DEVELOPMENT OF A NOVEL FERMENTED SNACK FOOD HAVING α-GLUCOSIDASE, ANGIOTENSIN CONVERTING ENZYME INHIBITORY AND OTHER FUNCTIONAL PROPERTIES

A Thesis
Submitted in the partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE
IN
MICROBIOLOGY

Under the guidance of:

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in this thesis entitled "**Development of a novel fermented snack food with α-glucosidase, Angiotensin Converting Enzyme inhibitory and other functional properties**" in partial fulfilment of the requirement for the award of the degree of Masters of Science in Microbiology, Department of Biotechnology and Environmental Sciences (DBTES), Thapar university, Patiala, is an authentic record of my work during the period of six months from January, 2013 to June 2013, under the guidance of Dr. Abhijit Ganguli, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled "**Development of a novel fermented snack food with α-glucosidase, Angiotensin Converting Enzyme inhibitory and other functional properties**" submitted by Arkadeep Mukherjee in partial fulfilment of the requirement for the award of Degree of Masters of Science in Microbiology to Thapar University, Patiala, is a record of student's own work carried out by him. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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To my parents, for all the support and motivation they have given me throughout my life.....

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Contents

Abstract

This study aimed to develop a fermented snack food having α-glucosidase and Angiotensin Converting Enzyme (ACE) inhibiting bioactive substances along with other functional properties. First, the snack food which comprised of a mixture of flour of *Vigna mungo* and chickpea were made into a batter, fermented with a *Lactococcus lactis* strain (previously characterized in the laboratory for probiotic attributes) and then steamed. Extract concentration of 200 mg/ml exhibited 13.88 ± 0.36 % inhibition of α-glucosidase having IC_{50} value of 3.804 mg/ml of the total peptide which is almost 21 times increase in the percent inhibition with respect to control which was prepared without addition of the strain. Inhibition of ACE was about six folds from the control exhibiting 26.85 ± 1.166 %. Sensorial analysis based on 9 point hedonic scale scored adequately for general acceptability both for adults and elderly. Both sensory and nutraceuticals produced were retained over 48 hours at 4°C. The results of this study suggest a potential of applicability of this functional snack food for both adults and the elderly. However stability of these peptides in acidic environment (gut) and clinical studies are of utmost importance prior to application of this functional food.

List of Abbreviations

Abs	Absorbance
ACE	Angiotensin Converting Enzyme
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BP	Blood Pressure
CEP	Cell envelope protease
cfu	Colony forming units
CLA	Conjugated linoleic acids
CVD	Cardiovascular Diseases
DM	Diabetes mellitus
EC	Enzyme Commission
G6P	Glucose 6-Phosphate
GABA	ϒ-aminobutyric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHL	Hippuryl-L-Histidyl-L-Leucine
IBD	Inflammatory Bowel Disease
IC	Inhibitory Concentration
LAB	Lactic Acid Bacteria
LDL	Low-density lipoprotein
M	Molar
mg	Milligram
min	Minute
ml	millilitre
mM	milimolar
MRS	De Mamm, Rogosa and Sharpe
MUFA	Monounsaturated fatty acids
N	Normal
NADP	Nicotinamide adenine dinucleotide phosphate
PB	Phosphate buffer
PNPG	para-Nitrophenyl-α-D-glucopyranoside
PUFA	Polyunsaturated fatty acids
RDA	Recommended Dietary Allowance
R_f	Retardation factor
rpm	Revolutions per minute
SCFA	Short chain fatty acid
sec	Seconds
SHR	Spontaneously Hypertensive Rats
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography
µg	microgram
µl	microlitre

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Introduction

1. Introduction

Lactic acid bacteria (LAB) comprise a taxonomically diverse group of Gram positive, non spore forming rods and cocci with major applications in food processing and industrial fermentation. Although most of the probiotic bacteria belong to the *Lactobacillus* and *Bifidobacterium* genera, there are also other species of the genera *Lactococcus, Enterococcus Propionibacterium* which are also considered as probiotic bacteria (Dunne, 1999). LAB constitutes an integral part of the healthy gastrointestinal (GI) microecology and influence host metabolism (Fuller, 2000). Hence there are a number of factors which alters the gastrointestinal microflora like diet, age, environmental conditions, host genotype and also by factors like antibiotic and stress. LAB imparts tremendous health benefits in cases of intestinal disorders, lactose intolerance and altered vitamin content of normal diet. Antagonistic effect against various pathogenic organisms, anti-hypertensive and anti-diabetic effects are some of the properties which are gaining fast recognition. Member of LAB are widely-used probiotics in fermented foods and beverage industry and also contributes to the sensory qualities of the food and also in the prevention of spoilage (Sgouras, 2004).

Prolongation of life: Optimistic Studies, a book written nearly a century ago (1906) by Elie Metchnikoff emphasised on the pressing issue on ageing. He postulated lactobacilli fermented food would improve health which he related to the longevity of the Caucasians who consumed fermented milk products with *Lactobacillus bulgaricus*. This evoked a series of probes in creating a new generation of health foods in human nutrition. These health foods were termed as 'probiotic' by Fuller (1989). A unanimous definition was achieved after several modifications and amendments (Schrezenmeir, 2001). They now define probiotics as 'a preparation or product containing viable, defined micro-organisms in sufficient numbers that alter the microflora in a compartment of the host and that exerts health effects in the host'.

1.1 Functional food: Nutraceuticals and Bioactivity

When food is being cooked or prepared using “scientific intelligence" with or without knowledge of how or why it is being used, the food is called "functional food." Thus, functional food provides the body with the required amount of vitamins, fats, proteins, carbohydrates, etc, needed for its healthy survival.

