CHAPTER 1

INTRODUCTION

1.1 Milk consumption and composition:

Bovine milk and dairy products have used traditionally for human nutrition. The significance of milk is reflected in our northern mythology where a primal cow named Audhumla was evolved from the melting ice. She had horn and milk was running as rivers from her teats. This milk was used as the food for Ymer, the first creature ever existing [1]. The consumption of milk and milk products among milk consuming regions vary considerably from about 180 kg/yr per capita in Iceland and Finland to less than 50 kg per capita in Japan and China. In the western societies, the consumption of milk has decreased during the last decades [2]. This trend may partly be explained by the negative claims on health effects that have been attributed to milk and milk products. This criticism has arisen especially because milk fat contains a high fraction of saturated fatty acids assumed to contribute to heart diseases, weight gain and obesity [3]. The association between food and health is well established and recent studies have shown that modifiable risk factors seem to be greater significance for health than previously anticipated [4]. Prevention of disease may be just as important as treatment of diseases in the future. Indeed, many consumers of today are highly aware of health-properties of food, and the market for healthy food and special health benefits is in the increasing trend due to the consumer awareness of health properties of food.

Bovine milk contains the nutrients needed for growth and development of the calf, and is a resource of lipids, proteins, amino acids, vitamins and minerals. It contains immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes and other bioactive peptides. The lipids in milk are emulsified in globules coated with membranes. The proteins are in colloidal dispersions as micelles. The casein micelles occur as colloidal complexes of protein, salts of primarily calcium [5]. Lactose and most minerals are in solution. Milk composition has a dynamic nature,
and the composition varies with stage of lactation, age, breed, nutrition, energy balance and health status of the udder. Specific milk proteins are involved in the early development of immune response, whereas others take part in the non-immunological defence (e.g. lactoferrin). Milk contains many different types of fatty acids [6]. All these components make milk a nutrient rich food item.

1.2 Components in milk and their health effects:

1.2.1 Protein:

Bovine milk contains about 32 g protein/l. The milk protein has a high biological value, and milk is therefore a good source for essential amino acids. In addition, milk contains a wide array of proteins with biological activities ranging from antimicrobial ones to those facilitating absorption of nutrients, as well as acting as growth factors, hormones, enzymes, antibodies and immune stimulants [7]. The nitrogen in milk is distributed among caseins, whey proteins and non-protein nitrogen. The casein content of milk represents about 80% of milk proteins. Caseins biological function is to carry calcium and phosphate and to form a clot in the stomach for efficient digestion. The milk whey proteins are globular proteins that are more water soluble than caseins, and the principle fractions are beta-lactoglobin, alpha-lactalbumin, bovine serum albumin and immunoglobulins. Whey is the liquid remaining after milk has been curdled to produce cheese, and it is used in many products for human consumption, such as ricotta and brown cheese. Concentrated whey is an additive to several products e.g. bread, crackers, pastry and animal feed. The rate at which the amino acids are released during digestion and absorbed into the circulation may differ among the milk proteins, and whey proteins are considered as rapid digested protein that gives high concentrations of amino acids in postprandial plasma [8].

Some of the milk proteins (e.g. secretary immunoglobulin A, lactoferrin, 1-antitrypsin, β-casein and lactalbumin) may be relatively resistant to digestive enzymes, and the whole protein or peptides derived from it, may exert their function in the small
intestine before being fully digested [9]. As several bioactive proteins and peptides derived from milk proteins are potential modulators of various regulatory processes in the body, some of these are produced on an industrial scale, and are considered for application as ingredients in both 'functional foods' and pharmaceutical preparations. Although the physiological significance of several of these substances is not yet fully understood, both the mineral binding and cytomodulatory peptides derived from bovine milk proteins are now claimed to be health enhancing components that can be used to reduce the risk of disease or to enhance a certain physiological functions [10]. Milk protein composition may differ among breeds. For example the concentration of beta-casein A1 is low in cow milk in Iceland and in New Zealand. It has been speculated that this proteins may have a role in the development of diabetes and cardiac disease. However, later it was concluded in a review article that there is no convincing evidence that the A1 beta-casein of cow milk has any adverse effect in humans [11].

