Olivares et al. (2006) aimed to evaluate the antimicrobial activity against some pathogenic bacteria of four lactobacilli (*Lactobacillus salivarius* CECT5713, *Lactobacillus gasseri* CECT5714, *Lactobacillus gasseri* CECT5715, *Lactobacillus fermentum* CECT5716) isolated from human breast milk. In the conclusion; the four lactobacilli and particularly *Lactobacillus salivarius* CECT5713 showed antibacterial activity. These results suggest that these lactobacilli strains from human breast milk could play a part of anti-infective protection in neonates and would be good strains to develop probiotic products for infant.

Human milk is an important food for neonates during some months to grow them up and protect the infants against some infectious. The high concentration of LAB in milk from healthy mother may play an important biological role during the first months of life. Studies on this biological fluid indicate that human milk is a challenging Source for potential probiotic bacteria.

### 2.2 MASTITIS:

Mastitis is defined as an inflammation of the mammary gland or udder of the ewe. The term mastitis is from the Greek word mastos, for breast, and it is, for inflammation of. The response to injury to the udder of sheep is called inflammation. Mastitis is the reaction of milk – secreting tissue to injury produced by physical force, chemicals introduced into the gland or most commonly from bacteria and their toxins. To clarify the discussion on mastitis, the following definitions are given. (Carrol et al., 1973).

#### 2.2.1. UDDER INFECTION

The udder cavity is invaded by microorganisms which cause inflammation. (Carrol et al., 1973).

#### 2.2.2. SUBCLINICAL MASTITIS

No swelling of the udder is detected nor are there observable abnormalities in the milk. Special screening tests, however, such as the California Mastitis Test (CMT), Wisconsin Mastitis Test (WMT) and the catalase test will show changes in the milk. This type of mastitis is referred to as “hidden”. It is based on an estimation of somatic cell counts. (Carrol et al., 1973).

#### 2.2.3. CLINICAL MASTITIS
Can be mild or acute, and there is the presence of leukocytes (white blood cells) in the milk. (Carrol et al., 1973).

2.2.4 MILD CLINICAL MASTITIS

Involves abnormality in the milk such as flakes, clots, and a watery or other unusual appearance. A hot or sensitive udder may be slight or absent, however there may be signs of swelling. (Carrol et al., 1973).

2.2.5. SEVERE CLINICAL MASTITIS

Involves a hot, hard sensitive udder that is quite painful to the ewe. The onset is sudden and the ewe may become ill showing signs of fever 105° - 107° F), rapid pulse, depression, weakness and loss of appetite. When the whole system of the ewe is affected, the condition is referred to as acute systemic mastitis or bluebag. Milk production by a ewe with a bluebag has usually ceased and the lambs will need to be reared as orphans or grafted on another ewe. (Carrol et al., 1973).

2.2.6. CHRONIC MASTITIS

A persistent udder infection exists most of the time in the sub clinical form occasionally can develop into the clinical form before returning to the sub clinical. The results are hard lumps in the udder from the “walling off” of bacteria and the forming of connective tissue. (Carrol et al., 1973).

2.3. MAMMARY GLAND SYSTEM

Among domestic animals, the cow, sheep and goat have the simplest mammary gland system. Development of the mammary gland in the sheep and goat is identical to that of the cow (Schalm et al., 1971). The teat has a single opening leading into a teat sinus which is continuous with the gland sinus above which a number of large ducts empty.

2.3. GENETIC FACTORS

There are a limited number of studies on the influence of heredity on resistance or susceptibility to mastitis in the cow, goat or sheep. Genes are known to influence the shape and structure of the teat (Hickman, 1962). Mastitis histories of two cow families in different geographical locations revealed significant difference which led to the conclusion that heredity played a part in the infection rate. Dam-daughter comparisons based on data derived from field surveys cite the influence of heredity on mastitis (Randel et al., 1964).

2.4. MICROORGANISMS-PRIMARY CAUSE OF MASTITIS
The primary cause of mastitis in cattle, goats and sheep are well-recognized groups of microorganisms, Streptococcus sp., Staphylococcus sp., Pasteurella sp, and coliforms, Escherichia coli, Enterobacter sp., and Klebsiella sp, Recent studies at the University of Missouri collected data on the incidence of sub clinical mastitis in ewes and identified Staphylococcus, sp., Streptococcus sp, and Micrococcus sp., found in bacterial cultures (Andrews et al., 1985). Nineteen microorganisms have been identified as causative agents of mastitis in cattle. Yeast and fungus have also been found frequently infecting the udder, but usually go unnoticed because they produce a mild or sub clinical mastitis. (Alsemgeest et al., 1994).

2.5 CONTROL OF MASTITIS

One of the most important keys to controlling mastitis in ewes is good management practices. The incidence of mastitis is greater in closely confined flocks. Bedding material in barns should be clean, especially before and after lambing. Microorganisms thrive in dark, wet, warm bedding. When the ewes lay down to rest, the bacteria in dirty bedding can easily enter the teat when the udder is full of milk. Microorganisms can enter the teat canal. Dirty bedding and crowding will make this possible. Lambs from infected ewes will often nurse other ewes, spreading the microorganisms to others in the flock. Isolating ewes suspected of chronic or acute mastitis will help reduce the incidence of mastitis in a flock. Ewes should be fed in bunks rather than on the ground. (Burvenich et al., 2003)

2.6 MANAGEMENT

Ewes with udders that show obvious signs of acute or chronic mastitis should be separated from the flock and treated with antibiotics. Then lambs often need to be bottle fed. Milk production may be decreased significantly or slightly depending upon the degree of infection. (Falbo et al., 1992).

