BIOACTIVE PEPTIDES FROM FERMENTED MILK

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CERTIFICATE

This is to certify that the project entitled **"Bioactive peptides from fermented milk products"** which is being submitted by **Subhanshi Agarwal and Neha Sharma** in partial fulfillment for the award of degree of B. Tech in Biotechnology from Jaypee University of Information Technology is the record of candidate's own work carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Gunjan Goel

Dr. Gunjan Goel

Designation: Sr lecturer

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Summary

Viability and metabolic activities are important characteristics of probiotic lactic microorganisms. They give rise to therapeutic benefits as well as increase physiological activity of cultured products through liberation of a number of bioactive peptide. The main focus of this project was to assess the performance of lactic cultures as single strain starters and in mixed strain in regard to viability, metabolic activity and ability to produce bioactive compounds. For this, five lactic cultures namely, *Lb. casei, Lb rhamnosus, Lb. delbrueckii ssp. Bulgaricus, S. thermophilus* and *Lb. helveticus* were assessed for their performance in skimmed milk as fermentation media. The fermentation was carried out for different time intervals of 6h upto 24 h to achieve maximum free amino acid content and proteolysis. Most of the lactic cultures attained the similar growth pattern, the cfu counts ranging from 10^8-10^9cfu/ml with a pH drop of 2.0 and acidity in range of 0.7-0.8% (lactic acid equivalent), except *Lb rhamnosus* which was found to be weak acid producer. The yoghurt starter (*Lb. delbrueckii ssp bulgaricus* and *S.thermophilus*) in combination of 1:1 were found be the fastest acid producer with maximum proteolysis (255µg/ml of serine equivalent). The skimmed milk hydrolysate obtained via fermentation of yoghurt starter showed the highest radical scavenging activity (44%) as estimated by DPPH method. The fraction also possess antidiabetic potential when assessed by inhibition of α - amylase (48%). Most of the skimmed milk hydrolysate possessed antibacterial activity against Gram positive bacteria.

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Chapter 1

Introduction

The role of proteins as physiologically active components in the diet is being increasingly acknowledged. Many of the proteins that occur naturally in raw food materials exert their physiological action either directly or upon enzymatic hydrolysis in vitro or in vivo. In recent years it has been recognized that dietary proteins provide a rich source of biologically active peptides. Such peptides are inactive within the sequence of the parent protein and can be released in three ways: (a) through hydrolysis by digestive enzymes, (b) through hydrolysis by proteolytic microorganisms and (c) through the action of proteolytic enzymes derived from microorganisms or plants.

Bioactive peptides generally consist of between 3 and 20 amino acids and are encrypted within the primary structure of a dietary protein. It is now well established that physiologically active peptides are produced from several food proteins during gastrointestinal digestion and fermentation of food materials with lactic acid bacteria.

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health. Upon oral administration, bioactive peptides, may affect the major body systems— namely, the cardiovascular, digestive, immune and nervous systems depending on their amino acid sequence. For this reason, the potential of distinct dietary peptide sequences to promote human health by reducing the risk of chronic diseases or boosting natural immune protection has aroused a lot of scientific interest over the past few years.

These beneficial health effects may be attributed to numerous known peptide sequences exhibiting, e.g., antimicrobial, antioxidative, antithrombotic, antihypertensive and immunomodulatory activities. The activity is based on their inherent amino acid composition and sequence. The size of active sequences may vary from two to twenty amino acid residues, and many peptides are known to reveal multifunctional properties.

Today, milk proteins are considered the most important source of bioactive peptides and an increasing number of bioactive peptides have been identified in milk protein hydrolysates and fermented dairy products.

Increased public consciousness of diet related health issues has resulted in a consumers' orientation towards healthy foods. Numerous scientific studies have confirmed that many chronic

osteoporosis, cancer, coronary heart diseases and hypertension are linked to furthermore, some reported that milk and other dairy products have long been significant component of a balanced diet. Milk is a natural source which contains ingredients and biologically active compounds with potential health.

studies have reported that individuals who constantly consumed milk were after from heart attack than those who did not .Similarly some other studies who consumed dairy products had a lower incidence of diabetes type II. have been recognized as one of the most significant sources of bioactive

peptides with potent physiological activities may be liberated from milk proteolytic enzymes in the gut and thus influence the major body's nervous, digestive, cardiovascular and immune systems .

been isolated from many protein sources such as soy proteins, gelatine, gut milk proteins appear to be the most important sources of bioactive Milk is an excellent source of highly valuable proteins which are in teins and whey proteins. Caseins and whey proteins comprise g%, respectively, of total milk proteins. Numerous health advantages active peptides have been claimed for commercial interests in the ining-functional foods defined bioactive peptides as substances that cesses of the body functions with beneficial effects.

most important starter cultures used in traditional fermented milk ation mainly stems from two important properties: rapid utilization ing to fast acidification of milk as growth medium, and highly capable of supplying essential amino acids required by a fast of small and oligo-peptides with different physiological functions roteins through microbial proteolysis and has been well recognized scientific studies have confirmed 16 *L. helveticus* strains, in antihypertensive peptides from milk proteins, including Val-Pro-

Pro (VPP) and Ile-Pro-Pro (IPP) with demonstrated *in vivo* antihypertensive activity in a rat model and human studies. However limited work has been done on yoghurt starters.

Therefore, this investigation was carried out with following objectives:

1) Optimization of fermentation of skimmed milk with different lactic cultures
2) Functional attributes of skimmed milk hydrolysate.

CHAPTER 2

Literature Review

2.1. Bioactive peptides

2.1.1. Definition

Accordingly to a widely shared definition, a bioactive dietary substance is "a food component that can affect biological processes or substrates and, hence, have an impact on body function or condition and ultimately health".

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health. Upon oral administration, bioactive peptides, may affect the major body systems namely cardiovascular, digestive, immune and nervous systems. The beneficial health effects may be classified as antimicrobial, ant oxidative, antithrombotic, antihypertensive, antimicrobial or immunomodulatory. The activity of these bifunctional peptides is based on their inherent amino acid composition and sequence. The size of active sequences may vary from 2-20 amino acid residues.

2.2. Mechanisms of production of bioactive peptides

Milk-derived bioactive peptides, and more generally food bioactive peptides, are usually composed of 2-20 amino acids and become active only when they are released from the precursor protein where they are encrypted.

Different mechanisms can release the encrypted bioactive peptides from the precursor proteins:

(a) enzymatic hydrolysis by digestive enzymes

(b) food processing and

(c) proteolysis by enzymes derived from microorganisms or plants.

2.2.1. Bioactive peptides produced by Enzymatic hydrolysis

Bioactive peptides may be released in vivo during gastrointestinal digestion. These bioactive peptides are mostly the result of the degradation of casein with several proteases such as pepsin, trypsin or chymotrypsin. The peptide products resulting from milk proteins digestion with site-specific pancreatic proteases, such as trypsin or chymotrypsin are well investigated.

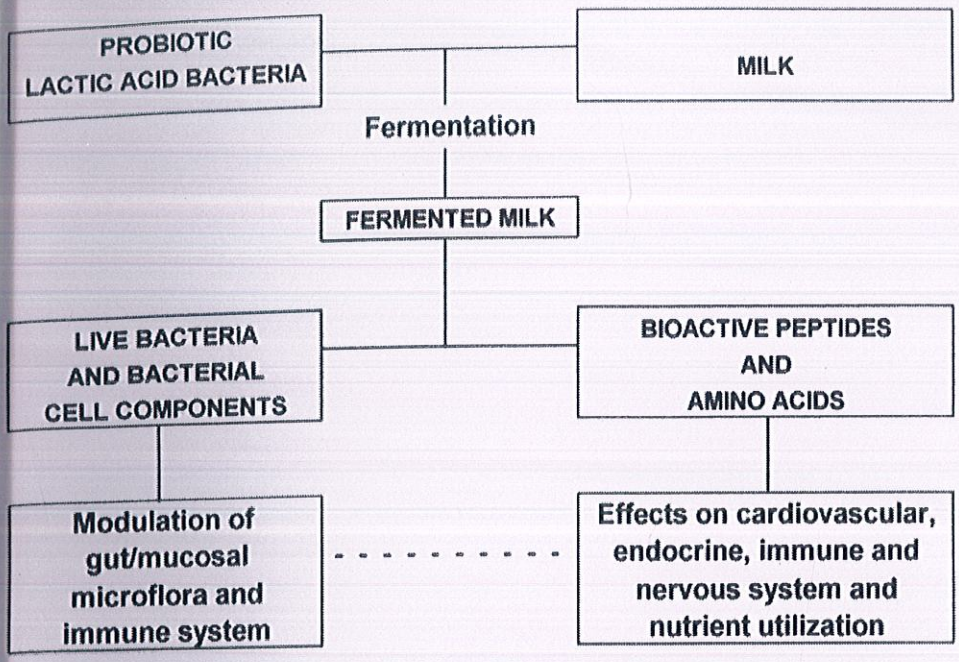


Fig.2.1 Bioactive peptides production by Fermentation.

The cleavage of latent bioactive peptides from milk proteins normally occurs during digestion by pepsin and pancreatic enzymes (trypsin, chymotrypsin, carboxy and amino peptidases), producing active peptide fragments in the gastrointestinal tract of the milk-consuming individual. The physiological effects of bioactive peptides depend on their ability to reach their target sites intact, which may involve absorption through the intestinal epithelium prior to travel to the peripheral organs.