1.2.2 Branched chain amino acids and other amino acids:

Milk is especially rich in essential amino acids and branched chain amino acids. There is several evidence that these amino acids have unique roles in human metabolism; in addition to provide substrates for protein synthesis, it also suppresses protein catabolism and serve as substrates for gluconeogenesis, they also trigger muscle protein synthesis and promote protein synthesis [12]. The stimulated insulin secretion caused by milk, is suggested to be caused by milk proteins, and as shown by Biller et al., 1995 [13] a mixture of leucine, isoleucine, valine, lysine and threonine resulted in glycemic and insulinemic response resembling the response seen after ingestion of whey. A combination of milk with a meal with high glycaemic load (rapidly digested and absorbed carbohydrates) may stimulate insulin release and reduce the postprandial blood glucose concentration [14]. A reduction in postprandial blood glucose is favourable, and it is epidemiological evidence suggesting that milk may lower risk of diseases related to insulin resistance syndrome.
1.2.2.1 Taurine:

The concentration of taurine is high in breast milk (about 18 mg/l) and in colostrum from cow [15]. Goat milk is however very rich in taurine: 46–91 mg/l. Taurine is an essential amino acid for preterm neonates, and specific groups of individuals are at risk for taurine deficiency and may benefit from supplementation, e.g. patients requiring long-term parenteral nutrition (including premature and newborn infants); diabetes patients, those with chronic hepatic, heart or renal failure. It is suggested that during parenteral nutrition, supplementation of 50 mg taurine per kg body weight may be required. It is implicated in numerous biological and physiological functions: bile acid conjugation and cholestasis prevention, antiarrhythmic/inotropic/chronotropic effects, central nervous system neuromodulation, retinal development and function, endocrine/metabolic effects and antioxidant/anti-inflammatory properties [16]. Taurine has been shown to have endothelial protective effects, it may function principally as a negative feedback regulator, helping to dampen immunological reactions before they cause too much damage to host tissues or to the leukocytes themselves, and it is shown to be analgesic.

1.2.2.2 Glutathione (GSH):

Fresh milk may be a good source of glutathione, a tripeptide of the sulphur amino acid cysteine, plus glycine and glutamic acid. In the organism glutathione has the role as an antioxidant. Glutathione can be oxidized forming GSSG (oxidized glutathione), and in this reaction it may remove reactive oxygenspecies (ROS), thereby regulating the level of ROS in the cells. Glutathione appears to have different important roles in leukocytes, as a growth factor, as an anti-apoptotic factor in leukocytes and to regulate the pattern of cytokine secretion [17]. GSH, moreover, is also central for antioxidative defence in the lungs, which may be very important in connection with lower respiratory infections including influenza [18].
1.2.3 Lipids:

1.2.3.1 Fatty acids:

In average, milk contains about 33 g of total fat/l.

1.2.3.2 Saturated fatty acids:

More than half of the milk fatty acids are saturated, accounting to about 19 g/l whole milk. The specific health effects of individual fatty acids have been extensively studied [19].

1.2.3.3 Unsaturated fatty acids:

Oleic acid (18:1c9) is the single unsaturated fatty acid with the highest concentration in milk accounting to about 8 g/litre whole milk. Accordingly milk and milk products contribute substantially to the dietary intake of oleic acid in many countries.

1.2.3.4 Trans vaccenic acid (VA):

The main trans 18:1 isomer in milk fat is vaccenic acid, (18:1, 11t, VA), but trans double bounds in position 4 to 16 is also observed in low concentrations in milk fat.

1.2.4 Phospholipids and glycosphingolipids:

Phospholipids and glycosphingolipids accounts to about 1% of total milk lipids. These lipids contain relatively larger quantities of polyunsaturated fatty acids than the triacylglycerols. They have functional roles in a number of reactions, such as binding cations, help to stabilize emulsions, affect enzymes on the globule surface, cell-cell interactions, differentiation, proliferation, immune recognition, transmembrane signalling and as receptors for certain hormones and growth factors. Gangliosides are one of these components found in milk. Gangliosides (with more than one sialic acid moiety) are
mainly found in nerve tissues, and they have been demonstrated to play important roles in neonatal brain development, receptor functions, allergies, for bacterial toxins etc...

1.2.5 Minerals, vitamins and antioxidants in milk:

Milk contains many minerals, vitamins and antioxidants. The antioxidants have a role in prevention of oxidation of the milk, and they may also have protective effects in the milk-producing cell, and for the udder. Most important antioxidants in milk are the mineral selenium and the vitamins E and A. As there are many compounds that may have anti-oxidative function in milk, measurement of total anti-oxidative capacity of milk may be a useful tool.

1.3.5.1 Calcium in milk:

The calcium concentration in bovine milk is about 1 g/l. In human nutrition adequate calcium intake is essential. Getting enough calcium in the diet gives healthy bones and teeth.