Chronic mastitis can be prevented by a good management program. Before weaning cut out all grain feeding for 3-5 days. Feed a lower quality substitute such as grass hay at this time. Reducing water and all feed 12-24 hours before weaning is sometimes practiced. Reducing the volume of milk by reducing grain and feeding low quality hay will help prevent under distension and fever. Microorganisms will have more difficulty infecting a flaccid udder. In addition, the tissue in the udder will not be damaged, preventing vulnerability to microorganisms. (Fang et al., 1996).

2.7 TREATMENT OF MASTITIS
Disinfect the teat end with alcohol and infuse a tube of mastitis antibiotic through the teat canal. Give the ewe an injection of a combination of penicillin, dihydrostreptomycin, dexamethasone and an antihistamine. The antibiotics should affect the microorganisms and the dexamethasone and antihistamine should help the tissue heal and reduce inflammation. (Haddad et al., 2001).

2.8. PLASMID ISOLATION

40% of isolates from mastitis milk sample were found to contain enteropathogenic Escherichia coli isolates such as 026, 055, 0111, and 0119 out of 57 isolates 20 were found to contain plasmid (Correa et al., 2002). From 1999 to 2001, 3625 food samples were examined for the presence of Escherichia coli 0157 all strains possessed the attaching and effacing gene, the enterohemorrhagic plasmid and verotoxin (vt) genes (Tutenel et al., 2002). Studies used plasmid profile as an epidemiological tool for the discrimination of Escherichia coli isolates (Mini et al., 2005). Escherichia coli isolates obtain from milk samples of dairy cattle suffering from sub clinical mastitis were typed according to plasmid and protein patterns (Uckuns et al., 2005)

2.9. POLYMERASE CHAIN REACTION:

E. coli strains isolated from cases of clinical mastitis have a great variability in genotype and the E. coli isolated from separate episodes of inflammation were is most instances of the same serotype and had the same DNA amplification pattern (Len et al., 1995). Episodes of mastitis caused by Escherichia coli having the same genotype in different quarters were detected by PCR (Dopfer et al., 1999). Escherichia coli strains were investigated for the presence of total of 17 virulence associated genes describe for diarrheagenic (stx ½, eae, hly EHEC, estI, astA, cdtB), septicemic (hlyA, papC, cnfl/2, FyuA, IRP2) and pathogenic E. coli (iucD, tsh, fimc, hilE and stx2f) fim C was detected in highest prevalence in 92.7% of the isolates (Traute, 2001). Shiga toxigenic Escherichia coli serotype 0157 were found to carry the gene for shiga toxin type1 (stx1), stx2, intimin (12.9) encoding gene eae (14.5%) and enterohemolysin encoding gene hlyA by multiplex PCR assay (Adwan et al., 2002). On examination of Escherichia coli isolates from bovin mastitis by pcr 49% of finish isolates and 42% of Israeli isolates had at least one virulence gene (tral cnf2, cnfl, aer, sfa, pap, afa8E) but genes other traT were present in only 24% of finnish and 5% of Israeli isolates (Kaipainen et al., 2002). Escherichia coli was isolated from the milk sample of dairy cattle showing mastitis plasmid isolation by alkaili lysis method and to multiplex PCR for the detection of stx1 stx2 and eae genes (Lira et al., 2004).
Escherichia coli was isolated from the milk of 34 symptomatic cows associated with bovin mastitis and were screened by DNA amplification for the following *E. coli* virulence genes, cnf1, cnf2, eae, eagg, einv, itx1, stx2, and vt2e. The most common virulence gene detected was stx1 (31%) followed by cnf2 (7.5%) vt2e (6.25) and eae (4%) (bean, 2004). The primer set eaeA was able to produce and amplification product from each *E. coli* serotype except 0128; H7 and most sensitive for real time PCR for the detection of pathogenic *E. coli* (barak et al., 2005). Escherichia coli 0157;H7 was isolated from the hide sample and subjected to multiplex PCR assay for the amplification of the genes stx1, stx2, eaeA, flic, rfbE0157 and hiy A (childs et al., 2006). 16S RNA based method was developed for rapid identification of *E. coli* using single strand confirmation polymorphism (sscp) analysis the bands of the single strand (ss) DNA in these patterns corresponded to the mobility equivalent to 983 base pairs (bp) to 379 base pair (bp) of double stranded (ds) DNA ladder (Anand et al., 2007). Two multiplex PCR assay were used to detect the presence of the genes encoding shiga toxins 1 and 2 (stx1 and stx2) in timin (eaeA), heat stable enterotoxina (sta) F5(K99), F41 and F17 fimbriae. The results demonstrated that none of the potential virulence factors investigated was commonly observed in *E. coli* isolates from bovin clinical mastitis. Shiga toxin producing *E. coli* (STEC) which is pathogenic to humans was not detected by PCR (leylaceuler et al., 2007).

### 2.10. BOVINE MASTITIS

Mastitis is complex; there is no simple solution to its control. Some aspects are well understood and documented in the scientific literature. Others are controversial, and opinions are often presented as facts. The information and interpretations presented here represent the best judgments currently accepted by the National Mastitis Council. To simplify understanding of the mastitis complex, it is useful to consider that three major factors are involved in this disease: the microorganisms as the causative agent, the cow as host, and the environment, which can influence both the cow and the microorganisms. Hill et al., 1981).