Although nutraceutical and functionality of foods are very much related, the lies a significant difference between them. When functional food aids in the prevention and/or treatment of disease(s) and/or disorder(s), it is called a nutraceutical (Kalra, 2003). Thus when diet supplementation is an essential property of nutraceutical enriched food, the functionality of food lies with its bioactivity in eliciting significant effects in ameliorating diseased condition. Nutraceuticals are represented for use as a traditional food or as the sole item of meal or diet.

Functional food contains certain bioactive compounds derived from various natural sources have attracted growing interest in the food and pharmaceutical industries in view of their potential to be exploited in many industries. They can find applications in nutraceuticals promoting health, in special dietary preparations preventing microbial infections in humans and farm animals, in biopreservation of foods and feeds, as well as in pharmaceutical preparations replacing synthetic drugs (Rokka, 2011)

1.2 Production of bioactive compounds by probiotic LAB

Lactic acid bacteria growing in milk develop a proteolytic system that releases peptides from casein. (Yamamoto, 1997). They are thus gaining importance as nutraceuticals that benefit aspects of health and nutrition. Technologies involving food processing such as using heat, pH changes, or the ability of microbial enzymes to hydrolyze proteins, such as during fermentation of milk by LAB, can be utilized for generation of biopeptides. Moreover, certain diseases and disorders that do not have convincing treatment strategies or 100% cure can benefit from the proteins and peptides derived from the fermentative processes. These antidisease effects can be classified into antiproliferative, antimutagenic, anti-inflammatory, anticancerous, or antioxidative properties that are manifested in many diseases including cancer, diabetes, inflammatory disorders and hypertension (Mine, 2006).

With the emergence of new detection platforms these diseases have become increasingly prevalent in the last decade. Such diseases have vicious cascades involving interplay of several molecules that not only act on their own but also trigger their partners or adjoining cells and molecules and cause damage to tissue. Many drugs have been discovered and modelled to reduce the ill effects of certain pathogenic molecules participating in these disease processes. Unfortunately they cannot be administered to the masses on a large scale because they are expensive, and moreover they only delay the disease progression; they do not completely cure the disease. A cheaper alternative is to identify foods or food constituents

having functional properties so that they can be consumed as a prescribed or a surrogate to expensive drugs on a large scale if proven therapeutic. Thus, evaluating compounds present in foods for biological activities such as anticancer and antimutagenic among others, can prove effective as they not only can be recommended for subjects who are already on treatment for cancer but also as a preventive measure for those genetically predisposed to diseases.

Peptides are considered fragments of proteins that, upon digestion using specific proteolytic enzymes or fermentation, impart positive functions or benefits that influence human health. They normally remain dormant until they are acted upon by specific proteases. Gastrointestinal proteolytic enzymes release the peptides, and the small fraction released and absorbed is sufficient for imparting biological functions (Mine, 2006).

1.3 Antidiabetic peptides produced by LAB

Diabetes mellitus is a chronic metabolic disorder characterized by a congenital (DM1) or acquired (DM2) inability to transport glucose from the bloodstream into cells (Gunawan-Puteri, 2010), associated with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (WHO, 2006). It is widely accepted that the most challenging goal is the management of patients with diabetes mellitus is to achieve blood glucose levels as close to normal as possible (Mooradian, 1999).

Carbohydrates are the major constituents of the human diet. α- Glucosidase (EC 3.2.1.20), located in the brush-border surface membrane of intestinal cells, has drawn a special interest of the pharmaceutical research community because it was shown that the inhibition of its catalytic activity led to the retardation of glucose absorption and the decrease in postprandial blood glucose level (Braun, 1995). Lactic acid bacteria are known to produce such inhibitors of the enzyme in the form of peptides in various amounts during fermentation. On administering α-glucosidase inhibitors with carbohydrates, they compete with the binding of oligosaccharides and prevent their cleavage to monosaccharides, thereby slowing the digestion process (Fonseca, 2003)

1.4 Antihypertensive peptides produced by LAB

Hypertension is a significant health problem in today's society. There can be no doubt that its presence has far-reaching implications, as it is so widespread, and also because it is a

principal risk factor in cardiovascular disease. It is, in fact, the primary cause of death in developed countries. Antihypertensive peptides have been obtained from food protein of both animal and plant origin. The principal ones are antihypertensive peptides derived from milk proteins. Proteolysis by naturally occurring enzymes in milk or by microbial enzymes, especially from LAB, generates such bioactive peptides during milk fermentation, thereby enriching the fermented product. Once produced, these bioactive peptides may act in the body as regulatory compounds with a hormone-like activity (Gobbetti, 2000). Blood pressure regulation is partially dependent on the rennin-angiotensin system. The angiotensin-I converting enzyme (ACE) regulates peripheral blood pressure and its inhibition can exert antihypertensive effects (Meisel, 1997). ACE hydrolyzed an inactive form of decapeptide, Angiotensin I, to an octapeptide, Angiotensin II, is a potent vasoconstrictor, and inactivated catalytic function of bradykinin, which is a depressor (Abubakar, 1998).

Proteinases of lactic acid bacteria may hydrolyze more than 40% of the peptide bonds of αs1- and β-CNs, producing oligopeptides of 4 to 40 amino acid residues (Kunji, 1996). These inhibitory peptides with various amino acid sequences have been found in hydrolysates from other food proteins digested with different proteases under different hydrolysis conditions (J´erˆome, 2002).