1.3.5.2 Selenium in milk:

The selenium concentration in body fluids and tissues are directly related to selenium intake. It has a role in the immune- and antioxidant system and in DNA synthesis and DNA repair.

1.3.5.3 Iodine in milk:

Iodine is an essential component of the thyroid hormones. These hormones control the regulation of body metabolic rate, temperature regulation, reproduction and growth.
1.3.5.4 Magnesium in milk:

Magnesium is ubiquitous in foods, and milk is a good source, containing about 100 mg/l milk. Magnesium has many functions in the body, participating in more than 300 reactions.

1.3.5.5 Zinc in milk:

Zinc is an essential part of several enzymes and metalloproteins. Zinc has several functions in the body, in DNA repair, cell growth and replication, gene expression, protein and lipid metabolism, immune function, hormone activity, etc...

1.3.5.6 Vitamin E in milk:

Vitamin E concentration in milk is about 0.6 mg/l. Recommended intake is 15 mg/day. Observational studies indicate that high dietary intake of vitamin E are associated with decreased risk for cancer and coronary heart disease.

1.3.5.7 Vitamin A in milk:

Milk is a good source of retinoids, containing 280 ug/l. The recommended daily intake is 700–900 µg/day. Vitamin A has a role in vision, proper growth, reproduction, and immunity, cell differentiation, in maintaining healthy bones as well as skin and mucosal membranes.

1.3.5.8 Folate in milk:

Bovine milk contains 50 µg folate/l. Recommended intake of folate is 400 µg/day for adults. Many scientists believe that folate deficiency is the most prevalent of all vitamin deficiencies.
1.3.5.9 Riboflavin in milk:

Milk is a good source of riboflavin, 1.83 mg riboflavin/l milk. Daily recommended intake is 1.1 and 1.3 mg for women and men, respectively.

1.3.5.10 Vitamin B₁₂ in milk:

Milk is also a good source of vitamin B₁₂, being 4.4 µg/l. The daily recommendation is 2.4 µg. Vitamin B₁₂ is found only in animal foods and its deficiency may cause megaloblastic anemia and breakdown of the myelin sheath.

1.4 Bacterial flora of milk in milk:

Milk samples from normal healthy mammary glands contain many strains of bacteria. To prevent diseases caused by pathogenic bacteria in milk and to lengthen the shelf life of milk, treatment such as cooling and pasteurization or membrane filtration is needed. To preserve milk, addition of selective, well-documented strains of starter cultures for fermentation is a method that has been used for centuries.

1.5 Intolerance to milk components:

The public "belief" that milk causes an inflammatory process and an increase in mucus production has not been confirmed. It has been shown that respiratory symptoms were not associated with milk intake, and concluded that consumption of milk does not seem to exacerbate the symptoms of asthma, but in a few cases people with cow's milk allergy may have asthma-like symptoms after milk consumption. However in cells from another tissue; mucin producing cells of gastric mucosa, alpha-lactalbumin stimulates mucin synthesis and secretion. The intolerance is generally not observed for fermented milk. Researchers found out that patients who had developed intolerance to milk components did not develop the same level of intolerance to fermented milk components.
1.6 Intolerance to milk proteins:

There has been speculation if milk proteins may have a role in Attention Deficit Hyperactivity Disorder (ADHD), autism, depressions and schizophrenia in some cases. There are major supports to the hypothesis that ADHD may be linked to increased levels of neuroactive peptides and increased urinary peptide levels. A diet free of milk, milk products and gluten may in many cases give reduced ADHD symptoms. Further, opioid peptides derived from food proteins (exorphins) have been found in urine of autistic patients [18]. This area of investigation is important and large scale, good quality randomised controlled trials are needed.

1.7 Physiologically active milk peptides:

In addition to providing immunodefence systems, milk also contains other major peptide fractions that elicit behavioral, neurological, physiological, and vasoregulatory responses. Often, the peptide displays multifunctional properties. Several articles reviewing this topic have already been published [20, 21, 22, and 23]. Here, we categorize classifications of physiologically active peptides based on their primary biofunction.