Well over 100 different microorganisms can cause mastitis, and these vary greatly in the route by which they reach the cow and in the nature of the disease they cause. Cows contract udder infection at different ages and at different stages of the lactation cycle. Cows also vary in their ability to overcome an infection once it has been established. Therefore, the cow plays an active role in the development of mastitis. The cows’ environments influence both the numbers and types of bacteria they are exposed to and their ability to resist these microorganisms.
However, through appropriate management practices, the environment can be controlled to reduce this exposure and enhance resistance to udder disease. Practical measures are now available to maintain common forms of mastitis at relatively low and acceptable levels in the majority of herds. While continued research is needed to control the less common forms of intramammary infection, herd problems are often the result of failure to apply the proven mastitis control practices consistently and to consider all aspects of the disease problem. Mastitis is complex; there is no simple solution to its control. Some aspects are well understood and documented in the scientific literature. Others are controversial, and opinions are often presented as facts. The information and interpretations presented here represent the best judgments currently accepted by the National Mastitis Council. To simplify understanding of the mastitis complex, it is useful to consider that three major factors are involved in this disease: the microorganisms as the causative agent, the cow as host, and the environment, which can influence both the cow and the microorganisms. (Werling et al., 1996).

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2.11. ECONOMIC LOSS

Economic loss to mastitis in the United States is estimated to be approximately $185/cow annually. If we assume the same milk price and this value is multiplied by the total number of milking cows (9.5 million head), the total annual cost of mastitis is about $1.8 billion. This is approximately 10% of the total value of farm milk sales, and about two-thirds of this loss is due
to reduced milk production in sub clinically infected cows. The average production loss per lactation for one infected quarter is about 1,600 pounds. Other losses are due to discarded abnormal milk and milk with held from cows treated with antibiotic, costs of early replacement of affected cows, reduced sale value of culled cows, costs of drugs and veterinary services, and increased labor costs. (Wang et al., 2003).

2.12. MILK PRODUCTION, COMPOSITION AND QUALITY

Mastitis reduces milk yield and alters milk composition. The magnitude of these changes in individual cows varies with the severity and duration of the infection and the causative microorganisms. Mastitis is almost always caused by bacteria. These microorganisms produce toxins that can directly damage milk-producing tissue of the mammary gland, and the presence of bacteria initiates inflammation within the mammary tissue in an attempt to eliminate the invading microorganisms. The inflammation contributes to decreased milk production and is primarily responsible for the compositional changes observed in milk from infected quarters and cows. In general, compositional changes involve an increase in blood components present in milk and a decrease in normal milk constituents. (Vandeputte et al., 1993).

2.12.1. PRODUCTION

The Dairy Herd Improvement Association (DHIA) has adopted an SCC scoring system that divides the SCC of compositive milk into 10 categories from 0 to 9 known as linear scores. The DHIA programs determine the SCC on each milking cow each month and report either the SCC or the linear score. Linear scores can be used to estimate production losses, but the average linear score for the lactation most accurately reflects reduced milk yield. Cows with higher lactation average SCC scores produce less milk. Production losses in older cows are about double those of first lactation cows. Determining the exact amount of milk lost at a specific SCC or linear score or for any one cow is not possible. However, the fact remains that elevated SCC results in major losses to dairy producers, and elevated SCC is almost always due to the presence of intramammary infection. (Vandeputte et al., 1993).

2.12.2. COMPOSITION

Changes in milk composition accompany the increase in SCC following infection of the mammary gland. Elevated SCC is associated with a decrease in the content of lactose and fat in milk because of a reduced ability of the mammary gland to produce these components. Some studies have shown no change in fat percentage, yet total fat production declines with the
decrease in milk production. Although there may be little change in the total protein content as a result of sub clinical mastitis, there are marked and significant changes in the types of proteins present. The major milk protein is casein. This protein has high nutritional qualities and is very important in cheese manufacturing. Casein content of milk with a high SCC is reduced, but lower quality whey proteins increase in concentration, resulting in similar total protein content. The lower quality whey proteins are blood serum proteins such as serum albumin, immunoglobulins, and transferring, which increase in milk as a result of the destruction of membranes that normally prevent blood serum proteins from entering milk. Sodium and chloride increase in high SCC milk due to increased passage of these minerals from blood into milk. Potassium, normally the predominant mineral in milk, declines due to its passage out of milk to lymph between damaged secretory cells.

Most of the calcium in milk is associated with casein, and disruption of casein synthesis results in reduced calcium levels in milk from mastitic cows. These alterations in mineral content affect the pH and conductivity of milk. pH of normal milk is generally around 6.6, but may increase to 6.9 or higher in milk from mastitic quarters. Others important compositional changes include increases in enzymes originating from damaged mammary tissue, the blood stream, or milk somatic cells. Many of these enzymes negatively impact milk quality. An increase in the enzyme lipase can raise the content of free fatty acids, which produce off-flavors in milk from mastitic cows. An additional example is the enzyme plasmin, which may double in concentration in high SCC milk. Plasmin attacks casein and can markedly reduce the casein content, resulting in lower yields of cheese and other manufactured products and off-flavors in milk. (Vandeputte et al., 1993).