Many of the known bioactive peptides have been produced *in vitro* using gastrointestinal enzymes, usually pepsin and trypsin. ACE-inhibitory peptides and CPPs, for example, are most commonly produced by trypsin. Other digestive enzymes and different enzyme combinations of proteinases including alcalase, chymotrypsin, pancreatin, pepsin and thermolysin as well as enzymes from bacterial and fungal sources have also been utilized to generate bioactive peptides from various proteins.

2.2.2. Bioactive peptides produced by Food Processing

The structural and chemical changes that occur during the processing of food proteins may result in the release of bioactive peptides. In particular, heat and/or alkali treatment can generate additional inter-and intramolecular covalent bonds that are resistant to hydrolysis. Such processing conditions also promote the racemic conversion of L-amino acids to D-isomers and consequently, lead to indigestible peptide bonds. The potential formation of indigestible peptide sequences during food processing is noteworthy, because this may promote both formation and absorption of bioactive peptides that do not occur naturally in the precursor protein. Such bioactive peptides can be generated during manufacture of several milk products and may thus be ingested as food components.

For example, partially hydrolyzed milk proteins for hypoallergenic infant formulae and for clinical applications in nutrition consist exclusively of peptides and contain bioactive peptides. Cheese contains phosphopeptides as natural constituents and secondary proteolysis during cheese ripening leads to formation of various ACE inhibitory peptides.

2.2.3. Bioactive Peptides produced by Microbial fermentation

Many industrially utilized dairy starter cultures are proteolytic to some extent. Bioactive peptides can, thus, be generated by the proteolytic activities of the strains of starter and non-starter bacteria e.g. *Lactobacillus helveticus, Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus, Lactococcus lactis, Streptococcus thermophilus and Lactobacillus casei* used in the manufacture of fermented dairy products. The proteolytic system of lactic acid bacteria (LAB) is well characterized. This system consists of a cell wall-bound proteinase and a number of distinct intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases and dipeptidases. Extracellular proteinases cause degradation of casein into oligopeptides. The longer chain oligopeptides may be a source of bioactive peptides when further degraded by intracellular peptidases of lysed-lactic acid bacteria.

The single most effective way to increase the concentration of bioactive peptides in fermented dairy products is to ferment or co-ferment with highly proteolytic strains of LAB. The choice of

strains influences the release of effective bioactive peptides. The strain should not be too proteolytic otherwise the product will be destroyed and must have the right specificity to give high concentrations of active peptides. Various bioactive peptides including ACE-inhibitory or Antihypertensive peptides, Anti-diabetic, Immunomodulatory, Antioxidative and Antimicrobial peptides have been released from milk proteins through microbial proteolysis .The best known ACE-inhibitory peptides, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), have been identified in milk fermented with strains of *Lb. helveticus* and *Saccharomyces cerevisiae.* In addition to live microorganisms, proteolytic enzymes isolated from LAB have been successfully employed to release bioactive peptides from milk proteins.

2.3. Sources of Bioactive peptides

2.3.1. Milk

Milk is an excellent source of well balanced nutrients and also exhibits a range of biological activities that influence digestion, metabolic responses to absorbed nutrients, growth and development of specific organs, and resistance to disease. These biological activities are mainly due to the peptides and proteins in milk. However, some of the biological activity of milk protein components is latent, and is released only upon proteolysis action. Bioactive peptides are produced during digestion of milk in the gastrointestinal tract, and also during fermentation and food processing.

Milk is a rich source of protein. Casein and whey proteins are the two main protein groups in milk, caseins comprises about 80 percent of the total protein content in bovine milk and are divided into α-, ß- and κ-caseins. Whey protein is composed of *ß*-lactoglobulin, α-lactalbumin, immunoglobulins (IgGs), glycomacropeptides, bovine serum albumin, and minor proteins such as lactoperoxidase, lysozyme and lactoferrin. Each of the subfractions found in casein or whey has its own unique biological properties. Milk proteins can be degraded into numerous peptide fragments by enzymatic proteolysis and serve as source of bioactive peptides. It has been well established that a number of *Lactobacillus* sp. grow well in skim milk (Gilbert et al., 1996)

It is now well documented that bioactive peptides can be generated during milk fermentation by the proteolytic activity of starter cultures. As a result, peptides with various bioactivities can be found in the end-products, such as various cheeses and fermented milks. These traditional dairy products may under certain conditions have specific health effects when ingested as part of the daily diet.

Table 2.1 Examples of the identified bioactive peptides in fermented milk and their corresponding physiological activity

Micro-organisms used	Precursor proteins	Peptide sequence	Bioactivity	References
Lb. helveticus	Beta-cn	Lys-Val-Leu-Pro-Val-Pro-(Glu)	ACE inhibitory	Maeno et al. (1996)
Lb.helveticus	Whey proteins	Try-Pro	ACE inhibitory	Yamamomoto et al. (1999)
Lb. delbrueckii subsp.*bulgaricus*	Beta-cn	Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile	ACE inhibitory	Ashar and Chand (2004)
Streptococcus thermophilus+Lc. lactis subsp.lactis biovar.*diacetylactis*	Beta-cn	Ser-Lys-Val-Tyr-Pro	ACE inhibitory	Pan et al. (2004)

References: Korhone & Pihlanto. Bioactive peptides: Production and functionality (2006).

Table 2.2 Occurrence of peptides in fermented milk products:

Product	Examples of identified bioactive peptides	Bioactivity	References
Sour milk	b-cn f(74–76, f(84–86), k-cn f(108–111)	Antihypertensive	Nakamura et al. (1995)
Emmental Fermented milks	Active peptides not identified	ACE inhibitory	Parrot et al. (2003)
Yoghurt	Active peptides not identified	Weak ACE-inhibitory	Meisel et al. (1997)
Dahi	Ser-Lys-Val-Tyr-Pro	ACE inhibitory	Ashar and Chand (2004)
Gouda	as1-cn f(1–9), b-cn f(60–68)	ACE inhibitory	Saito et al. (2000)

Ref: Korhone & Pihlanto. Bioactive peptides: Production and functionality. (2006)

The occurrence of various bioactive peptides in fermented milks, e.g. yoghurt, sour milk and ''Dahi'', has been reported in many studies, as shown in Table 2. ACE inhibitory, immunomodulatory and opioid peptides, have been found in yoghurt and in milk fermented with a probiotic *Lb. caseissp. rhamnosus* strain. Most studies have employed strongly proteolytic *Lb. helveticus* strains for the production of antihypertensive peptides in fermented milk products . At present, at least two fermented sour-milk products containing the ACE-inhibitory tripeptides VPP and IPP have been launched commercially in Japan and Finland, respectively.

Table 2.3 Commercial dairy products with health or function claims based on bioactive peptides.

Brand name	Claimed functional bioactive peptides	Health/function claims	Manufacturers
Calpis	Val-Pro-Pro, Ile-Pro-Pro,derived from b-casein and k-casein	Reduction of blood pressure	Calpis Co. Japan
BioZate	b-lactoglobulin fragments	Reduction of blood pressure	Davisco, USA
C12	Casein derived peptide	Reduction of blood Pressure	MV International, Netherlands
Vivin Alpha	Whey derived peptide	Aids relaxation and sleep	Borculo Domo Ingredients, Netherland

Ref: Korhone & Pihlanto. Bioactive peptides: Production and functionality (2006).

The Japanese product ''Calpis'' is fermented with a culture containing *Lb. helveticus* and *S.cerevisiae* and the Finish product ''Evolus'' contains the same tripeptides produced by *Lb.helveticus*. In animal model studies, single oral administration of these products has been shown to have an antihypertensive effect and ''Evolus'' has also been demonstrated to prevent the development of hypertension.

An increasing number of ingredients containing specific bioactive peptides based on casein or whey protein hydrolysates have been launched on the market within the past few years or are currently under development by international food companies. Such peptides possess anticarcinogenic , antihypertensive , mineral-binding and stress-relieving properties.

2.3.2. Soybeans

Soybean-based foods contain an array of biologically active compounds that can confer important health benefits such as antioxidant effects (Setchell, 1998; Tsai & Huang, 1999). These phytochemicals include saponins, phytates, protease inhibitors, phenolic acids, and lecithin, all known for their anticancer potential (Cohen et al., 2000; Messina & Flickinger, 2002); phytosterols, which have hypocholesterolemic effects; isoflavones, which are known for several health benefits (Fukui et al., 2002); and omega-3 fatty acids, which have well recognized cardioprotective effects. Among these compounds, isoflavones have attracted the most attention.

The proteins β-conglycinin (7S globulin) and glycinin (11S globulin) constitute up to 90% of the total soy protein (Gianazza et al., 2003). Evaluation of these dietary proteins is very interesting because their hydrolysis by proteases produces peptides with biological activities.

Soybean, an important source of food proteins, has received increasing interest from the public because of its reported health benefits. These health benefits are attributed to its components, including isoflavones, saponins, proteins, and peptides. Lunasin, Bowman-Birk inhibitor, lectin, and 3-conglycinin are some of the biologically active peptides and proteins found in soybean.

Hydrolysates of soy proteins contain antioxidant peptides (Chen et al., 1998). These peptides show high activity against the peroxidation of linoleic acid, paraquat-induced oxidative stress in rats, and scavenging effects on peroxynitrite, active oxygen, and free radical species (Takenaka et al., 2003). They may therefore help prevent some free radical-related diseases.