1.7.1 Antihypertensive Peptides (ACE Inhibitors):

Antihypertensive peptides inhibit the angiotensin converting enzyme (ACE) [24, 25]. ACE is a peptidylidipeptidase that cleaves dipeptides from the carboxy terminal end of the substrate. ACE converts angiotensin I to angiotensin II, increasing blood pressure and aldersterone, and inactivating the depressor action of bradykinin. ACE inhibitors derived from casein, or casokinins, have been identified within the sequences of human β- and κ-CN [26]. They are also generated by tryptic digestion of bovine αs1- and β-CN [27]. The C-terminal tripeptide sequence is the primary structural feature governing this inhibitory response [28], and reports indicated that the ACE binding pocket exhibited a preference for hydrophobic amino acids at each of these sites [29]. A second
characteristic of ACE inhibitory casokinins is the presence of a positively charged lysine or arginine at the carboxy terminal end [30]. It was shown that removal of this critical amino acid residue from bradykinin, an endogenous ACE inhibitor, resulted in production of an analogue that was essentially inactive [31]. ACE inhibitory peptides are also derived from both αs1- and β-CN that are generated by the hydrolysis of sour milk with the *Lactobacillus helveticus* CP790 extracellular protease. These peptides exhibited antihypertensive activity in spontaneously hypertensive rats as monitored by systolic blood pressure [32]. A synthetic seven amino acid peptide, equivalent to a segment found in the β-CN hydrolysate, exhibited potent antihypertensive activity in these rats over an 8-h interval after oral administration [33]. A third subclass, β-lactorphins, are sequestered within the primary amino acid sequence of bovine β-LG and released by trypsin [34]. Lastly, novel angiotensin-I converting enzyme (ACE) inhibition was detected in synthetic peptides that corresponded to sequences within both β-LG and α-LA.

### 1.7.2 Antithrombotic Peptides:

Antithrombotic peptides are present in milk. Early on, it was learned that the mechanisms involved in milk clotting, defined by the interaction of κ-Casein (CN) with chymosin and blood clotting processes, defined by the interaction of fibrinogen with thrombin, were comparable. In this regard, the C-terminal dodecapeptide of human fibrinogen γ-chain (residues 400 to 411) and the undecapeptide (residues 106 to 116) from bovine κ-CN are structurally and functionally quite similar. This casein-derived peptide sequence, termed casoplatelin, affected platelet function and inhibited both the aggregation of ADP-activated platelets and the binding of human fibrinogen λ-chain to its receptor region on the platelets’ surface [35]. A smaller κ-CN fragment (residues 106 to 110), casopiastrin, was obtained from trypsin hydrolysates and exhibited antithrombotic activity by inhibiting fibrinogen binding [36]. A second segment of the trypsin κ-CN fragment, residues 103 to 111, inhibited platelet aggregation but did not affect fibrinogen binding to the platelet receptor [37]. Later, it was reported that biologically active peptides, isolated from both casein and lactotransferrin, inhibited platelet function [38].
Antithrombotic peptides have also been derived from κ-caseinoglycopeptides that were isolated from several animal species. Bovine κ-caseinoglycopeptide, the C-terminal end of κ-CN (residues 106 to 169), inhibited von Willebrand factor-dependent platelet aggregation [39]. Two antithrombotic peptides, derived from human and bovine κ-caseinoglycopeptides, have been identified in the plasma of 5-d-old newborns after breast-feeding and ingestion of cow’s milk based formula, respectively [40]. The C-terminal residues (106 to 171) of sheep κ-casein, or κ-caseinoglycopeptide, decreased thrombin- and collagen-induced platelet aggregation in a dose dependent manner [41]. Lastly, thrombin-induced platelet aggregation was inhibited with pepsin digests of sheep and human lactoferrin. A single peptide peak containing this activity was obtained by reverse-phase chromatography of the hydrolysate [42].

### 1.7.3 Casein phosphopeptides: (CPP)

Casein phosphopeptides (CPP) have been identified after trypsin release from αs1-, αs2-, and β-CN [43]. The phosphate residues, which are present as monoesters of serine, occur mainly in clusters. Most CPP contain three serine phosphate clusters followed by two glutamic acid residues, form soluble organophosphate salts, and probably function as carriers for different minerals, especially calcium [44]. These fractions exhibit different degrees of phosphorylation, and a direct relationship between the degree of phosphorylation and mineral chelating ability has been described [45]. In this event, αs2-CN > αs1-CN > β-CN > κ-CN; however, the distribution of their phosphoserine clusters is not uniform. It was further demonstrated that the specific amino acid composition associated with the phosphorylated binding site also influences the degree of calcium binding [46].