2.12.3. QUALITY

Mastitis not only reduces dairy producer profits but also results in important and costly losses to processors due to poor quality milk. Reduced quality is detected with herd milk at 400,000 cells/ml. A variety of dairy products are affected, including cheeses, powdered milk, fermented products, and fluid milk. Progressive milk plants pay on milk quality for obvious reasons, but quality premiums also pay big dividends to producers. For example, a 100-cow herd averaging 50lbs milk per cow per day and receiving $0.25 per hundred weight premium would get $375 more per month in milk receipts. The potential for invasion is greatly increased by bacteria that reside in or colonize the teat duct. Such colonization’s occur in both lactating and
dry cows, and the colonizing bacteria may survive for months, serving as sources of bacteria for infecting the gland. The practice of dipping teats with an effective bactericide both before and after each milking greatly reduces teat duct colonization. To better understand the important difference among mastitis causing organisms, the following list summarizes contagious and environmental mastitis. (Vandeputte et al., 1993).

2.13 DEVELOPMENT OF MASTITIS

A basic knowledge of mammary gland anatomy and physiology is necessary to understand how mastitis develops. The interior of each quarter is composed of a teat cistern, gland cistern, milk ducts, and glandular tissue. The glandular tissue or secretory portion contains millions of microscopic sacs called alveoli). Each alveolus is lined with milk producing epithelial cells and is surrounded by muscle cells and is surrounded by muscle cells that contract and squeeze milk from the alveolus during milking. Blood vessels bring nutrients to each alveolus where epithelial cells convert them into milk. Between milking, milk accumulates in the alveolar spaces, milk ducts, and cisterns. During milking, the accumulated fluid is removed through the teat ducts. (Sample et al., 1994).

2.14. INVASION OF THE UDDER

Mastitis results once bacteria pass through the teat duct and multiply in milk-producing tissues. Microorganisms breach the teat duct in several ways. Between milkings, microorganisms may pass through the teat duct by multiplying inside the duct, or by physical movement resulting from pressure placed on the teat end as the cow moves about. During machine milking, microorganisms may be propelled into or through the teat duct into the teat cistern. (Nolan et al., 2000).

2.15. CONTROLLING ENVIRONMENTAL MASTITIS

2.15.1. Prevention:

- Reduce the number of bacteria to which the teat end is exposed. (Paape et al., 2003)

2.15.2. Environment:

- Cow environment should be as clean and dry as possible.
  - Cow should not have access to manure, mud, or pools of stagnant water.
  - Dry cow environment is as important as lactating cow environment.
- Calving area must be clean.
• properly design and maintain free stalls. (Paape et al., 2003)

2.15.3. Bedding:
• Bacteria numbers in bedding depends on available nutrients, amount of contamination, moisture, and temperature.
• Inorganic materials (such as crushed limestone or sand) are low in nutrients and moisture, and thus bacteria.
• Finely chopped organic bedding (such as sawdust, shavings, recycled manure, pelleted corncobs, various seed hulls, chopped straw) are frequently high in bacterial numbers. (Paape et al., 2003)

2.15.4. Teat dipping:
• Post milking teat dipping with a germicidal (germkilling) dip is recommended.
• Controls the spread of contagious mastitis.
• Exerts no control over coliform infections.
• Barrier dips are reported to reduce new coliform infections; however, they do not appear to be as effective against environmental streptococci and the contagious pathogens.
• Attempts to control environmental mastitis during the dry period, using either germicidal or barrier dips, have been unsuccessful. (Paape et al., 2003)

2.15.5. Dry cow therapy:
• Recommended for all quarters of all cows at drying off.
• Helps control environmental streptococci during the early dry period.
• Has little or no value in controlling coliforms.
• Not effective during the period prior to calving. (Paape et al., 2003)

2.15.6 Backflushing milker claws between cows:
• Will not control environmental mastitis. (Paape et al., 2003)

2.15.7. Proper milking procedure:
• Proper milking procedure is important.
• Wash teats, but not the udder. Clean and dry teats before attaching the milking machine.
• Milking wet udders will likely increase mastitis. (Paape et al., 2003)

2.15.8. Predipping:
• A germicidal teat dip reduces environmental mastitis during lactation by 50%.
• Be sure teat dip is removed from teats before attaching milking machine to prevent contamination of the milk. (Paape et al., 2003)

2.15.9. Milking machine:
• Maintain and operate properly.
• Badly functioning milking machines result in frequent liner slips and teat end impacts will increase environmental mastitis. (Paape et al., 2003).

2.15.10. Nutrition:
• Proper nutrition will reduce the risk of environmental mastitis.
• Adequate levels of Vitamin E and selenium reduce the incidence of environmental mastitis.
• There are conflicting reports whether Vitamin A and B-carotene influence udder health.
• Ongoing research at the University of Kentucky indicates that copper may play a role in maintaining the immune system in dairy cattle.
• Feed dairy cattle a balanced ration. (Paape et al., 2003)

2.15.11. Vaccines:
• Not effective in preventing new infections.
• Research on vaccines to reduce Escherichia coli and staphylococcal mastitis infections looks promising. (Paape et al., 2003)

2.16. PREVENTION OF NEW INTRAMAMMARY INFECTIONS

Between regions of the world, major differences remain in the standard approaches used for pre-milking udder preparation. A summary of the literature and extension information in the area of milking management would suggest that a wide variety of methods can lead to the efficient and appropriate harvesting of milk. In some systems, pre-milking udder preparation is important for minimizing bacterial contamination, avoiding disinfectant residues and properly stimulating milk letdown. Assessment of the efficiency of the milking process remains as a
substantial challenge. Use of the Lactocorder instrument has recently been described for objectively assessing milk flow relative to milking management methods and automatic machine take-off settings19. This method shows considerable promise as a practical tool to evaluate current milking management, and to demonstrate the impact of proposed changes.