Soybean is a valuable source of inhibitors of the angiotensin-converting enzyme (Ahn et al.,2000; Shin et al., 2001). Inhibitors of this enzyme are now widely used as antihypertensive agents, causing a fall in blood pressure comparable to that produced by thiazides, and calcium antagonists (Pool et al., 1989). Many peptides isolated by the hydrolysis of food proteins have inhibitory activity against the enzyme and reduce blood pressure after oral administration (Ahn et al., 2000; Je et al., 2004; Yamamoto, 1997). Daily use of food with such peptides may be effective in maintaining blood pressure at the normal level.

2.3.3. Fish

The muscles, skeleton, skin, and internal organs of fish can be used as a source of biologically active peptides. Enzymatic hydrolysis of these materials can provide biologically active peptides with different physicochemical properties. These peptides can demonstrate antihypertensive, anticoagulant, immunomodulative, antioxidant, and other pharmacological properties.

Peptides extracted from the protein hydrolyzate of walleye pollock (*Therragra chalcogramma*) skeleton were shown to significantly inhibit the effects of the angiotensin I converting enzyme (ACE). An enzymatic hydrolysis of the fillet of the Pacific hake, *Merluccius productus*, using Protamex protease yields a protein hydrolyzate that has an inhibitive activity against ACE in vitro. An enzymatic alkaline hydrolysis of proteins from fillets of catfish yielded several fractions of hydrolyzates with different degree of hydrolysis. The anticoagulant and antithrombotic effects of fish protein hydrolyzates found in experiments in vitro indicated that the fish peptides may affect blood coagulation at the stage of the activation of the coagulation pathway .

A peptide fraction with a molecular weight of approximately 10 kDa isolated from the protein hydrolyzate of cod skeletons demonstrated high antioxidative activity. Antiskeletons of yellow fin sole, *Limanda aspera* ,tuna and the hoki, *Johnius belengerii*.

2.4. Properties of lactic acid bacteria

The lactic acid bacteria are defined as Gram-positive cocci or rods with a low-GC. These are acid-tolerant, generally non-spore forming bacteria and associated by their common metabolic and physiological characteristics. Furthermore, LAB can be found in spoiling plants and lactic products which produce lactic acid as the major metabolic end product as a result of carbohydrate fermentation. Several strains of LAB produce proteinaceous bacteriocins that create an additional hurdle for spoilage and pathogenic microorganisms.

Moreover, it seems that lactic acid and other metabolic activity products contribute to the organoleptic and textural profile of a food item. Due to their ubiquitous presence in fermented foods and their contribution to the intestinal microflora of human mucosal surfaces, the industrial importance of the LAB is more manifested by their generally recognized as safe (GRAS) status.

The genera that comprise the LAB are at its core *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus* as well as the more peripheral *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*; these belong to the order *Lactobacillales* (Sonomoto, 2011).

Two main pathways for hexose fermentation have been used to classify LAB genera. Under certain conditions (excess glucose and limited oxygen), homolactic LAB catabolize one mole of glucose in the Embden-Meyerhof-Parnas path to produce two moles of pyruvate. Intracellular redox balance can be maintained through the oxidation of NADH, associated with pyruvate reduction to produce lactic acid. This process yields two moles of ATP per 33 mole of glucose consumed. Representative homolactic LAB genera include *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*.

Heterofermentative LAB use the second pathway which is called pentose phosphoketolase pathway. In this pathway, one mole of glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and afterward decarboxylated to yield one mole of CO2. Pentose-5-phosphate formed is split into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP also subjected to further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. In theory, end-products (including ATP) are produced in equimolar quantities from the catabolism of one mole of glucose. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, *Weissella* (Sonomoto, 2011).

2.5. Proteolytic activity of LAB

Lactic acid bacteria isolated from fermented dairy products, need from 4 up to 14 amino acids for growth depending on the strain (Chopin, 1993). It has been confirmed that the quantity of free amino acids and short peptides in milk is very low. Therefore, LAB use developed proteolytic system allowing for degradation of milk proteins for their growth (Juillard et al., 1995b). Caseins are composed of all amino acids required for the growth of 37 lactic acid bacteria in milk to high cell density. Nevertheless, only less than 1% of the total casein

constituents, is actually required (Kunji et al., 1996). It has been well established that a number of *Lactobacillus* sp. grow well in skim milk (Gilbert et al., 1996b).

Amino acids and peptides produced by enzymatic hydrolysis of milk proteins by LAB proteolytic system and utilization of these amino acids are a central and integral part of their metabolic activity. During fermentation, milk cannot supply all essential amino acids required for LAB growth in free form therefore, LAB have developed ability to degrade milk proteins, mainly caseins, by their proteolytic system producing initially peptides, and then amino acids needed for their growth (Savijoki et al., 2006). Milk proteins during fermentation are subjected to slight proteolytic degradation resulting in a number of potentially bioactive peptides which may vary between 2-20 amino acid residues.

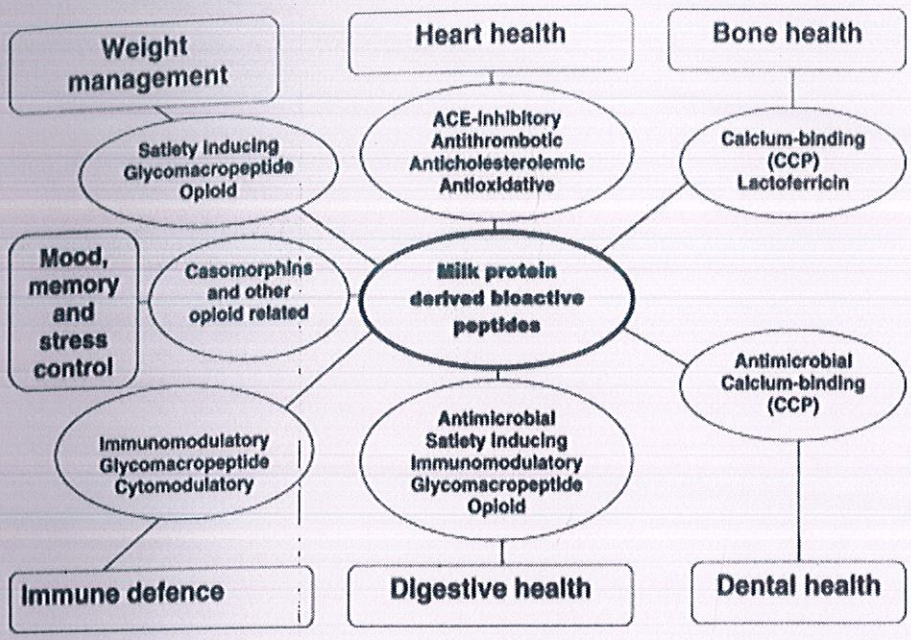
Lactobacillus helveticus have been especially reported as a species with very high proteolytic and peptidolytic activity in comparison to other LAB strains presumably due to the structure of their proteolytic system consisting of proteases, peptidases and a transport system.

2.6. Physiological functions of dairy derived bioactive peptides

Fermented dairy foods, in addition to providing energy and nutrients, also are a source of physiologically important peptides that have a positive impact on body's functions. These potential health benefits may be due to the production of microbial metabolites, such as cell wall components (Nakajima et al., 1995), bacteriocins (Hernández et al., 2005) and the hydrolysis of cell-free extracts containing proteinase and peptidase activities on milk proteins substrates (Pan et al., 2005).

They have been found to have specific activities, such as antihypertensive, antioxidative, antimicrobial, immunomodulatory, opiod or mineral-binding activities.

Fitzgerald and Meisel (2003) reported that some of the well-recognized activities of known bioactive peptides are antihypertensive and immunomodulatory activities. Moreover, risks of acquiring some of chronic diseases and metabolic disorders that are associated with unbalanced diet may be reduced by consumption of fermented dairy products. Some of the explored risk lowering effects involved cancer, osteoporosis, coronary heart diseases, hypertension and obesity.



.4 Physiological functions of milk derived bioactive peptides.

l. Anti-microbial peptides

nicrobial peptides (also called host defense peptides) are an evolutionarily conserved ›onent of the innate immune response and are found among all classes of life. These des are potent, broad spectrum antibiotics which demonstrate potential as novel therapeutic s. Antimicrobial peptides have been demonstrated to kill Gram negative and Gram positive ›ria (including strains that are resistant to conventional antibiotics), mycobacteria ıding *Mycobacterium tuberculosis*), enveloped viruses, fungi and even transformed or ›rous cells.

›ad spectrum of antimicrobial proteins protects the gastrointestinal tract against pathogenic ria and viruses. They act indirectly by stimulating the growth of beneficial microorganisms › gut or directly by exerting an antimicrobial activity or neutralizing the mechanisms of ıment or invasion of pathogens. Further, bioactive proteins can inhibit the growth of ›gens by withholding nutrients which are essential for the proliferation of bacteria.

›ferrin is an iron-binding glycoprotein that forms the antibiotic fragment lactoferricin during tion. Another mechanism of the antimicrobial activity of lactoferrin is the growth inhibition

due to vasoconstricting action . Inhibition of ACE exerts an antihypertensive effect through a decrease of angiotensin II and an increase of bradykinin.

The primary structural feature governing this inhibitory response is the C-terminal tripeptide sequence and thus these peptides may interact with subsites at the active site of ACE (Hong et al., 2008). ACE preferentially interacts with substrates and inhibitors containing hydrophobic amino acid residues in the three C-terminal positions (Cheung et al., 1980). The positive charge of Arg or the ε-amino group of Lys at the C-terminus has also been shown to contribute to the ACE-inhibitory potential of several peptides (Cheung et al., 1980).