CPP are mostly resistant to enzymatic hydrolysis in the gut and most often found in a complex with calcium phosphate [47]. This complex formation results in an increased solubility which, in turn, provides enhanced absorption of calcium across the distal small intestines of animals fed casein diets in comparison to control animals fed
soy-based diets [48]. This passive transport system is the primary means of calcium absorption under physiological conditions and provides calcium required for bone calcification. Caseinophosphopeptides also inhibit caries lesions through recalcification of the dental enamel. Hence, their application in the treatment of dental diseases has been proposed.

1.7.4 Immunomodulatory Peptides:

Immunomodulatory milk peptides affect both the immune system and cell proliferation responses. As discussed previously, β-casokinins inhibit ACE enzymes that are responsible for inactivating bradykinin, a hormone with immune enhancing effects. Thus, this chain of events indirectly produces an overall immunostimulatory response. Peptides derived from casein hydrolysates were shown to increase phagocytic activity of human macrophages against aging red blood cells and augment phagocytosis of sheep red blood cells by murine peritoneal macrophages in vitro [49]. Immunostimulatory activity against *Klebsiella pneumoniae* was demonstrated in vivo using rats treated intravenously with a hexapeptide obtained by hydrolysis of human β-CN.

Most recently, lactoferricin B, obtained by hydrolysis of lactoferrin with pepsin, was found to promote phagocytic activity of human neutrophils via dual mechanisms that may involve direct binding to the neutrophil and opsonin-like activity. Small peptides, corresponding to the N-terminal end of bovine α-LA (dipeptide) and κ-CN (tripeptide), significantly increased proliferation of human peripheral blood lymphocytes [100], while the C-terminal sequence of bovine β-CN (amino acid sequences 193 to 209), obtained by hydrolysis with pepsin-chymosin, induced a similar response in rats. Bioactive peptides in yogurt preparations actually decreased cell proliferation with IEC-6 or Caco-2 cells. This report may explain, in part, why consumption of yogurt has been associated with a reduced incidence of colon cancer. In general, the mechanisms by which these milk-derived peptides exert either their immunopotentiating effects or influence proliferative responses are not currently known; however, one example suggests that the opioid milk
peptide, β-casomorphin, may exert an inhibitory effect on the proliferation of human lamina propria lymphocytes in vitro via the opiate receptor [50]. This antiproliferative response was reversed by the opiate receptor antagonist, naloxone.

1.7.5 Opioid Milk Peptides:

The major opioid peptides are fragments of β-CN, called β-casomorphins, due to their exogenous origin and morphine-like properties; however, they have also been obtained from pepsin hydrolysis of bovine αs1-CN fractions (43, 54, 66, and 102). Similar peptides have been reported from human β-CN fractions (24, 98), and the Y-P-F sequence, which is common to bovine β-casomorphin, was also found to be present in the primary structure of human β-CN. Various synthetic derivatives have been made and among these, Y-P-F-V-NH2 (valmuceptin) and Y-P-F(D)-V-NH2 (D-valmuceptin) show high affinity for their receptor [51]. Schuster and co-workers in 1980 reported opioid activity from synthetic tetra- and pentapeptide fragments of human β-casein [52]. Opioid peptides have been generated in vitro by enzymatic digestion of β-caseins from cows, water buffalo, and sheep. In general, the α- and β-CN fragments produce agonist responses, while those derived from κ-CN elicit antagonist effects. Opioid peptides may be further subdivided into classifications according to the specific milk protein from which they were derived. It is noteworthy that bioactive peptides are generated from most of the major proteins in both bovine and human milk.

a. Structure and function

“Typical” opioid peptides, or endorphins, are derived from proenkephalin, propiomelanocortin, and prodynorphin and exhibit a definite N-terminal sequence Y-G-G-F. Milk derived peptides, generated by hydrolysis of various precursor proteins such as α- and β-CN, α-LA, and β-LG are called “atypical,” exomorphic, agonist peptides and exhibit morphine-like activity [53]. Their primary structure (i.e., Y-X1-F or Y-X1-X2-F or Y) differs from the amino terminal sequence of the “typical” endogenous opioid peptide defined above. With the exception of αs1-CN, most share a common sequence
feature, defined by a N-terminal tyrosine residue, that is absolutely essential for activity. Typically, a second aromatic amino acid residue, such as phenylalanine or tyrosine, is also present in the third or fourth position. This structural motif fits well into the binding pocket of the opioid receptor. One of the most potent milk-derived opioid peptides, β-casomorphin-4-amide (or morphiceptin), also contains a proline that is crucial for its function. This residue reportedly maintains the proper orientation of the tyrosine and phenylalanine side chains. Exorphins have been isolated from peptic hydrolysates of α-casein fractions as well. In general, their structures differ considerably from those of β-caseinomorphins. Active fractions were shown to be a mixture of two separate peptides derived from α-casein fragments #90–95 and #90–96. The sequences were determined as listed, [R90-Y-L-G-Y-L95-(E96)], in which case the latter peptide proved to be more effective. The N-terminal arginine residue was also reported to be essential for activity.