Implementation of post-milking teat disinfection is almost unanimous throughout the North American dairy industry for the control of contagious pathogens. It is recommended that all teats of all cows be dipped in an effective post-milking teat disinfectant after every milking. A bibliography of peer-reviewed publications that document the efficacy of teat disinfection products is published annually. Nevertheless, there continues to be major regional differences throughout the world with respect to the importance of this milking management practice. It remains important that each dairy herd develop and document a milking management protocol that is efficient, and that achieves the udder health goals of the herd. (Mattila et al., 1986).

Over the past decade, there have been many noteworthy developments with respect to machine milking of dairy cows. Both small automated milking systems and large rotary parlour installations have become commonplace. Whatever the system, it must be well-designed and properly maintained. Consistent standards for the evaluation of milking equipment function have been documented13. Dynamic system testing is essential for an accurate assessment of equipment function. Standard evaluation procedures and forms are available, including video documentation of the evaluation process. There remains a great challenge for dairy producers to keep abreast of the developments in milking systems, while ensuring that their current equipment is functioning well and being used appropriately. (Linggood et al., 1987).

Over the past decade, there has been an overwhelming increase in awareness concerning the ergonomics, comfort and welfare of dairy cattle housing system1. Furthermore, workers in this field have made effective use of time-lapse video recording to investigate the impact of facility design on cow behavior. The use of this technology has assisted the development of free-stall and tie-stall designs that allow cows to express their natural behavior. Unimpeded eating, drinking and resting should result in improved resistance to disease, and better udder health status. However, scientific documentation of these benefits from improved stall design in controlled studies is difficult to conduct, and is not abundant. There is a need for controlled research in this area, in particular on the relationships between cow comfort and udder health. (Kulberg et al., 2002)
2.17. ELIMINATION OF EXISTING INFECTIONS

The management of cases of clinical mastitis during lactation is a complex issue. The basic options for a clinical mastitis therapy protocol include treating all cows with antibiotics, treating no cows with antibiotics, or treating only certain cows with antibiotics. Different mastitis organisms require different treatment regimens and control strategies. Thus, timely and accurate milk bacteriological culture results would benefit producers and veterinarians. Many coliform cases could be managed conservatively, particularly in vaccinated cows.

Cows with gram-positive infections could receive antibiotic therapy in a more pharmacological appropriate approach than is currently used. There remains a definite need for new, rapid ways to determine the bacterial cause of current cases of clinical mastitis. To this point, cow-side test systems have not been extremely successful at improving our ability to classify clinical matitis cases as to etiologic agent in time to affect the treatment protocol. Recently, an evaluation of Petrifilm for mastitis diagnosis and treatment protocol has been reported18. It was concluded that these products could be used to guide treatment decisions for clinical mastitis, but adequate training of farm personnel is essential. There is a recent trend for large farms in the United States to develop and implement a targeted therapy program based upon actual milk bacteriology using a selective media tri-plate or quad-plate, and a large volume of milk, cultured on-farm. These protocols use interpretive charts, decision flow diagrams and usually call for a 16 to 24 hour delay in antimicrobial therapy, until the causative organism is determined. The refinement of these or other test systems hold great promise for benefits from rational antibiotic use in clinical mastitis therapy. (Dopfer et al., 1999).

Udder health management during the dry period is an integral period for elimination of existing, and prevention of new IMI. There has recently been a great deal of interest in shorter dry period length, such as 35-40 days, and preliminary results of effects on metabolism and milk production are very encouraging. As of yet, the effects of short dry period length on udder health have not been elucidated. (Breazile, 1998).

Intramammary administration of dry cow antibiotic therapy (DCT) has remained as a consistent recommendation for all quarters of all cows following abrupt cessation of milking. In North America, there is significant adoption of this process10. However, some regions of the world promote the use selective DCT, and others use very little DCT at all. When the control of contagious pathogens has been successful, the focus of DCT shifts from elimination to
Two periods of increased susceptibility to new IMI have been identified during the dry period. These two periods occur in the early dry period immediately after drying-off and at the end of the dry period just prior to calving. A landmark study has recently evaluated the association of milk production, and other important cow and quarter-level factors during late lactation, on new IMI during the dry period. Cows that were producing higher levels of milk on the day before dry-off were significantly more likely to develop a new IMI during the dry period. In addition, teat-end scores, and the rate of teat closure, influenced new IMI. Quarters with a cracked teat-end were 1.7 times more likely to develop new IMI. At the end of six weeks, 23.4% of teats were still classified as open. Quarters that closed during the dry period were 1.8 times less likely to develop a new IMI. These recent field studies provide strong evidence for the importance of management strategies aimed at enhancing teat canal closure. The use of an external dry cow teat sealant applied at drying-off for reduction of the new infection rate over the dry period has been evaluated. (Galland et al., 2001).