Thus inhibition of ACE has is considered to be a useful therapeutic approach in the treatment of hypertension. Therefore, in the development of drugs to control high blood pressure, ACE inhibition has become an important activity. Many studies have been attempted in the synthesis of ACE inhibitors such as captopril, enalapril, alcacepril and lisinopril, which are currently used in the treatment of essential hypertension and heart failure in humans (Ondetti, 1977). However, these synthetic drugs are believed to have certain side effects such as cough, taste disturbances, skin rashes or angioneurotic edema all of which might be intrinsically linked to synthetic ACE inhibitors. (Atkinson & Robertson, 1979). Therefore search for novel, safer and economic ACE inhibitors for the remedy of hypertension. Hence an extensive effort has been made to characterize such compounds from natural sources and microbial action on food products.

1.5 Objectives

In the last few decades there has been a considerable increase in consumption of functional food enriched with various probiotic strains. This is a result of general awareness about the health beneficial properties of probiotic. Chronic non communicable diseases like diabetes and hypertension have had a cure since ancient times mostly from botanical sources. Therefore substantial research has been carried out as plants continue to be the source of treatment for such diseases. Various proteinaceous sources were hydrolysed enzymatically to produce inhibitors of ACE rendering the process extremely expensive and unpopular.

This study was undertaken to provide a popular food source and incorporate microbial enrichment of the food matrix with remedies for diabetes and hypertension through the process of fermentation and to provide essential nutraceuticals in the form of vitamins, polyphenols etc. Traditionally prepared fermented food is widely consumed as a staple/snack food throughout Asian countries. Development of such food to a level of high functionality having nutraceuticals enriched by probiotics is important which could be consumed not only to alleviate malnutrition but also provide nutraceuticals having positive effects on aggravated conditions of hyperglycemia, hypertension and gastroenteritis.

Accordingly, the following objectives were framed:

1) Development of a novel fermented food which will provide wide range of bioactive compounds.

2) Optimization of process for product development including fermentation time and inoculum size.

3) Stability of the bioactive compounds throughout storage period.

4) Estimation of other nutraceuticals like Vitamin C and B_2 content

Review of literature

2. Review of literature

2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are group of Gram-positive bacteria that are devoid of cytochromes and preferring anaerobic conditions, fastidious, acid-tolerant and strictly fermentative. They are usually non-motile and non-sporulating which are oxidase, catalase, and benzidine negative bacteria and produce lactic acid. This bacterial group contains both rods (*Lactobacilli* and *Carnobacteria*) and cocci (*Streptococci*). Different species of lactic acid bacteria (such as *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Enterococcus*, *Vagococcus*, *Lactobacillus*, *Carnobacterium*) have adapted to grow under widely different environmental conditions. They are found in the gastrointestinal tract of various animals, dairy products, seafood products, soil and on some plant surfaces (Ring, 1998). Although lactic acid bacteria are not dominant in the normal intestinal microbiota, several trials have been undertaken to induce an artificial dominance of lactic acid bacteria (Verschuere, 2000a). Based on their carbohydrate metabolism LAB are divided into two distinct groups. The homo-fermentative group utilizes the Embden-Meyerhof-Parnas (glycolytic) pathway to transform a carbon source chiefly into lactic acid. Hetero-fermentative bacteria produce equimolar amounts of lactate, CO2, ethanol or acetate from glucose exploiting phosphoketolase pathway. Homo-fermentative group consist of *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Streptococcus*. Hetero-fermentative group include *Leuconostoc*, *Weisella* (Vasiljevic, 2008)

Orla and Jensen also divided the Lactobacilli into the three groups (the **Thermobacteria**, **Streptobacteria**, and the **Betabacteria**) based on growth temperature and biochemical reactions. Although those three groups have been replaced for the most part, the three names are still in common use and are defined according to growth temperature, ability to ferment pentoses, ability to produce carbon dioxide from glucose or gluconate, requirement for thiamine, formation of lactic acid as a major product of fermentation, Homofermentative or Heterofermentative type of fermentation, reduction of fructose to mannitol, and hydrolysis of arginine.

Table 1: Few families of order Lactobacillales

Family: *Lactobacillaceae*	
Genus: *Lactobacillus* Strictly fermenative with complex nutritional requirements; grow in and are associated with many different habitats; aciduric or acidophilic, produce pH 4.0 in foods containing a fermentable carbohydrate; often suppress growth or kill other bacteria; important in fermented food manufacture (dairy, meats, sourdough, beer and wine) as well as spoilage.	
L.plantarum	Synonyms: *L. arizonensis*, *Lactobacterium plantarum*, *Streptobacterium plantarum*, *L. arabinosus*, *L. plantari*, *L. arizonae*. Often found in association with cabbage products and with Cheddar cheeses; can cause spoilage of pickles, acidity and off-flavor in wine. Ferments lactose, maltose and mannitol ("typical Streptobacteria"); grows predominantly at 15 °C; forms small chains with short round ends.
L.delbrueckii	Forms an important complex of homofermentative lactics that previously had species status: *L. delbrueckii subsp. lactis* (synonyms: *L. lactis*, *L. lactis-acidi*, *Lactobacterium caucasicum var. lactis*, *Thermobacterium lactis*, *Bacterium lactis acidi*, *Bacillus lactis acidi*), *L. delbrueckii subsp. bulgaricus* (synomyms: *L. bulgaricus*,*Thermobacterium bulgaricum*), *L. delbrueckii subsp. delbrueckii*, and *L. delbrueckii subsp. indicus*. *L. delbrueckii subsp. lactis* is used as starter culture in the manufacture of Swiss-type cheeses (because of its heat tolerance). *L. delbrueckii subsp. bulgaricus* is used as starter culture for yoghurt manufacture.
L. casei	Synonyms: *L. casei casei, Streptobacterium casei,Bacillus casei a, Bacillus a*. Found in association with milk products. Ferments lactose, maltose and mannitol ("typical *Streptobacteria*"). Forms short to long rods with square ends. Probiotic, used in yoghurts.
L. helveticus	Synonyms: *L. suntoryeus, L. helveticum, Plocamobacterium helveticum, Thermobacterium helveticum, Caseobacterium e, Bacillus casei e,Bacillus e*. Belongs to *Thermobacteria*; important probiotic, used in production fermented dairy products, can cause spoilage of beer