b. Opioid agonists

β-Casein peptides were among the first reported opioid peptides. β-Casomorphins are fragments corresponding to the 60 to 70th amino acid residues of bovine β-CN, considered the “strategic zone,” and are classified as μ-type receptor ligands [54]. Three exorphins, derived from bovine αs1-CN, were shown to be selective for δ-receptors. Certain proteolytic bacteria, such as Pseudomonas aeruginosa and Bacillus cereus, also produce high levels of β-casomorphins when inoculated and grown in milk. β-Caseinomorphins are resistant to enzymes of the gastrointestinal tract and have been detected in vivo in the intestinal chyme of mini pigs [55] and human small intestines. Because their absorption in the gut has not been observed in adults, it is generally concluded that the physiological influences are limited to the gastrointestinal tract with important effects on intestinal transit time, amino acid uptake, and water balance. Once they enter the bloodstream, they are rapidly degraded. In contrast, passive transport of β-caseinomorphins across intestinal mucosal membranes does occur in neonates, which may experience physiological responses such as an analgesic effect on the nervous system resulting in calmness and sleep in infants. A precursor of β-casomorphin was
reported in the plasma of newborn calves and infants after ingestion of bovine milk. In pregnant or lactating women, β-casomorphins originate in the milk pass through the mammary tissue, and possibly influence the release of prolactin and oxytocin. More recently, it was shown that many peptides derived from αs1-, β-, or κ-CN, and κ-caseinoglycomacropeptide can be detected in the stomach of adults after consumption of milk or yogurt [54].

Casomorphins, as opioid ligands, modulate social behavior increase analgesic behavior prolong gastrointestinal transient time by inhibiting intestinal peristalsis and motility, exert anti-secretary (anti-diarrheal) action, modulate amino acid transport, and stimulate endocrine responses such as the secretion of insulin and somatostatin. Opioid-like milk peptides also play a regulatory role regarding appetite by modifying endocrine activity of the pancreas, resulting in an increase of insulin output. Presently, data suggest that intracerebro ventricular β-casomorphin1-7 stimulates uptake of a high fat diet in rats fasted overnight. Enterostatin inhibited this effect, as did naloxone, a general opioid antagonist. Ligand binding studies indicated that at high dosages, β-casomorphin1-7 andenterostatin may interact with the same low affinity receptor to modulate intake of dietary fat [55].

c. Opioid antagonists

Opioid antagonists suppress the agonist activity of enkephalin. Mensink et al., 1992 [56] reported that a chloroform and methanol extract from a peptic digest of bovine κ-CN bound to opioid receptors of rat brain. The peptide was methylated at the C-terminal end and exhibited antagonist effects selective for the μ- and κ-type of opioid receptor. The peptide was thus named casoxin. Casoxins A and B have been chemically synthesized and correspond to amino acid sequences within both bovine and human κ-CN. Casoxin C is an opioid antagonist, obtained from tryptic digests of bovine κ-CN, that also functions as an agonist for C3a receptors. Lastly, casoxin D, purified from human αs1-CN fractions, elicits an opioid antagonist response. In general, the chemically
modified casoxins are more active than their non-methylated derivatives. Lactoferroxins are antagonists generated from human lactoferrin. Initially, a chloroform and methanol extract from a peptic digest of lactoferrin was assayed for activity, and the results indicated that the opioid properties were similar to those of naloxone, a known antagonist ligand. Peptides derived from pepsin digestion, alone, were minimally effective, while those purified from a methyl-esterified fraction were significantly more potent. HPLC analyses resulted in purification of three separate active fractions designated lactoferroxin A, B, and C, respectively. It was determined that the α-carbonyl group of each was methyl esterified based on comparison of bioactivity measurements and HPLC retention times to those of corresponding synthetic peptides. Like casoxins, the chemically modified peptides may not actually exist in vivo. Lactoferroxin A, residues 318 to 323, showed a preference for μ-receptors. On the other hand, lactoferroxin B and C, derived from residues 536 to 540 and 673 to 679, respectively, exhibited a higher propensity for κ-receptors.