2.18. CLINICAL CHARACTERISTICS AND OUTCOME OF E. COLI MASTITIS:

Despite the numerous studies carried out on mastitis caused by Escherichia coli, many open questions remain concerning the pathogenesis and clinical course of the disease. When studying the aspects affecting severity of E. coli mastitis, the first problem encountered centres around the definition of the term severity; it can be based on clinical signs or outcome of mastitis (Pyorala et al., 1994). The intensity of clinical signs reflects host response at the time of observation, and varies during the course of mastitis. The final outcome, as rapid elimination of bacteria, prolonged infection or death of the cow due to endotoxin shock, describes more the ability of the cow to limit deleterious inflammatory reactions and to clear the infection (Pyorala et al., 1994).

The host response may be more aggressive because of a particularly virulent bacterial strain invading the udder or a vast number of bacteria, which results in large amounts of released endotoxin. Host response may also be intense in a case of a more alert immune system. The outcome may become serious or fatal if the cow’s immune system can not kill the bacteria due to their high virulence or excessive number, or an impairment of the immune system. Serious consequences may also occur if the cow’s immune system is not suppressed after the bacteria have been cleared from the udder. Different methods have been introduced for classifying the clinical course of E. coli mastitis. Cows can be divided into severe or mild responders based on
intensity of general and local clinical signs, changes in appearance of milk and loss of milk production during mastitis (Hirvoven et al., 1999).

Cows can also be grouped by outcome or the ability to return to production. In experimental mastitis, the severity of some clinical signs, such as heart rate and rumen motility, has been shown to correlate with a high number of bacteria in the milk and with loss of milk production. The clinical characteristics and outcome of E. coli mastitis vary from mild mastitis, where cows have only local signs in the udder and the duration of the infection is short, to very severe or even fatal forms (Dosogne et al., 2002).

E. coli mastitis typically has a sudden onset, which leads to changes in milk appearance, first to serous to yellow, and later to clotty and thick. Milk somatic cell count (SCC) increases to very high numbers. The udder becomes hard, swollen and tender. The cow also has systemic signs, generally including high fever, increased pulse frequency, reduced rumen contractions, lack of appetite, depression and decreased milk production. Studies using experimental E. coli mastitis models have shown that the first signs are usually noticed at the local level, at approximately 8 h post-challenge (PC), and fever and other systemic signs peak at 12 h. In mild or moderate cases, the systemic signs vanish within 48 h and local signs within 7 days. In severe cases, the cow may not survive or systemic signs may be prolonged, with milk production being lost permanently. In experimental E. coli mastitis, bacteria can be detected in the milk until 5-7 days after inoculation; endotoxin is simultaneously detected. However, a large variation exists in the duration of infection between experimental studies due to the different bacterial strains and doses used in the challenge. Bacteraemia or endotoxaemia occur seldom and only in very severe cases. Bacteraemia was found significantly more often in cows with severe clinical signs only trace amounts of endotoxin have been detected sporadically in the blood of cows with experimental E. coli mastitis. The release of inflammatory mediators, rather than the absorption of endotoxin, causes the systemic signs of E. coli mastitis (Burvenich et al., 2003)

2.19. **E. Coli BACTERIA:**

E. coli is a Gram-negative, non-spore-forming rod, which belongs to the family Enterobacteriaceae. The cell wall of Gram-negative bacteria typically consists of three layers, the cytoplasmic membrane and the outer membrane, separated by a peptidoglycan layer. The outer cell membrane contains phospholipids, membrane proteins and lipopolysaccharide (LPS). LPS comprises lipid-A, the lippolysaccharide core and repeated polysaccharide units called O-
antigens (Cullor, 1996). Lipid-A is the lipophilic, inner part of LPS. The toxic effects of LPS also known as endotoxin are caused by lipid-inner part of LPS. The toxic effects of LPS also known as endotoxin are caused by lipid –A (Cullor, 1996). Here, the terms LPS and endotoxin are used synonymously. On the outer surface, bacteria may have fimbrias which protrude from the cell wall. The surface may be covered with a thick polysaccharide layer called a capsule. Based on the different antigenic structures of O-antigens, K-antigens (capsular) and H-antigens (flagellar), E. coli strains can be divided into O: H: K serotypes (Cullor, 1996). Escherichia coli is part of the normal intestinal flora of humans and animals. It is the most common facultative anaerobic bacterial species in the gut and is constantly excreted in the faeces to the environment. Pathogenic E. coli bacteria can cause intestinal and extra intestinal infections in mammalian and avian hosts (Cullor, 1996). Infections of the gastrointestinal tract may lead to various kinds of diarrhoeic diseases, which, in the case of shiga toxin, may even progress to systemic hemolytic uremic syndrome in humans and oedema disease in pigs (Cullor, 1996). E. coli is the predominant causes invasive diseases, such as bacteraemia and meningitis, in humans and animals. In avian species, E. coli is an important cause of respiratory and ovarian tract infections (Cullor, 1996).

2.20. VIRULENCE FACTORS;

Pathogenic E. coli bacteria are typically specific to the type of disease they cause and to the animal species infected. They produce virulence factors involved in pathogenesis of specific diseases (Chinna et al., 1999). Bacterial virulence factors are required to colonize and infect the host and to fight host defense mechanisms. Major groups of E. coli virulence factors include toxins, adhesions, proteins secreted into host cells, polysaccharide capsules and O-antigens, and other mechanisms to resist killing by complement or to scavenge iron. Bacteria do not produce virulence factors constantly but only upon receiving certain signals from the host or environment (Chinna et al., 1999). The genes for virulence factors may be present in the bacterial genome or may reside extra chromosomally on plasmids, even though the virulence factor is not produced (Harel et al., 1999).