L.fermentum	Synonyms: *Lactobacterium fermentum*, *Bacillus casei d*, *Bacillus d*. Can grow at temp. 45 °C; belongs to arginine and ribose fermenting Betabacteria; important spoilage microorganism of beer.
Family: *Streptococcaceae*	
Genus: *Lactococcus* *Lactic streptococci*. The genus was proposed by Schleifer et al. in 1985 to re-classify some species of the genera Streptococcus and Lactobacillus. *Lactococci* are are homofermentative and exclusively produce L-lactate; generally found on plants and the skins of animals (Casalta E, 2008). *Lactococci* are used widely in dairy industry. The principal concern is reliability and stability of their starter cultures because many of the desirable traits of the *lactococci* are plasmid-dependent and can be unstable.	
L. lactis	Synonyms: *Streptococcus lactis*, Bacterium *lacti*. The sunspecies of *L. lactis* are the most important of the commercially used LAB; commonly isolated from plant material, but the most recognised habitat is dairy products

2.2 Biochemistry of lactic acid bacteria

Lactic acid bacteria are chemotrophic, they find the energy required for their entire metabolism from the oxidation of chemical compounds. The oxidation of sugars constitutes the principle energy producing pathway. Lactic acid bacteria of the genera *Lactobacillus*, *Leuconostoc* and *Pediococcus*, the important bacteria to winemaking, assimilate sugars by either a homofermentative or heterofermentative pathway.

2.2.1 Homofermentative Metabolism of Hexoses

Homofermentative bacteria transform nearly all of the sugars they use, especially glucose into lactic acid. The homofermentative pathway includes a first phase of all the reactions of glycolysis that lead from hexose to pyruvate. The terminal electron acceptor in this pathway is pyruvate which is reduced to lactic acid. See Figure 1. In fermentation, pyruvate is decarboxylated to ethanal, which is the terminal electron acceptor, being reduced to ethanol.

2.2.2 Heterofermentative Metabolism of Hexoses

Bacteria using the heterofermentative pathway, which includes *Leuconostoc* (the most important bacterium in enology) use the pentose phosphate pathway. In this pathway, NADPH is generated as glucose is oxidized to ribose 5-phosphate. This five-carbon sugar and its derivatives are components of important biomolecules such as ATP, CoA, NAD+, FAD, RNA and DNA. NADPH is the currency of readily available reducing power in cells (NADH is used in the respiratory chain). This pathway occurs in the cytosol. After being transported into the cell, a glucokinase phosphorylates the glucose into glucose 6-P (glucose 6-phosphate). Its destination is completely different from the glucose 6-P in the homofermentative pathway. Two oxidation reactions occur: the first leads to gluconate 6-P and the second, accompanied by a decarboxylation, forms ribulose 5-P. See Figure 2. In each of these reactions a molecule of NADP+ is reduced. Ribulose 5-P can then be epimerized either to ribose 5-P or to xylulose 5-P. Xylulose 5-P is then cleaved into acetyl-phosphate and glyceraldehydes 3-phosphate. See Figure 3. The glyceraldehyde 3- phosphate is metabolized into lactic acid by following the same pathway as in the homofermentative pathway. The acetylphosphate has two possible destinations, depending on environmental conditions. This molecule can be successively reduced into ethanal and ethanol, in which case the molecules of the coenzyme NADPH formed during the two oxidation reactions of glucose at the beginning of the heterofermentative pathway, are reoxidized.

This reoxidation is essential for regenerating the coenzymes necessary for this pathway. The final products are then lactate and ethanol. Or the acetyl-phosphate can produce acetate (acetic acid) through the enzyme acetate kinase. This reaction also yields a molecule of ATP. The final products of this pathway are then lactate and acetate. Bacteria of the genus *Leuconostoc* preferentially produce lactate and ethanol in a slightly aerated environment and lactate and acetate in an aerated environment.