1.7.6 Miscellaneous Peptides:

Physiologically active peptides that directly affect gastrointestinal functions have also been documented. Casomorphins slow gastric motility and emptying in non-ruminants, while caseinomacropeptide, a 64-amino acid glycopeptide released from κ-CN by gastric proteases, exerts its effects on digestive function by inhibiting gastric acid secretions. Several other milk-derived peptides have been described in the literature. Atrial natriuretic factor, or atriopeptin, is a peptide found naturally occurring in human milk. This peptide functions as a strong diuretic, natriuretic, and vasorelaxant, and plays an important role in circulatory adaptation to extrauterine life. More recently, a peptide, obtained by in vitro proteolysis of bovine β-LG, was found to exert its effect on smooth muscle.
1.8 Global probiotic food market in the industrialized nations:

The most active area within the functional foods market in Europe has been probiotic dairy products, in particular, probiotic yogurts and milks. In 1997 these products accounted for 65% of the European functional foods market, valued at US$889 million, followed by spreads, valued at US$320 million and accounting for 23% of the market. Probiotic dairy products are expected to command the highest market share among all the probiotic foodstuffs, accounting for almost 70% in the year 2009 and reaching a market size of almost $24 billion by the end of 2014. The biggest markets for these products are Europe and Asia; the U.S. market has slowly but surely opened up to these products in the recent past and is expected to grow at a CAGR of 17% from 2009 to 2014, the biggest contributor being probiotic cultured drinks followed by probiotic yogurts. Though the market base of probiotic products is comparatively lesser in the US, the market is expected to grow at an astounding rate of almost 14% in the same period driven by the large scale acceptance of - the probiotic yogurts in spoonable single serve packs, probiotic cultured drinks in single shot packaging form and probiotic dietary supplements.

1.9 Indian probiotic market:

Indian probiotic market is valued at $2 million as per 2010 estimates and it is poised to quadruple by 2015 due to the advent of Indian and Multinational companies coming in to the fray. With their advent, the Indian probiotic market turnover is expected to reach $8 million by the year 2015. The existing probiotic market in India majorly comprises of three segments, urban chain, young adults and people with special needs such as pregnancy, lactation, immunodeficiency, geriatric etc… India at present accounts for less than 1% of the total world market turnover in the probiotic industry and it is a huge deficit considering the fact that India has the highest cattle population and India being the world’s highest milk producer.
Probiotics in India generally comes in two forms, milk and fermented milk products with the former occupying 62% of the market share and later having 38% market share (Indian consumer survey, 2010). Indian probiotic products currently are Dahi (Indian yoghurt), flavoured milk and butter milk. Major pharmaceuticals companies have become active in this space and are devising newer drugs and products, however current drugs are predominant in the area of nutraceuticals.

1.10 Current players in Indian probiotic market:

1.10.1 Yakult Danone:

Yakult Danone India Pvt Ltd (YDIPL), is a 50:50 joint venture between Japan’s Yakult Honsha and The French- Danone Group, with its offering Yakult, a probiotic drink made from fermented milk, *lactobacillus* and some sugar. Yakult is a world leader in probiotic drinks and has a rich heritage dating back to 1935. Yakult was launched in India in the late 2007. The brand was initially available only in Delhi. Now Yakult is being launched nationally in a phased manner. Yakult is fermented milk that contains healthy bacteria *Lactobacillus casei* strain Shirota. According to the brand site, a 65 ml Yakult bottle contains 6.5 bn probiotic bacteria.

Yakult has been testing its marketing strategy for around a year and is now ready for the national roll out. The brand is currently available in Delhi, Mumbai, Chandigarh and Jaipur. The entry of Yakult is expected to increase the visibility and growth of probiotic category in India. Yakult is also available in supermarkets. Another interesting fact is about the pricing strategy of Yakult. The 65ml bottle is priced at Rs 10 and the product is available in a pack of 5. The price sounds reasonable for those consumers who are health conscious. The main challenge for this product is to make the consumers believe that the product is delivering benefit to them. Most of the health foods have the problem of giving measurable visible results to the consumers. Yakult primarily targets those consumers who are health conscious and is aware of the importance of functional
foods like probiotics. It has adopted the right marketing strategy to educate the consumers and also encourage them to make regular use of this product.