Lactic acid bacteria possess the proteolytic system that hydrolyses milk proteins which present a potential for release of ACE-inhibitory peptides (Yamamoto et al., 1993).

2.6.3. Anti-Oxidative Peptides

Antioxidant activity of bioactive peptides can be attributed to their radical scavenging, inhibition of lipid peroxidation and metal ion chelation properties of peptides. It also has been proposed that peptide structure and its amino acid sequence can affect its antioxidative properties.

Antioxidants may function by preventing the formation of radicals or by scavenging radicals or hydrogen peroxide and other peroxides. Milk contains several antioxidant factors, like vitamins and enzymes. Peptides generated from the digestion of milk proteins are reported to have antioxidative activities. Milk-derived antioxidative peptides are composed of 5–11 amino acids including hydrophobic amino acids, proline, histidine, tyrosine or tryptophan in the sequence. Antioxidant activity of the hydrolysates seems to be inherent to the characteristic amino acid sequences of peptides derived, depending on the protease specificity.

Oxidative metabolism is crucial for the survival of human cells (Pihlanto, 2006a). However, the risk associated with this activity is that the production of free radicals may cause oxidative changes. Free radicals have also been linked with many other pathological conditions such as atherosclerosis, diabetes, rheumatoid arthritis. Inhibition of the free radicals formed in the living body and foodstuff is an important way to protect body from these serious diseases.

Many artificial antioxidative agents are prohibited in some countries because of the risk associated with their consumption (Pihlanto, 2006). Peptide produced from β-casein f (177–183),

for example, which known asACE-I, it has been reported to have antioxidant activity . Furthermore, a potent antioxidant activity was found in the peptide Tyr–Phe–Tyr–Glu-Pro–Leu. Casein hydrolysates were reported to have higher concentration of histidine, lysine, proline and tyrosine, which are able to react with free radical and serve as scavengers (Suetsuna et al., 2000). However, few antioxidant peptides have been observed in microbial fermented milk.

2.6.4. Anti-Diabetic peptides

From time to time, research suggests that consumption of milk and dairy products may help prevent weight gain or obesity or even prevent type 2 diabetes. Whey protein accounts for 30 percent of total protein in milk and the remaining 70 percent is casein. Whey protein isolate and whey protein concentrates are commonly used by bodybuilders.

Recent studies have shown whey protein has beneficial insulin otropic and glucose-lowering properties in both healthy and type 2 diabetes individuals.

Whey protein releases bioactive peptides and amino acids upon being digested in the gastrointestinal tract. These amino acids and peptides promote the release of several gut hormones including cholecystokinin, peptide YY and the incretins gastric inhibitory peptide, and glucagon-like peptide 1 that potentiate insulin secretion from β-cells and are linked with regulation of food intake.

Additionally, these whey protein derived bioactive peptides may also function as endogenous inhibitors of dipeptidyl peptidase-4 (DPP-4) in the proximal gut, preventing incretin degradation, which is good for type 2 diabetes management.

Whey protein in forms of whey protein hydrolysates and whey protein concentrate is available as a dietary supplement. In addition to their potential benefits for people with type 2 diabetes, these products can help maintain muscle health including help repair muscle damage induced during physical exercise. For individuals who do not use animal products yet want to maintain muscle health, they may use supplements of branched chain amino acids, leucine, isoleucine and valine. These amino acids are helpful when a person uses a protein-restricted diet.(*Journal of Nutritional Biochemistry, published online Sept 2012).*

2.7. Bioactive peptides: Current status in India

The Fermented dairy products (FDP) at National Dairy Research Institute has extablished antifungal peptides from fermented milk using lactic cultures. FDP laboratory is also engaged in production of antimicrobial bioactive peptides from milk whey & casein, imparts training to entrepreneurs as well as provides consultancy to dairy industries through Consultancy Cell of the Institute. Work has also being done at SRM University on isolation, identification and characterization of bioactive peptides and determination of its gastroprotective, immunomodulatory and anti-genotoxic activities. The milk was fermented with *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* to obtain Casein Phosphopeptide (CPP) with gastroprotective, immunomodulatory and anti-genotoxic activity. At CFTRI,Mysore, the research is on progress on stabilization of peptides in cosolvents. Two peptides with multifunctional properties from alpha (S2)-casein were stabilized in presence of cosolvents for their biological activities like ACE inhibition activity and antioxidant activity. These bioactive peptides in cosolvents were also thermostable. Infra red spectra of peptides in cosolvents reveal no change in the secondary structure in presence of cosolvents. Correlation between sequence, structure and composition of peptides on biological activities were studied. Another group at Sardar vallabh bhai patel university of agriculture and technology , Meerut (U.P.). are working on In silico study for prediction of structures of bioactive peptides by using homology approach and different bioinformatics tools as Basic Local Alignment Search Tool (BLAST) Swiss Model workspace repository and template were applied. The homology model comprises four main steps find out the homology model against query protein identification of structural template(s) alignment of target sequence and template structure(s) model building and model quality evaluation. The feasible structures of antimicrobial antithrombotic casein derived immunomodulatory and mineral binding peptides were designed.

2.8. Future perspectives for bioactive peptides

Fermented dairy products and other foods containing bioactive peptides would appear to have the potential to offer specific health benefits to consumers. While there is a need for further basic research to clarify why these peptides have physiological effects, commercial products

containing bioactive peptides are now commercially available. Food and pharmaceutical companies are actively considering how to exploit bioactive peptides in both human nutrition and in health promotion.

Bioactive peptide preparations have the potential to be used in the formulation of functional foods, cosmetics and as potent drugs having well defined pharmacological effects. With the rise of consumer concerns about the deleterious effects of chemical preservatives and the increasing preference for natural components, milk derived bioactive substances may have value in food preservation and nutraceuticals. Application of enrichment protocols such as membrane processing and chromatographic isolation may also be an area of future interest in the extraction of potent biofunctional peptides from fermented dairy products and their subsequent utilization as functional food ingredients. Molecular studies are required to study the mechanisms by which the bioactive peptides exert their activities. Ultimately this research may be helpful in understanding, preventing and treating life-style related diseases such as cardiovascular disease, cancers, osteoporosis, stress and obesity.

2.8.1. Food and pharmaceutical companies

There is a mounting worldwide interest in the therapeutic potential of bioactive peptides. Bioactive peptides trigger certain useful functionalities such as antioxidative, antimicrobial, antihypertensive, and immunomodulatory activities in the living body system. With the needed research and development, there exist enormous opportunities to effectively harness these diverse functionalities of bioactive peptides for the treatment and prevention of different medical conditions, and this can be a profitable commercial focal point of operation for the production of novel blockbuster products in biopharmaceutical manufacturing industries.

2.8.2. Formulation of functional foods, and nutraceutical

Functional foods and nutraceuticals provide an opportunity to improve the human health and reduce health care costs. The phrase "Let food be the medicine and medicine be the food," coined by Hippocrates is receiving a lot of interest today as food scientists and consumers realize the many health benefits of certain foods. The challenges and opportunities, motivating the

development and regulations of functional foods and nutraceuticals. The examples of the functional foods and nutraceuticals and their health benefits, like probiotics and prebiotics, proteins and peptides.

2.8.3. Formulations in cosmetic industry

Recent studies have shown that peptides with antioxidative properties can be released from caseins by hydrolysis with digestive enzymes and by proteolytic LAB in fermented milks (Korhonen and Pihlanto, 2003a). Most these were derived from α_s-casein and have been shown to possess free radical-scavenging activities and to inhibit enzymatic and non-enzymatic lipid peroxidation . In the future, antioxidative peptides may find applications as ingredients in different fields, e.g. in the prevention of oxidation in fat-containing foodstuffs, cosmetics and pharmaceuticals. More research is needed to demonstrate if peptides produced during fermentation can prevent oxidative damage *in vivo* .

2.8.4. Food preservation

Food safety is a growing concern of great importance worldwide. The consumption of processed foods with chemical preservatives has led to increased consumer concern and the demand for more natural and minimally processed foods. As a result, researchers have shown a growing interest in natural antimicrobial agents such as certain peptides. Peptides with antimicrobial properties are used as the first chemical barrier against microbial attack, being synthesized in response to bacterial infections. They are produced by almost all species of life, from microorganisms, plants and animals, to humans (St Georgiev 1990; Hancock and Diamond 2000). In animals, antimicrobial peptides are produced mainly in those tissues exposed to adverse conditions such as skin, eyes, and lungs, which are more likely to be in contact with microorganisms (Zasloff 2002; Papo and Shai 2003). Antimicrobial peptides have found many applications, including those in biomedical devices, food processing equipment, and food preservation. In food preservation, peptides can be incorporated into materials to create antimicrobial packaging (Appendini and Hotchkiss 2002). In this way, antimicrobial packaging plays an important role in maintaining the safety and quality of food, since the aim is to prolong food shelf life and to reduce bacterial growth on the product surface (Soares and others 2009a).

CHAPTER 3

Materials and Methods

Chemicals

3.1 Sodium dodecyl sulphate (SDS) , Di-Sodium tetra decahydrate, O- phthalaldehyde, Serine, Dithiothritol, Sodium bi-carbonate, Sodium Potassium tartarate, Copper sulphate, Folin & Ciocalteu's phenol reagent, Bovine serum albumin, Casein enzyme hydrolysate , Sodium acetate, Ascorbic acid, Tween 80, 2,3,5triphenyltetrazolium chloride, Crystal violet solution, Grams iodine solution, Safranine.