Figure 1: Homofermentative metabolism of LAB

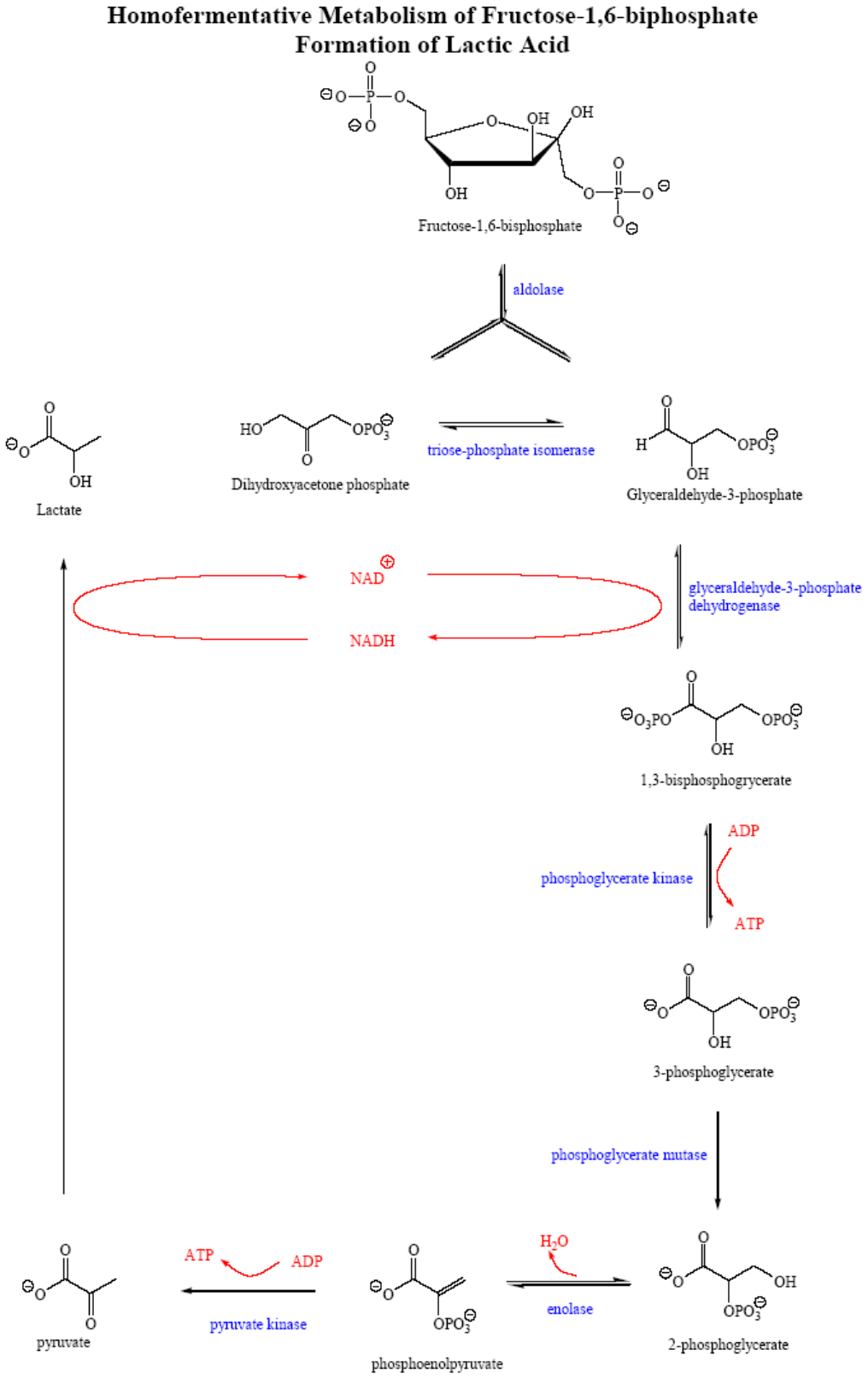
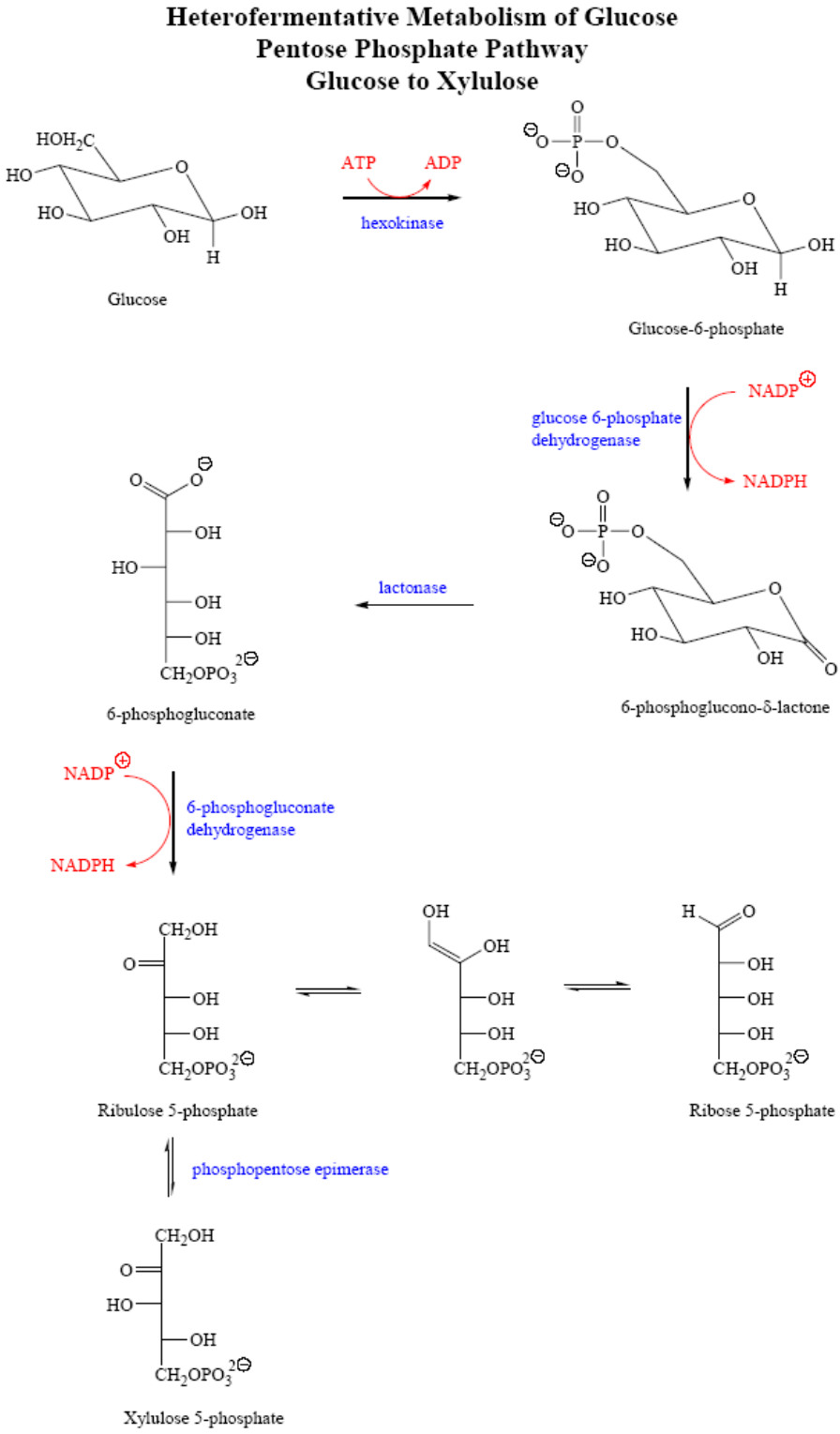
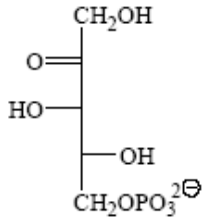