1.10.2 Amul:

Amul was the first to foray into the category with its probiotic ice creams Prolife in February 2007. Amul, on the other hand, having tasted success in the probiotics category with its ice cream in February earlier this year, is already in the process of test-marketing pouched lassi (sweetened curd) in Gujarat and some parts of Maharashtra, with plans of introducing it in the other parts of the country soon. Probiotic products contribute to 10 per cent to its ice-cream sales and 25 per cent of its Dahi (Indian yoghurt) sales.

1.10.3 Nestle:

Nestle, having recently declared dairy as its key area of growth, is all set to introduce probiotics in its other dairy products as well. The total packaged curd market in India is estimated at 40,000-60,000 tons per annum, of which Nestle has a 30 per cent market share. Internationally, the average contribution of probiotic products to total dairy products is estimated between 10-20 % depending on the country and business. Nestle also has introduced flavoured milk varieties of probiotic nature.

1.10.4 Mother dairy:

Mother Dairy – Delhi was set up in 1974 under the Operation Flood Programme, a wholly owned subsidy of the National Dairy Development Board (NDDB), whose current chairman is Dr. Amrita Patel. Currently, it is one of the largest milk (liquid/unprocessed) plants in Asia selling more than 25 lakh liters of milk per day, thereby enjoying a market share of 66% of the branded milk sales in New Delhi, capital of India. Other important markets include Mumbai, Saurashtra and Hyderabad. Mother dairy ice-cream was launched in the year 1995 and has shown continuous growth over the years, and today it boasts approximately 62% market share in Delhi and NCR. b-Activ Probiotic Dahi, b-
Activ Probiotic Lassi, b-Activ Curd and Nutrifit (Strawberry & Mango) are the company’s probiotic products. Probiotic products are contributing to 15 per cent of the turnover of their fresh dairy products. Over the next 3-4 years, the contribution is expected to go up to 25 per cent and the urban acceptance is making the company to increase their focus on probiotic products.

11 Fermented milk:

Historically, the seasonal variation in milk production made it necessary to preserve milk. The Nordic countries including Iceland have a long tradition for using fermented milk, and the consumption of fermented milk is about 20 kg per person. During fermentation bacteria and yeasts convert lactose in the milk to various degradation products depending on the species present. Lactobacilli and Streptococci give rise to lactic acid and monosaccarides (especially galactose). Bifidobacteria give rise to lactic acid, acetic acid and monosaccarides, while yeasts, present only in some few fermented milk products, produce CO₂ and ethanol. Different bacteria may be used for fermentation, giving products of special flavour and aroma, and with several potential health beneficial metabolites. The bacteria contain cell wall components that bind Toll-like receptors on dendritic cells (and also other leucocytes) found in the mucosa of the small intestine and colon, thus stimulating the Th1 immune response [57].

It has been shown that fermented milk stimulates the Th₁ immune response, and down-regulates the Th₂ immune response. The immune system may thus be strengthened against cancer, virus infections and allergy. Bacterial DNA has also a similar effect, binding to Toll-like receptor-9. Some bacteria can also improve the intestinal microbial balance, and the fermented milk may have positive health effects both in the digestive channel and in metabolism. During the fermentation of milk, lactic acid and other organic acids are produced and these increase the absorption of iron [57]. If fermented milk is consumed at mealtimes, these acids are likely to have a positive effect on the absorption of iron from other foods. Lactic acid is also a poorer substrate for growth of pathogenic
bacteria than glucose and lactose. The low pH in fermented milk may also delay the gastric emptying from the stomach into the small intestine and thereby increase the gastrointestinal transit time. Also, full-fat milk has been shown to increase the mean gastric emptying half-time compared to half-skimmed milk, and accordingly it might be favourable to gastric emptying and thus may have an effect on appetite regulation.

1.12 Motivation and Problem statement:

Dairy industry is one of the biggest consumer oriented industries in India. Our country being the largest producer of milk and having the highest cattle population is poised for greater growth in the near future. The potential of peptides found in milk and fermented milk has been little utilized and the distinction of milk and fermented milk peptides has not been done entirely. Fermented milk peptides hold a lot of potential in treatment of various gut oriented ailments for which antibiotics are extensively used now. Antibiotics produce a lot of side effects which is totally absent in the case of fermented milk peptides. CPP have been largely used as an anti-hypertensive, but CPP can also be used as an Immunomodulatory agent enhancing the immune system of the humans. CPP can also be used as an anti-genotoxic agent who has anti-genotoxic property. Establishment of these facts about the fermented milk peptides can direct the change over in antibiotics based medicinal system for various diseases and also will serve as an anti-genotoxic agent against low ionizing radiation.