Procurement of Milk & Cultures

3.2 Amul skimmed milk was obtained from Shimla market. The pure lactic cultures were obtained from the Microbiology lab, JUIT. Following strains were used:

- *Streptococcus thermophilus* (NCDC-144)
- *Lactobacillus helveticus,* (NCDC-292)
- *Lactobacillus delbrueckii* ssp. b*ulgaricus* , (NCDC-144)
- *Lactobacillus rhamnosus*, (MTCC-1408)
- *Lactobacillus casei (MTCC-1423)*
- *Yoghurt culture* (NCDC 144) were obtained from National Collection of Dairy Culture,
- Karnal

Maintenance of lactic culture

3.3 The lactic cultures were maintained by inoculating each culture individually in MRS broth and were incubated at 37^0C for 24 hrs.

Preparation of chalk litmus milk

3.3.1 Chalk litmus milk tubes were prepared by adding a pinch of calcium carbonate and litmus to 20 ml of skimmed milk. The tubes were then sterilized at 121^0C for 15 min and kept at 4^0C until use.

3.3.2 Propagation of cultures

An aliquot of inoculums (50µl) of lactic culture and yoghurt culture was added to 20ml of sterilized skimmed milk and incubated for 24hr at 37°C. After incubation the tubes were checked for setting of the curd and presence of any gas (indicates contamination).

3.3.3 Confirmation of lactic cultures by microscopy

A drop of curd/fermented milk was spread on slide and stained following the procedure of Grams stain and observed at 100x under microscope.

3.4 Optimization of fermentation of milk

The optimization of fermentation of milk to achieve maximum proteolysis was done in three test tubes of skimmed milk (20 ml each). The tubes were inoculated with respective lactic cultures and incubated at 37^0 C for different time intervals. The incubated tubes were observed at 6, 12 and 24 h for pH , titratable acidity, wheying off, total protein content and degree of hydrolysis as per the methods described below.

3.5 Preparation of fermented milk

A 100ml of skimmed milk was added in flask and sterilized at 121°C at 37°C for 15 minutes. The flasks were inoculated with different lactic cultures and yoghurt cultures (*Lb. delbrueckii ssp. bulgaricus* and *S.thermophilus*, 1:1) individually and were incubated for 24 and 4 hrs respectively at 37°C .

3.6 Processing of fermented milk

Yoghurt and Fermented milk were taken and centrifugation was done at 7000rpm for 15 min. The supernatant of the samples was taken and lyophilized to obtain powdered form.

3.7 Analysis

3.7.1 pH :-pH of yoghurt (1% & 2%) and fermented milk was estimated with help of pH meter .

3.7.2 Acidity:-Acidity of the samples were estimated by adding few drops of phenolphthalein to yoghurt (1% & 2%) and fermented milk samples and titrated against 0.1N NaOH .

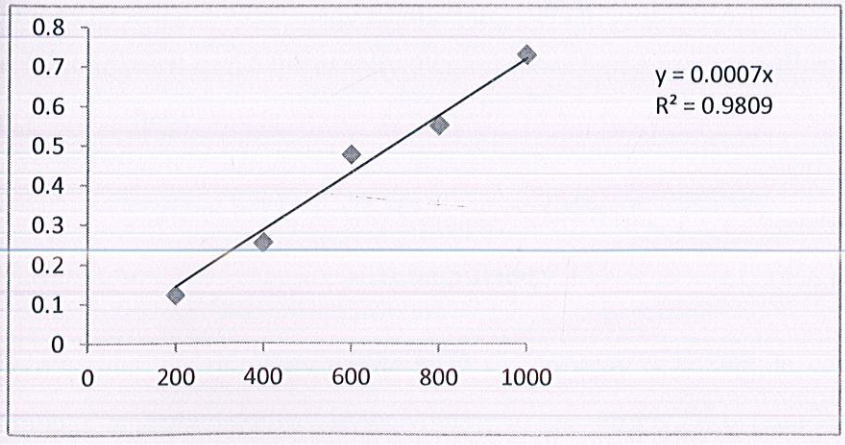
3.7.3 Estimation of Protein content (Lowry's assay):-

Standard –BSA(1mg/ml)

0.2, 0.4, 0.6, 0.8 and 1mL of the working standard was pipetted into a series of test tube. Preparation of lyophilized sample was done by taking 1mg of sample in 1 ml of distilled water and dilutions were made (1/10 , 1/50, 1/100). 5ml of alkaline solution was added and vortexed it and kept at 37°Cfor 15 mins. 0.5 ml of folin phenol reagent (1N) was added and incubated it for 30 min in dark and absorbance was taken at 750nm.

Sample measuring :- The sample solution was prepared as follows:

1mg of lyophilized protein samples(1%, 2% yogurt /fermented milk) in 1ml of distilled water. And following dilutions of sample were made (1/10 , 1/50 , 1/100). 5ml of alkaline solution was added and vortexed it and kept at 37°Cfor 15 mins. 0.5 ml of folin phenol reagent (1N) was added and incubated it for 30 min in dark and absorbance was taken at 750nm.



3.7.4 Estimation of proteolytic activity

Proteolytic activities of cultures used in the production of all batches of yoghurts were assessed by measuring liberated amino acids and peptides using the o-phthaldialdehyde(OPA) method.

The OPA reagent was prepared as follows:

7.620 g di- Natetraborate decahydrate and 200 mg Na-dodecyl-sulfate (SDS) were dissolved in 150 mL deionized water. The reagents were completely dissolved before continuing. 160 mg o-phthaldialdehyde 97% (OPA) was dissolved in 4 mL ethanol. The OPA solution was then transferred quantitatively to the above-mentioned solution by rinsing with deionized water.176 mg dithiothreitol 99% (DTT) was added to the solution by rinsing with deionized water. The solution was made up to 200 mL with deionized water.

Procedure:-

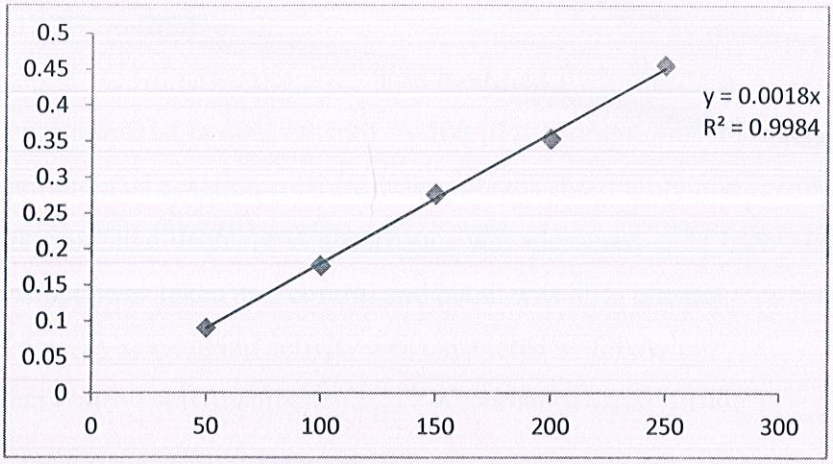
Standard :- Serine 1mg/ml

Serine standard was made using dilutions of 50ul,100ul,150ul,200ul,250ul. 3mL OPA reagents was added and mixed for 5 s. The mixture stood for exactly 2 min before being read at 340 nm in the spectrophotometer. Note the readings.

Sample measuring :- The sample solution was prepared as follows: 0.01gm of lyophilized protein samples (1%, 2% yogurt /fermented milk) in 1ml of distilled water. And following dilutions of sample were made (25ul,50ul ,100ul). 3mL OPA reagents was added and mixed for 5 s. The mixture stood for exactly 2 min before being read at 340 nm in the spectrophotometer. Note the readings.

$$\text{Serine-NH}_2 = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})/(\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}) \times 0{\cdot}915 \text{ meqv l}^{-1} \times S \times D/(P/V),$$

where serine-NH2 is meqv serine NH2 g)1 protein; S is sample volume in litre; D is dilution volume; P is protein content in the volume of the sample; V is sample volume in assay;



assay; β, α and h_{tot} are constants for whey 0·4, 1·0 and 8·8.

$$DH\% = h/h_{tot} \times 100\%,$$

where $h = (\text{serine-}NH_2 - \beta)/\alpha$meqv/g/protein.

3.8 Estimation of Functional attributes of skim milk hydrosylates.

3.8.1 Estimation of Anti-oxidative activity

3.8.1.1 ABTS method

The scavenging activity was estimated according to the procedure of Pellegrino et al. (1993) ABTS (7mM in water) was prepared by mixing an stock solution with potassium per sulphate (2.45mM) in an equal quantities and left to stand for 12-16 h at room temperature in the dark until reaching a stable oxidative state. The $ABTS^{\cdot+}$ solution was diluted with 80% ethanol to an absorbance of 0.80 ± 0.05 at 734 nm. 100 µL of sample was mixed with 2.9 ml of the $ABTS^{\cdot+}$ solution and the mixture was allowed to stand at room temperature for 30 min. in dark condition. The absorbance was determined at 734 nm.