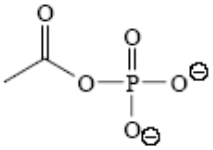
Figure 2: Heterofermentative metabolism of LAB



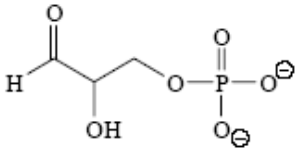


Xylulose 5-phosphate

xylulose 5-phosphate
phosphoketolase



acetyl-phosphate



glyceraldehyde-3-phosphate

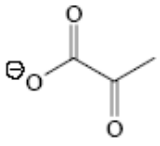
NADPH → $NADP^{\oplus}$

CH_3CHO
ethanal

NADPH → $NADP^{\oplus}$

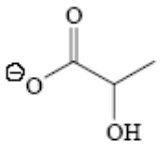
CH_3CH_2OH
ethanol

2ADP → 2ATP; $NADP^{\oplus}$ → NADPH



pyruvate

NADPH → $NADP^{\oplus}$



Lactate

same as homofermentative pathway

2.3 Probiotics

Probiotics are defined as "living micro-organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition" (Guarner, 1998) but interest in this area was initiated by Metschnikov 100 years ago (Metschnikoff,1907). Most probiotic microorganisms belong to Lactic Acid Bacteria (LAB), such as *Lactobacillus sp*, *Bifidobacterium sp* and *Enterococcus sp*. The yeast *Saccharomyces boulardii* has been studied extensively (Elmer et al., 1999) and also other bacterial species, like *Bacillus sp* (Senesi, 2001) and *Clostridium butyricum* (Takahashi, 2004).

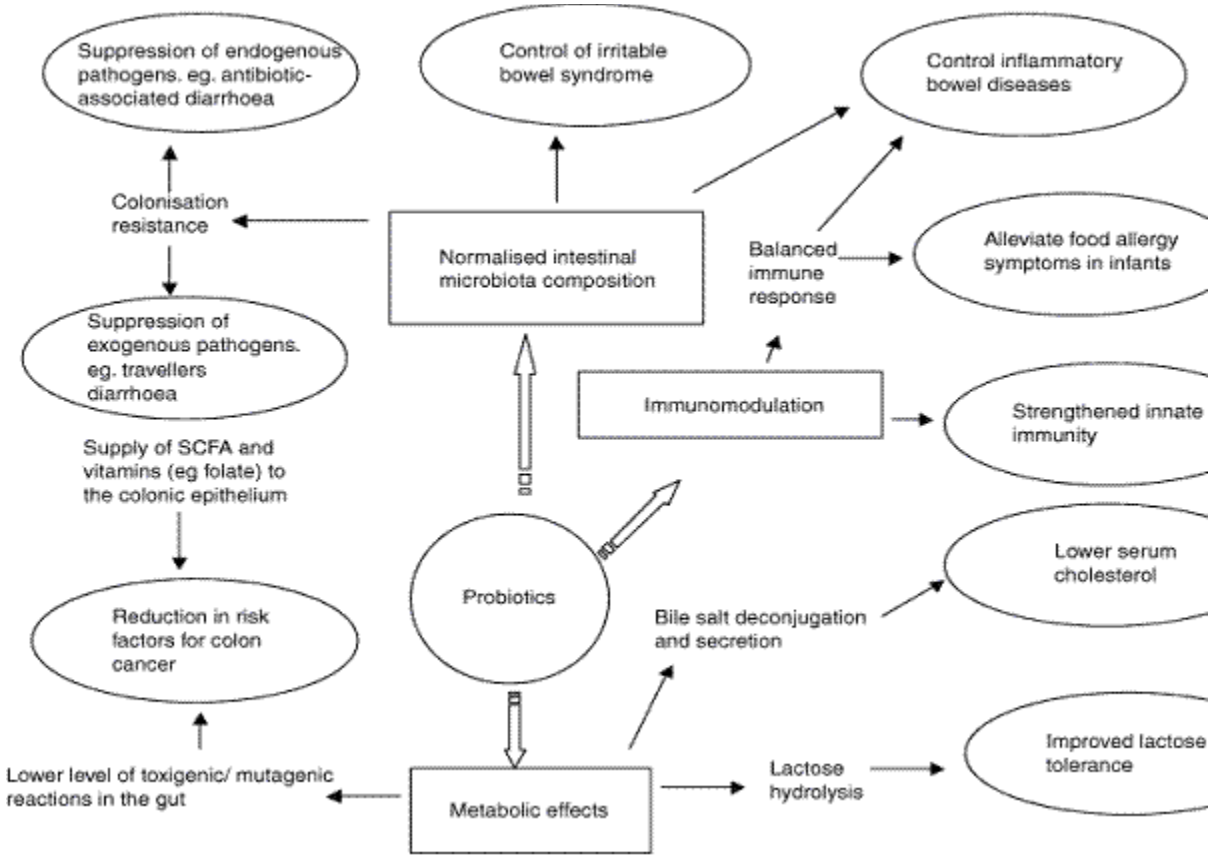
Most scientists agree that probiotic strains shall be able to survive transit through the gastric acid environment as well as exposure to bile and pancreatic juice in the upper small intestine to be able to exert beneficial effects in the lower small intestine and the colon, although there are convincing data on beneficial immunological effects also from dead cells (Mottet and Michetti, 2005). Best effect is achieved if the microorganisms colonise the intestinal surface mucus layer since they then can affect the intestinal immune system, displace enteric pathogens, provide antioxidants and antimutagens, and possibly other effects by cell signalling.