2.21. PATHOGENICITY OF E. COLI ISOLATED FROM MASTITIS:

E. coli is an opportunistic bacterium when causing mastitis (Valente et. al., 1988). Bovine mastitis resembles urinary tract infection; the infection in both is ascending and caused by bacteria from the environment. The source of mastitis- causing E.coli strains may be found in
the intestinal flora of the affected from the isolates causing mastitis, and E. coli mastitis is
definitely not caused by a limited number of specific pathogenic strains (Jung, 1999). However,
the existence of udder-adapted E. coli strains has been suggested, since the same serotype and
genotype of E. coli have been isolated from cases of recurrent mastitis (Dopfer et al., 1999).
Isolates from recurrent mastitis have been shown to invade mammary epithelial cells in vitro
faster and in larger numbers than the strains from occasional cases (Dopfer et al., 2000).

2.22. HOST RESPONSE:

Host response is a complicated process in which the non-specific and specific immune
systems act together to attack and destroy the invading pathogen and repair the damage the
pathogen or the immune system itself has caused to the host (van, 1995). The
First host defence against invading bacteria is the physiological barrier of the teat canal. Tight
and rapid closure after milking and during the dry period prevents influx of bacteria. After
invading the udder, the bacteria have to deal with humoral factors present in milk. During the
dry period a high concentration of lactoferrin is one of the most effective factors against
infections caused by coliform bacteria. Lactoferrin chelates iron and prevents growth of
coliforms, which require large amounts of iron. In the lactating period, the concentration of
lactoferrin in normal milk is not high enough to work efficiently and the elevated citrate
concentration further interferes with it (Kremer et al., 1996). During the middle of dry period the
cow is thus quite resistant to clinical E. coli mastitis. However, in early and late dry period there
may be acquisition of new E. coli infections, which become clinical during the first 100 days of
lactation (Bradley, 2000).

The bacteria killing ability of serum is thought to be an important defense mechanism
against E. coli. The serum killing effect is based on complement and its ability to cause lysis of
bacteria. The complement system also enhances PMN phagocytosis by attracting phagocytes,
opsonizing bacteria and priming the intracellular killing of phagocytes (Rainard, 2003).

The origin of complement components in bovine milk probably differs between normal
and mastitic milk. In normal milk, the transudation of liver-produced complement proteins from
the serum and the local synthesis of C3 may dominate (Rainard, 2003). Moreover, in this milk,
the killing function of the complement may not be as important as previously thought since the
amount of some complement compounds is low and other components of milk interfere with the
complement. (Rainard, 2003).
During inflammation the vascular permeability increases, causing exudation of complement proteins from serum, and the local synthesis by macrophages is simultaneously stimulated by proinflammatory cytokines (Rainard, 2003). This result in a different composition and a higher amount of compliment proteins in milk, enhancing the bactericidical activity. The most important defense mechanism against invading bacteria is the acute-phase response, which in E. coli mastitis is initiated by endotoxin released from bacteria. A great number of cytokines are produced by mammary macrophages, epithelial and endothelial cells and polymorph nuclear leucocytes (PMN) during the inflammatory process. The cytokines have various effects in initiating, mediating and reducing inflammation. In E. coli mastitis, the released endotoxin is bound by LPS-binding protein (LBP), and recognition of this complex is done by the membrane CD14 (mCD14) receptor of monocytes, macrophages and neutrophils, which are present in milk, causing the release of tumor necrosis factor alpha (TNFα) (Wang et al., 2003).

The soluble CD14 (sCD14) may also bind endotoxin, either directly or by binding the endotoxin-LBP complex (Wang et al., 2003). The endotoxin-LBP-sCD14 complex is recognized by the Toll-like receptors on the epithelial and endothelial cells of the mammary tissue, causing secretion of chemo attractants (Paape et al., 2003). Cytokines, interleukins (IL) and chemo attractants in turn act both locally and systemically, attracting neutrophils from the circulation to the infection site, opsonizing the pathogen and inducing production of acute phase-proteins (APP) in the liver (Paape et at., 2003).

After starting the acute-phase reaction, the speed of PMN efflux to milk and their ability to phagocytose the bacteria are critical to the development of mastitis. If the acute-phase reaction or bacteria killing ability of PMN is reduced, bacteria are able to multiply, and the damage caused to the host cells increases. On the other hand, an overreaction in the acute phase may also cause damage so the inhibiting and repairing actions of the acute-phase reactions have to be in balance. Differences in the production function and kinetics of cytokines and APP may partly explain the variation in local and systemic signs of individual animals with E. coli mastitis (Paape et al., 2003).

2.23. NEUTROPHIL FUNCTION:

In a healthy udder, the number of PMN in milk is low, <100 000 cells/ml. After any damage caused by bacteria or other pathogens or by physical injury, the number of PMN increases dramatically. This increase is mediated by a chemo attracting stimulus, and the degree
of the stimulus depends on the infecting pathogen. In *E. coli* mastitis, a high number of PMN is required at the infection site immediately, and mature circulating PMN present in blood migrate towards the infection site, leading to neutropenia. If the chemo attracting stimulus continuous, the reserve pool of mature PMN, located in blood vessels and in bone marrow, moves into the circulation and then to the infection site. Immature PMN are seldom normally present in bovine blood. They are seen if neutropenia, caused by migration, is corrected by the release of immature PMN from the bone marrow. PMN are produced in the bone marrow by granulopoiesis. Maturity of PMN has an impact on their function (Dosogne, 1998); immature PMN have impaired phagocytosis as well as decreased production of reactive oxygen species (ROS). Their migration to the infection site is also slower. Migration of PMN from blood circulation to the infection site depresses their functions; these cells have decreased phagocytosis because the migration utilizes the PMN energy sources.