Scavenging effect (%) = 1 – [(Absorbance $_{sample}$ / Absorbance $_{control}$) x 100

3.8.1.2 DPPH method

The assay was done according to the procedure modified by Shimada et al. (1992). 0.3mM of DPPH solution was prepared in 80% ethanol. A 500 µL of sample was allowed to react with 2.5 ml of DPPH solution. The reaction mixture was vortexes thoroughly and incubated at 30°C for 30 min. in a dark room and decrease in absorbance was measured at 517nm. The DPPH reagent only instead of sample was taken as a control and blank was 80% ethanol.

The free radical activity scavenging activity was calculated as following:

Scavenging effect (%) = 1 – [(Absorbance $_{sample}$ / Absorbance $_{control}$) x 100]

3.8.2 Estimation of Anti-diabetic activity:-

3.8.2.1 a-Amylase Inhibition Assay

Porcine pancreatic a-amylase (EC3.2.1.1) was purchased from Sigma Chemical Co. A total of 500 µl sample and of 0.02M sodium phosphate buffer (pH6.9) incubated for 10 min. After pre-incubation, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH6.9 with 0.006M sodium chloride) was added to such tube at timed intervals. The reaction mixture were then incubated at 25◦C for 10 min. The reaction was stopped with 1.0 ml of Di nitro salicylic acid colour reagent. The test tube were then incubated in a boiling water bath for 5 min, then cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water, and absorbance was measured at 540 nm

3.8.3 Estimation of Anti microbial activity :-

Anti microbial activity of bioactive peptides was estimated against *E.coli* , *Salmonella typhi* , *S.aureus* , *Bacillus sp.* Agar well method. Agar plates of individuals bacterial species were made and wells were inoculated with 200 ul of bioactive peptide

CHAPTER 4

Results and Discussion

4.1 Microscopic observation of lactic cultures:-

4.1.1 Confirmation of lactic cultures by microscopy

All the lactic acid bacteria's were tested for gram staining using gram staining technique. And the result was all of them were Gram positive in nature.

4.1.2 pH and acidity in fermented skimmed milk

The initial pH of the sterilized skimmed milk was 6.5. The lactic cultures reduced the pH to 4.5 when incubated for 24 h. The lactic cultures differed in their ability to reduce the pH of milk with *Lb.rhamnosus* being the weakest acid producer. Most of the cultures reduced the pH to 5.0 within 6 h of incubation. Nevertheless, the ΔpH of the strains were similar and ranged between 1.7 to 2.0, except for strain *L rhamnosus*, which had a ΔpH (24 h) of 1.00. The yoghurt culture in ration of 1:1 produced the lowest pH due to synergism between the two bacterial species. There was no whey separation upto 24 h of incubation and the coagulated skimmed milk was having thick consistency in all the test cultures

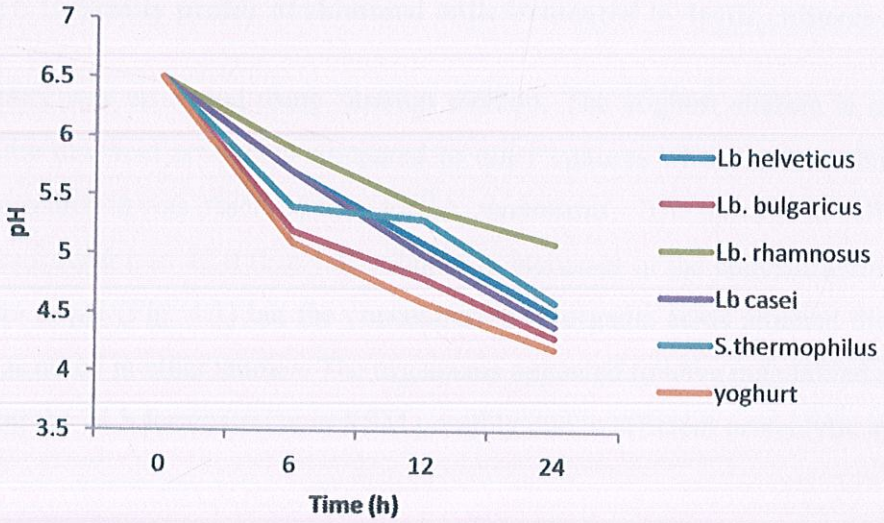


Fig 4.1: Changes in pH in skimmed milk fermented by lactic cultures

4.2 Titrable acidity

All the lactic cultures were evaluated for there acidic content with the help of titration technique. And then we had calculated the level of different organic acid content.

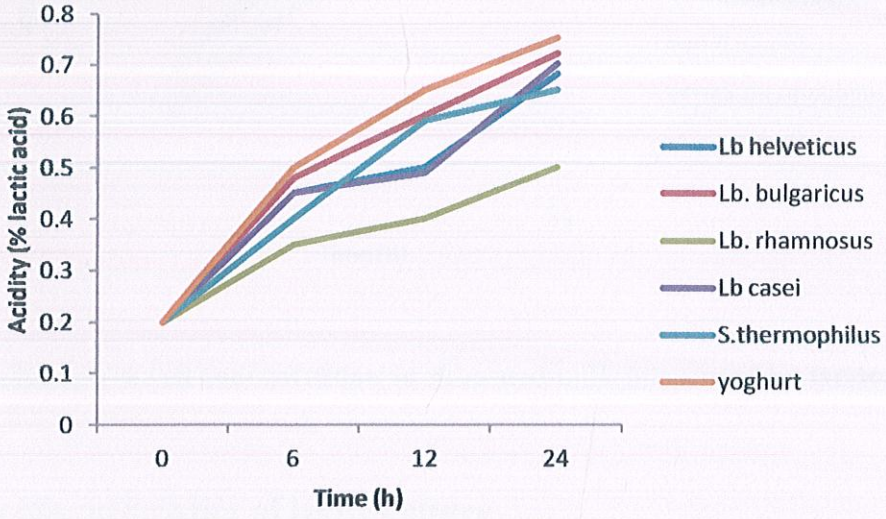


Fig 4.1: Acidity profile of skimmed milk fermented by lactic cultures

The titrable acidity was estimated using titration method. The yoghurt starters in combination were found be the fast acid producers compared to other cultures when used as single culture. The weak acid production was observed only for *Lb. rhamnosus*. The upsurge in cell growth for all lactic cultures from 6 h to 12 h (Fig.4.3) resulted in increases in the concentration of organic acids and decline in pH (Fig. 4.1) but the concentration of organic acids attained did not affect the cell growth as noted in other studies. The organisms appeared to have maintained appreciable cell counts during the 24 h fermentation in RSM possibly due to efficient proteolytic systems

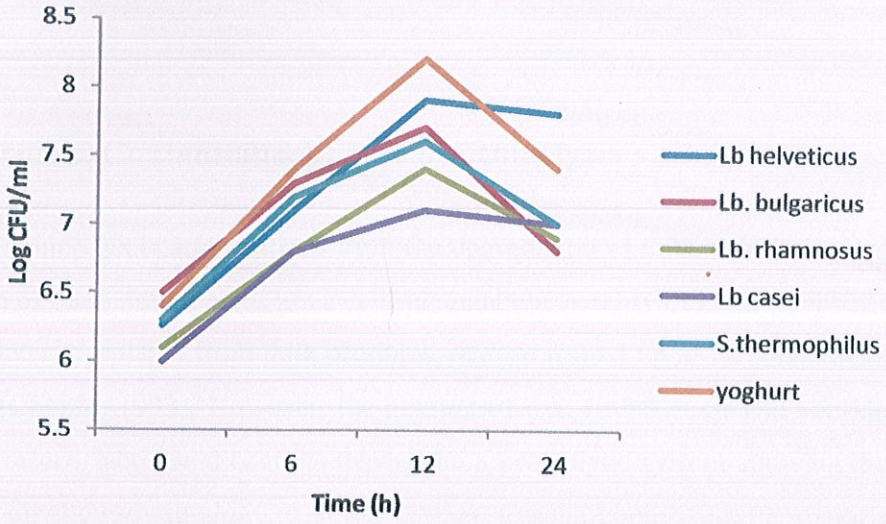


Fig 4.1: Changes in cell concentration in skimmed milk fermented by lactic cultures

4.3 Growth characteristics of lactic culture

Lactic acid bacteria are nutritionally fastidious organisms which require more free amino acids or peptides than present in milk. Thus proteolytic activity is important requirement for achieving a minimum level of 10^6 to 10^7 CFU/mL of lactic culture in a product to observe positive health effect. In our investigation, all strains grew well, although variable growth patterns were observed. The relatively high cell count at the beginning resulted most probably from the inoculation process used. The starting level was 10^6–10^7 CFU/ ml, except for *Lb. delbrueckii ssp. bulgaricus* and *Lb.casei*, which had 10^6 CFU/ ml. At the end of fermentation the number of LAB cells varied from 10^7to10^9 CFU/ ml and pH varied from 6.6 to 4.5. Fermentations with LAB combinations in yoghurt revealed similar growth parameters to those seen with single strain fermentations, the viable cell counts increased to 10^8 CFU/ ml. Although the cultures showed a consistent increase in cell concentration until 12 h, the required pH of 4.5 was not reached. It was observed that the individual cultures of *Lb. delbrueckii ssp. bulgaricus* and *S.thermophilus* grew well in fermented milk and did not produce organic acids as fast as with mixed starter cultures in yoghurt.