In recent years, multiple reports have described beneficial effects from various aspects on important diseases, like intestinal infections, inflammatory bowel disease (IBD), and allergy by addition of selected strains to food products, often together with fiber or a probiotic substance. In many countries, there are now several probiotic products on the market but the documentation is often based upon case reports, animal studies or uncontrolled small clinical trials. Furthermore, there is no general acceptance on how to characterize probiotic microorganisms, and few products declare the actual content of live microorganisms.

To qualify as a probiotic, certain criteria need to be met by a bacterium: a bacterial strain must be identified completely, be harmless for ingestion, adhere to mucosal membrane, able to colonize the gut epithelium, stable when stored, must survive the acid and bile salt concentration persisting in upper GI tract (Verna, 2010). Researchers have studied and used probiotics in a variety of medical conditions. However under ideal conditions of health and diet, the different strains of bacteria or micro-flora compete with and check the excessive number of any one strain.

A healthy condition can be achieved if a balance between the "good" and "bad" bacteria in the ratio of 85 percent to 15 percent.

Figure 3 : Health benefits of Probiotics



2.4 Indian traditional fermented foods as source of lactic acid bacteria

Traditional fermented foods are popular products since early history that have formed an integral part of the diet and it can be prepared in the household or in cottage industry using relatively simple techniques and equipment. Fermentation was evolved as a preservation technique during lean periods and prevention technique to counter spoilage of food products. It is one of the oldest and most economical methods for producing and preserving foods. In addition to preservation, fermented foods can also have the added benefits of enhancing flavor, increased digestibility, improving nutritional and pharmacological values (Jeyaram, 2011). Lactic acid bacteria perform

an essential role in the preservation and production of wholesome fermented foods. Homo-fermentative and the hetero-fermentative lactic acid bacteria are generally fastidious on artificial media but they grow readily in most food substrates and lower the pH rapidly to a point where other competing organisms are no longer able to grow. *Leuconostocs* and lactic *Streptococci* generally lower the pH to 4.0-4.5 and some of the *Lactobacilli* and *Pediococci* to about 3.5 (Steinkraus, 1983). Lactic acid bacteria (LAB) comprise large part of probiotic microflora. There are many LAB strains that have obtained ―generally regarded as safe‖ (GRAS) status and used commonly in commercial food products for human consumption. Probiotics are mono or mixed cultures of live microorganisms that might beneficially affect the host by improving the characteristics of indigenous microflora (Holzapfel, 1998). Lactic acid bacterial genera consist of *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Wesiella* etc. India is traditionally rich in fermented foods. In the Indian subcontinent, making use of fermented food using local food crops and other biological resources are very common. But the nature of the products and base material vary from region to region (Sekar, 2007). Fermented foods like *idli* and *dahi* were described as early as 700 BC. At present there are hundreds of fermented foods with different base materials and preparation methodology. Each fermented food is associated with unique group of microflora which increases the level of proteins, vitamins, essential amino acids and fatty acids. However, fermented foods are still produced traditionally by spontaneous fermentation and only limited knowledge has been obtained regarding the microflora of these products (Jeyaram, 2011).

2.4.1 Cereal based (with/without pulses) fermented foods

Cereal based fermented foods are considered as staple foods in their respective regions. Cereals such as rice (*Oryza sativum),* ragi flour *(Eleusine coracana),* wheat flour (*Triticum* spp.) and barley flour (*Hordeum vulgare)* are predominantly used and pulses such as black gram dhal, red gram, green gram dhal are used. These cereals and legumes are cultivated in India since Indus valley civilization (9000-5500 BC) period. They are considered as effective substrates for the production of probiotic-incorporated functional food, as they can be used as a source of non-digestible carbohydrates which stimulate the growth of *Lactobacilli* and *Bifidobacteria*. They contain water soluble fibres like β-glucan, arabinoxylan, galacto-oligosaccharides and

fructooligosaccharides, which are prebiotics (Swennen, 2006). Cereals and legumes are fermented by several groups of bacteria in the large intestine, yielding a variety of fermentation products, particularly short-chain fatty acids (SCFA). The resulting SCFA are known to provide an acidic environment in the large intestine, which stimulates the proliferation of probiotic cultures (Varadaraj, 2009). Mostly batter is prepared from these basic ingredients and batter is left overnight at room temperature for fermentation, occasionally sodium bicarbonate is added to provide anaerobic conditions for the growth of yeast and lactic acid bacteria. During the preparation of *Kallappam* fermented toddy is added as additional source of LAB. Fermented batter is either prepared as steamed cakes (*idli*) or pan cakes (*dosa, appam*) before it gets too soured. Predominant microflora isolated from batter of these foods include: *Weissella paramesenteroides, Lactobacillus fermentum, L. plantarum, Streptococcus faecalis, Pediococcus acidilactici, P. cerevisiae, Leuconostoc mesenteroides* *L. plantarum* AS1 isolated from south Indian fermented food *Kallappam* successfully prevented colonization of enterovirulent bacterium *Vibrio parahaemolyticus* in HT-29 cell line.