Ingestion of fat and other milk compounds also interferes with phagocytosis. The most important factor in intramammary defense against invading pathogens is the resident PMN. PMN recognize the pathogen with the help of complements and other opsonizing factors. After recognition, they are able to use two different mechanisms for killing invading bacteria: oxidative dependent and oxidative-independent mechanisms. The oxidative-independent mechanisms involve a variety of different enzymes and proteins in the non-azurophilic granules of PMN, such as lactoferrin, lysozyme, cytosine, elastase and fibronectin, which inhibit the growth of *E. coli* bacteria (Burvenich et al., 2003).

Production of lactoferrin by PMN is, however minimal compared with that by mammary tissue (Pfaffl et al., 2003). A more effective mechanism against *E. coli* bacteria is, however, the oxygen-dependent mechanism. It takes place when the plasma membrane of the PMN recognizes the bacteria and releases super oxide (O2-) and hydrogen peroxide (H2O2) in a respiratory burst (Paape et al., 2003). The oxygen-dependent mechanisms, which reside in the azurophilic granules of PMN, also involve the release of other ROS and nitrogen oxide after activation of myeloperoxidase during phagocytosis (Burvenich et al., 2003). ROS production of PMN can be measured by chemiluminescence’s (CL), which reflects the bactericidal capacity.

**2.24. TUMOR NECROSIS FACTOR ALPHA:**

TNFα belongs to the proinflammatory and inflammatory cytokines released from cells during inflammation (Shuster et al., 1996). The role of TNFα in the pathogenesis of *E. coli*
mastitis has been studied widely. It is supposed to have a pivotal role in initiating the effects of endotoxin during *E. coli* mastitis. TNF-α also causes systemic effects, seen in changes in hormone secretion, milk production and composition, inflammatory parameters and clinical signs (Kushiiki et al., 2003).

Some studies have found a correlation between severity of response and concentrations of TNF-α in blood or milk, while other studies have reported no direct correlation. TNF-α also affects PMN functions by increasing the size of PMN and enhancing oxidative burst activity (Paape et al., 2003). However, if excessive quantities of TNF-α are produced, numerous harmful effects will follow, including reduced blood pressure and tissue perfusion, intravascular thrombosis and severe metabolic disturbances, often leading to lethal shock (Paape et al., 2003).

**2.25. SERUM AMYLOID A:**

In cattle, serum amyloid A (SAA) is a sensitive APP (Boosman et al., 1989). It has also been shown to reflect the severity of mastitis (Eckersall et al., 2001). Cows with clinical mastitis have been reported to have increased levels of SAA in serum and milk compared with healthy cows (Pedersen et al., 2003); high levels have also been found in bovine colostrum (McDonald et al., 2001). SAA is mainly produced in the liver but also extrahepatically by macrophages, endothelial cells, mammary epithelial cells and smooth muscle cells (McDonald et al., 2001). Extrahepatically produced SAA consist of multiple isoforms; M-SAA3 may differ locally and systemically and may be concentration-dependent. Local and systemic production of SAA may also be governed by different control mechanisms (Eckersall et al., 2001).

**2.26. EFFECT OF AGE AND LACTATION STAGE:**

The clinical response and outcome of *E. coli* mastitis vary according to the age of the animal and the stage of lactation (Hill et al., 1979). Older cows are more susceptible to *E. coli* infections because of diminished PMN functions. It has been shown that *E. coli* intramammary infections can originate from the late dry period and become clinical in early lactation. Peracute *E. coli* mastitis with severe clinical signs most frequently occurs during the periparturient period and in early lactation (Hill et al., 1979). This is thought to be compromised immune mechanisms of the cows during the peripartum period (Mehrzad et al., 2001).

A compromised immune system may result for several reasons. The numbers of circulating PMN in the blood change according to lactation stage; they increase at parturition but then decrease rapidly during the puerperal period, reaching a lower level than seen in later
lactation (Kulberg et al., 2002). Cotisol levels may affect the number and function of PMN, and a positive correlation between PMN and cortisol has been detected. However, cortisol may interfere with the number and efficient function of PMN (Kulberg et al., 2002). In milk, cortisol increases at the time of parturition, but falls back to preparturition levels one day post-partum; in blood, no changes occur in cortisol levels. During the post-partum period the diapedesis of PMN into the infected quarters and phagocytosis are impaired with later lactation.

PMN functions may be altered due to hyperketonaemia, which is commonly seen during early lactation. Severe signs in experimentally induced *E. coli* mastitis were not associated with a mild negative energy balance, as indicated by elevated levels of non-esterified fatty acids or β-OH-butyrate (Kornalijnslijper et al., 2003), but an association was found with ketonaemia. The more severe response seen in early lactation may also be caused by a higher production of TNFα by mononuclear cells during this period. In endotoxin mastitis, TNFα was detected in plasma and milk of early-lactating cows but not in mid-lactating cows. However, in the last-mentioned study, the amount of endotoxin used for the induction of mastitis was much lower. (Kornalijnslijper et al., 2003).