4.4 Total Protein Content and Degree of proteolysis

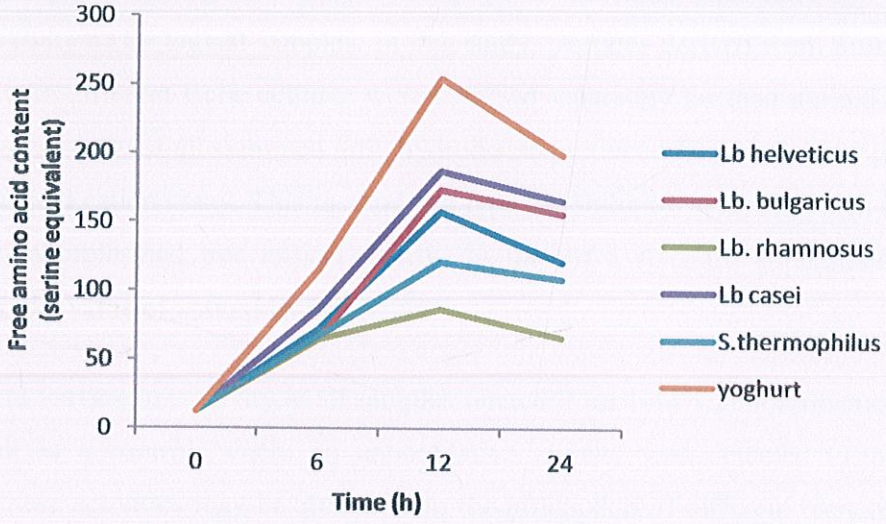
Production of amino acids and peptides from the degradation of milk proteins by LAB enzymes and utilization of these amino acids are a central metabolic activity of LAB (Gobbetti et al 2002). Lactic acid bacteria, isolated from milk products, require from 4 up to 14 amino acids depending on the strain (Chopin, 1993). However, the amount of free amino acids and peptides in milk is very low. Therefore, lactic acid bacteria depend on a proteolytic system allowing degradation of milk proteins for the growth (Juillard et al., 1995a). Casein, comprising the major part of milk proteins, contains all amino acids necessary for the growth of lactic acid bacteria in milk to high cell density, yet only a minor fraction, less than 1% of the total casein content, is actually needed (Kunji et al., 1996).

It has been well established that a number of *Lactobacillus* sp. grow well in skim milk (Gilbert et al., 1996). Proteolysis is a cascade of processes involving a number of steps including an extracellular proteinase initiating degradation of casein into oligopeptides, transport systems that translocate peptides and amino acids across the cell wall, various intracellular peptidases for further degradation of peptides into amino acids, and different enzymes that convert liberated amino acids into various components (Kunji et al., 1996).

Proteolytic activity of all fermented strains used in this study was assessed during 12 h of incubation time at 37°C and is shown in Fig 4.4. The free amino acid content in all fermented milk samples was variable due to proteolytic system of these microorganisms in the milk as the growth medium (Donkor et al., 2007). Proteolysis as assessed by the release of free NH3 groups by using OPA method, increased during the 12 h of the incubation time. The extent of proteolysis in the *L. helveticus* strains fermented milk was significantly higher than that of the other fermented strains in this study at the first half period of incubation time. The level of proteolytic activity remained substantially higher for yoghurt starters than those of *L. bulgaricus* and *S. thermophilus* strains, and that degree of proteolysis was depended significantly on incubation time and strain. These results were similar as those reported by Leclerc et al. (2002), who demonstrated a linear increase in the extent of proteolysis with fermentation time for *L. helveticus* among the species of LAB studied. The primary enzymes in LAB responsible for the

hydrolysis of the proteins are proteinases and peptidases (Law and Haandrikman, 1997, Shihata and Shah, 2000). *Lb. casei* achieved the highest proteolytic activity among the single starter strains studied followed by *Lb. delbrueckii ssp. bulgaricus*. These results indicate that the proteolytic activity *of Lb. casei* strains under the dropping of the pH, had a strong effect on bacterial growth compared to other strains examined (Leclerc et al., 2002).

The lactic strains having proteolytic enzymes is affected by decreasing the pH during fermentation time and thus might need longer time to adapt to the growth medium for proteolytic system development. These differences in the amounts of amino groups released during fermentation of milk observed could probably relate to the different proteinases and peptidases of the strains and appeared to be strain dependent (Shihata and Shah, 2000).



It was calculated from above table that Total Protein Content of Lactobacillus helvaticus was highest and Degree of Proteolysis was highest in yoghurt 2%.

4.5Anti-oxidative activity

Oxidative metabolism is crucial for the survival of human body's cells. The risk of this activity is that the production of free radicals causes oxidative changes (Pihlanto, 2006). Free radicals have been linked with many pathological conditions such as atherosclerosis, diabetes, rheumatoid arthritis (Abuja and Albertini, 2001, Gutteridge and Halliwell, 2000, Halliwell and Whiteman, 2004). Inhibition of the free radicals formed in the living body and foodstuff is the important way to protect body from above serious diseases.

1, 1-diphenyl-2-picrylhydrazyl (DPPH) is a popular indicator for showing free radical scavenging activity of biological samples. In this study, peptides derived from milk proteins by fermentation with different lactic cultures were assessed separately for their antioxidant activity. The antioxidant activity of all skimmed milk hydrolysate produced from milk by was determined by DPPH•+ radical cation assay. This method is a type of inhibition assay in which the extent of scavenging of a preformed free radical relative to that of a standard antioxidant compound corresponds to the value of antioxidant activity.

The free radical scavenging activity in all samples ranged from 4-44% inhibition comparing with untreated milk as a control, where no antioxidative activity was detected (Table:4.1). The variations of these activities may be attributed to the production of different bioactive peptides, which may or may not have antioxidant properties and it is likely to be strain dependent (Donkor et al., 2007). It has been reported that DPPH radical-scavenging activity of skim milk increased by fermentation with *L. casei* strain and also suggested that peptides produced in the fermented milk might be one of the factors enhancing radical scavenging activity (Nishino et al., 2000). However, we have not observed a higher anti-oxidative potential of *Lb. casei.*

Table 4.1: Anti oxidative activities of skimmed milk hydrolysate

Culture	Inhibition %
Lactobacillus rhamnosus	29

Lactobacillus casei	4
Lactobacillus delbruckii ssp bulgaricus	34
Lactobacillu helvaticus	19
Streptococcus thermophilus	39
Yoghurt (2%)	44
Control (skimmed milk)	Nil

Milk proteins have been suggested to have possible free radical scavenging by amino acids such as tyrosine and cysteine (Pihlanto, 2006). Peptides derived from casein hydrolysis have been reported to have antioxidant activity. We had estimated from our results that Yoghurt was showing highest percentage of inhibition i.e. 44%, whereas the monoculture inoculation by *S. thermophilus* and *Lb. delbruckii ssp bulgaricus* showed the highest inhibition rate. A k-casein derived peptide with DPPH radical scavenging activity has been found in milk fermented with *Lactobacillus delbrueckii ssp. bulgaricus* (Kudoh et al 2001) and *S. thermophilus* (Yn Lin et al 1999). A study published by Suetsuna et al (2000) also showed that some peptides derived from the pepsinic hydrolysate of casein demonstrated DPPH radical scavenging activities. Liu et al (2005) found that the DPPH radical-scavenging activity of milk-kefir and soymilk-kefir was significantly higher than that of milk and soymilk and they suggested that this activity may be, in part, attributed to the peptides deriving from degradation of milk and soybean proteins. These results of scavenging properties of free radicals by dairy cultures might be useful in food manufacturing and can present additional sources of health enhancing antioxidants.

4.6Anti-diabetic activity

This activity was estimated with the help of method a-Amylase Inhibition Assay. And respective results against various lactic bacteria's are as follows.

Samples	Inhibition %
Lactobacillus rhamnosus	22
Lactobacillus casei	11
Lactobacillus delbrueckii ssp. bulgaricus	30
Lactobacillu helveticus	15
Streptococcus thermophilus	40
Yoghurt (2%)	48

From the above table we had estimated that yoghurt showed highest inhibition i.e. 48%, whereas *Lactobacillus casei* showed least inhibition i.e. 11%.

The health-relevant functional benefits of *Lactobacillus bulgaricus* fermented milk and soymilk were investigated and targeted for management of hyperglycemia using *in vitro* models.Enzyme inhibitory activities linked to hyperglycemia (α – amylase and α–glucosidase) of fermented substrates were evaluated using *in vitro* assays. These activities were correlated to phenolic and lactic acid contents. In spite of total phenolic content decreasing over 24 h, inhibitory activity increased with fermentation, with higher activity in milk substrate. α–Amylase inhibitory activity was high in milk substrate throughout the fermentation and in soymilk it increased from a lower initial activity. This study provides insights that fermentation of milk and soymilk with specific lactic acid bacterial strains can potentially enhance functional properties relevant for hyperglycemia management linked to type 2 diabetes.

4.7Anti-microbial activity

The antimicrobial properties of milk have been widely acknowledged for many years. As early as 1930, it was reported that milk possessed active inhibitors that slowed the growth of streptococcal bacteria. The antimicrobial activity of milk is mainly attributed to immunoglobulins, and to non-immune proteins, such as lactoferrin (LF), lactoperoxidase and

lysozyme. This activity was estimated by agar well assay method and the respective results of various lactic cultures are in Table 4.3

Samples	*E.coli*	*Staphylocccus aureus*	*Bacillus sp.*	*Salmonella sp.*
Lactobacillus rhamnosus	-	+++	-	-
Lactobacillus casei	-	+++	++	-
Lactobacillus delbrueckii ssp. bulgaricus	++	++	++	-
Lactobacillus helveticus	++	+	-	-
Streptococcus thermophilus	++	++	++	+

- No activity, + weak antimicrobial activity, ++ moderate activity, +++ high antimicrobial acitivity

Food contamination by *Staphylococcus aureus* is a major problem for consumer's health in world,especially during the summer period. The use of bacterial interactions is a new way to limit the pathogenic germs growth. *Lactobacillus* strains are already reported to have a broad antimicrobial activity. The peptides generated via lactic acid fermentation exhibit antimicrobial activity against various Gram-positive and Gram -negative bacteria, e.g. *Escherichia, Helicobacter, Listeria, Salmonella* and *Staphylococcus*, yeasts and filamentous fungi. In the present study, the skimmed milk hydrolysate fraction had the antibacterial activity against gram positive pathogens.

Anti-microbial activity of *Lactbacillus rhamnosus* was reported against *Staphyloccus aureus* and *E.coli* (Arici et al 2010), and *Lactobacillus delbruckii ssp bulgaricus* and *Streptococcus thermophilus* and *Lactobacillus helveticus* showed moderate activities against *E.coli* and *S.aureus* (Gawad et al 2010).

The physiological importance of antimicrobial milk peptides remains to be established, although it has been suggested that they may modulate the intestinal microflora when formed during milk digestion in vivo and protect host against invading microorganisms

The most studied peptides are those with antimicrobial activity, characterized by their interaction with the cytoplasmic membrane of the microorganism regardless of the final target (Powers and Hancock 2003). Factors influencing the antibacterial activity are the electrostatic interactions between the peptide and positively charged and anionic lipids on the surface of the target microorganism. Also, the hydrophobicity of the peptide (factor required for insertion into the membrane) and peptide flexibility allow peptide interaction with the microbial membrane (Jenssen and others 2006). Although these characteristics are variable according to each peptide, all of them are essential to the function of peptides as antimicrobials. The exact mechanism of action of antibacterial peptides is not yet fully understood. However, there is a consensus among researchers regarding the first step in the initial interaction between peptide and the target cell (Reddy and others 2004).

5 References

1. Haque & Chand. Antihypertensive and antimicrobial bioactive peptides from milk proteins. Eur Food Res Technol (2008) 227:7–15 DOI 10.1007/s00217-007-0689-6
2. Korhone & Pihlanto. Bioactive peptides: Production and functionality. International Dairy Journal 16 (2006) 945–960
3. Judith Perez Espitia, Nilda de F´atima Ferreira Soares, Jane S´elia dos Reis Coimbra, N´elio Jos´e de Andrade, Renato Souza Cruz, and Eber Antonio Alves Medeiros. Bioactive Peptides: Synthesis, Properties, and Applications in the Packaging and Preservation of Food. 2012 Institute of Food Technologists® doi: 10.1111/j.1541-4337.2011.00179.x
4. Fields ,Falla, Rodan, ,Bush. Bioactive peptides: signaling the future 2009 Wiley Periodicals, Inc.
5. Sharma1, Singh, Rana . Bioactive Peptides: A Review. *INT. J. BIOAUTOMATION*, 2011, 15(4), 223-250
6. Hartmann and Meisel. Food-derived peptides with biological activity: Current Opinion in Biotechnology 2007, 18:163–169
7. FitzGerald R and Meisel.H Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme: British Journal of Nutrition (2000), 84, Suppl. 1, S33±S37
8. Yoshikawa M, Fujita H, Matoba N, Takenaka Y, Yamamoto T, Yamauchi R, Tsuruki H, Takahata H Bioactive peptides derived from food proteins preventing lifestyle-related diseases: Research Institute for Food Science, Kyoto University, Gokanosho, Uji, Kyoto 611-0011, Japan
9. Tsai, Tai-Jung Chen,Bonnie ,Pan, Shein-Da Gong, Mei-Yuh Chung Antihypertensive effect of bioactive peptides produced by protease-facilitated lactic acid fermentation of milk : Food Chemistry Volume 106, Issue 2, 15 January 2008, Pages 552–558
10. Makoto , Masashi , Yasunori , Toshiaki , Yukio Transepithelial Transport of the Bioactive Tripeptide, Val-Pro-Pro, in Human Intestinal Caco-2 Cell Monolayers: Bioscience, Biotechnology, and Biochemistry Vol. 66 (2002) No. 2 P 378-384

11. Parrot S, Degraeve P, Curia C, Gros In vitro study on digestion of peptides in Emmental cheese: Analytical evaluation and influence on angiotensin I converting enzyme inhibitory peptides : Food / Nahrung Volume 47, Issue 2, pages 87–94, 1 April 2003
12. Saito T, Nakamura T, Kitazawa H, Y. Kawai, Itoh T Isolation and Structural Analysis of Antihypertensive Peptides That Exist Naturally in Gouda Cheese : Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Tsutsumidori-Amamiyamachi 1-1, Aoba-ku, Sendai 981-8555, Japan
13. Chin Feng Liu and Tzu Ming Pan Beneficial Effects of Bioactive Peptides Derived from Soybean on Human Health and Their Production by Genetic Engineering : Department of Biochemical Science and Technology, College of Life Science, National Taiwan University Taiwan
14. Williamson H, Patricia S. PhD1; Flickinger,; Messina, Mark JEmpie, Mark
15. Isoflavone supplements containing predominantly genistein reduce hot flash symptoms: a critical review of published studies : The Journal of The North American Menopause Society september/October 2006 - Volume 13 - Issue 5 - pp 831-839
16. Maddalena F, Hans , Magda , Simona ,Pia , Ivano , Joel ,Vandekerckhove, Elisabetta Gianazza, Pietro Ghezzi Identification of proteins undergoing glutathionylation in oxidatively stressed hepatocytes and hepatoma cells : PROTEOMICS Special Issue: Multiplexing Technologies Volume 3, Issue 7, pages 1154–1161, July 2003
17. Chung Hung Lee, Lin Yang, Jin Ze Xu, Sai Ying Venus Yeung, Yu Huang, Zhen-Yu Chen a Relative antioxidant activity of soybean isoflavones and their glycosides: Food and Nutritional Sciences Programme, Department of Biochemistry, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, PR China
18. Tobias S, Claudia R, Shinji T , Baerbel R, Joachim Hr, and Holger Schulz Instillation of Six Different Ultrafine Carbon Particles Indicates a Surface Area Threshold Dose for Acute Lung Inflammation in Mice: Environ Health Perspect. 2006 March; 114(3): 328–333
19. Kwang Kon Koh, MD, FACC; Jeong Yeal Ahn, MD; Seung Hwan Han, MD; Dae Sung Kim, MD; Dong Kyu Jin, MD; Hyung Sik Kim, MD; Mi-Seung Shin, MD; Tae Hoon Ahn, MD; In Suck Choi, MD; Eak Kyun Shin, MD Pleiotropic effects of angiotensin II receptor blocker in hypertensive patients : J Am Coll Cardiol. 2003;42(5):905-910.

20. P. Collins, G.M.C. Rosano,C. Jiang, D. Lindsay, Prof P.A. Poole-Wilson, P.M. Sarrel Cardiovascular protection by oestrogen—a calcium antagonist effect : The Lancet Volume 341, Issue 8855, 15 May 1993, Pages 1264–1265.
21. Y Ahn, E Kwon, J E Shim, M K Park, Y Joo, K Kimm, C Park and D H Kim Validation and reproducibility of food frequency questionnaire for Korean genome epidemiologic study : European Journal of Clinical Nutrition (2007) 61, 1435–1441
22. Mohamed Ali Abdel-Rahman , Yukihiro Tashiro , Kenji Sonomoto Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria : Journal of Biotechnology Volume 156, Issue 4, 20 December 2011, Pages 286–301
23. Alain Chopin Organization and regulation of genes for amino acid biosynthesis in lactic acid bacteria : FEMS MicrobiologyReviews Volume 12, Issues 1–3, September 1993, Pages 21–37
24. Jean Guy LeBlanc, Aurelio Silvestroni, , Cristelle Connes, Vincent Juillard,, Graciela Savoy de Giori, Jean-Christophe Piard, and Fernando Sesma Reduction of non-digestible oligosaccharides in soymilk: application of engineered lactic acid bacteria that produce α-galactosidase : Genet. Mol. Res. 3 (3): 432-440 (2004)
25. Edmund R. S. Kunji, Gang Fang, C. Margot Jeronimus-Stratingh, Andries P. Bruins, Bert Poolman, Wil N. Konings Reconstruction of the proteolytic pathway for use of β-casein byLactococcus lactis: Molecular Microbiology Volume 27, Issue 6, pages 1107–1118, March 1998
26. C Gilbert, D Atlan, B Blanc, R Portailer, J E Germond, L Lapierre and B Mollet A new cell surface proteinase: sequencing and analysis of the prtB gene from Lactobacillus delbruekii subsp. bulgaricus.: J. Bacteriol. June 1996 vol. 178no. 11 3059-3065
27. Dorte Frees, Kirsi Savijoki, Pekka Varmanen, Hanne Ingmer Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria Molecular Microbiology Volume 63, Issue 5, pages 1285–1295, March 2007
28. M GUVEN, K YASAR, O B KARACA, A A HAYALOGLU The effect of inulin as a fat replacer on the quality of set-type low-fat yogurt manufacture: International Journal of Dairy Technology Volume 58, Issue 3, pages 180–184, August 2005.