2.4.2 Milk based fermented foods

Milk and milk based products are consumed most popularly due to their nutritive value. For the same reason milk is easily spoiled by pathogenic microorganisms, hence fermentation of milk using lactic acid bacteria is preferred for prevention. Lactic acid bacteria convert milk sugar lactose into lactic acid and produces antibacterial substance bacteriocin to suppress spoilage bacteria. *Dahi* or curd is most popular traditional Indian fermented product prepared by fermentation of milk by lactic acid bacteria. *Dahi* differs from yogurt in its use of mixed starters of mesophilic lactococci. A principle flavour-inducing metabolite is diacetyl, which is appreciated more by people of South Asian origin compared to the acetaldehyde flavour in yogurt. Yak (*Bos grunniens*; now *Poephagus grunniens*) is one of a few domesticated animals capable of surviving in extreme environmental conditions. It is mainly found in the highlands of the Nepalese Himalayas, India (Kashmir and Arunachal Pradesh), China (Tibetan highlands), Mongolia and Bhutan. The composition of yak milk is 16.9–17.7 g/l dry matter, 49–53 g/l protein, 55–72 g/l fat, 45–50 g/l lactose and 8–9 g/l minerals. Yak milk is processed into a number of dairy products such as fermented milk (*Kurut*), cheese (*Chhurpi*), *Chhur churpen,*

Churkham, Chhu, Philuk, Shyow and *Maa.* The chemical composition of yak cheese is around 68.2% of total solid (TS), 49.4% of butterfat on a dry matter basis and 1.37% of salt. It is largely consumed in the Himalayan highland and its industrial production is not yet standardized (Prashant Chauhan, 2009). LAB species isolated from fermented milk products include *Streptococcus cremoris, S. lactis, S. thermophilus, Lactobacillus bulgaricus, L. acidophilus, L. helveticus, L. cremoris, L. plantarum, L. curvatus, L. fermentum, L. paracasei* subsp. *pseudoplantarum, L. alimentarius, L. kefir, L. hilgardii*, *Enterococcus faecium, Leuconostoc mesenteroides, L. farciminis, L. brevis, Lactococcus lactis* subsp. *cremoris, L. casei* subsp. *casei* and *L. bifermentans.* There are reports that LAB isolated from *dahi* can be used to cure intestinal disease such as diarrhea (Agarwal, 2001), intake of dahi has anti-cholesteremic (Singh *et al*., 2012), anti-diabetic and angiotensin-converting enzyme inhibition effect.

2.4.3 Vegetable, bamboo shoot and unripe fruits based fermented foods

The lactic acid fermentation of vegetables, applied as a preservation method for the production of finished and half-finished products, is considered as an important technology because of its capability to improve the nutritive value, palatability, acceptability, microbial quality and shelf life of the fermented product. Moreover, this is a remarkable procedure to store the perishable vegetable in absence of cold-storage or refrigeration, where majority of rural people cannot afford canned or frozen foods. Certain fermented vegetable products (*gundruk, sinki, iniziangsang*) are said to be good appetizers and the ethnic people use these foods for remedies from indigestion (Tamang, 2009). Fermented bamboo shoot (BSs) products are consumed as a traditional food by ethnic people of North-Eastern states of India (Tamang, 2009). In India, BSs are harvested annually in Sikkim (26.2 t), Meghalaya (435 t) and Mizoram (426.8 t). Bamboo shoots are low in fat and cholesterol, but very high in potassium, carbohydrates and dietary fibres. Many nutritious and active materials (vitamins and amino acids) and antioxidants (flavones, phenols and steroids) can be extracted from bamboo shoots.

LABs are the dominant microorganisms in ethnic fermented vegetables and bamboo shoot products (Tamang *et al.,* 2009). *Pediococcus pentasaceous, Lactobacillus cellubiosus, L. plantarum, L. fermentum, L. brevis, L. mesenteroides, Lactococcus lactis, Enterococcus faecium,* and *P. acidilactici* are predominant LAB species found in fermented vegetables (Tamang, 2009)

determined the functional properties of lactic acid bacteria isolated from ethnic fermented vegetables (*gundruk, sinki, khalpi* and *inziangsang*) of the Himalayas. LAB strains showed strong acidification and coagulation activities. They showed antimicrobial activity, particularly a strain *L. plantarum* isolated from *inziangsang*, a fermented leafy vegetable product, was inhibitory towards *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Foods contain *Lactobacillus plantarum, L. brevis, L. corniformis, L. delbrueckii, L. fermentum, Leuconostoc fallax, Lactococcus lactis, L. mesenteroides, Enterococcus durans, Streptococcus lactis, L. casei* and *Tetragenococcus halophilus* as predominant LAB species, they also showed functional probiotic properties.

Table 2: Lactic Acid Bacteria Isolated from Indian fermented food

Fermented food	Usual Composition/Ingredients	Place of Origin/Usage	Lactic acid Bacteria Isolated	Reference
Cereal Based (with/without pulses) Fermented Foods				
Koozhu	*Eleusine coracana* (ragi) flour, boiled rice, non-fat yoghurt	Tamil Nadu	*Weissella paramesenteroides, Lactobacillus fermentum*	Satish *et al.*, 2010
Pazhaiya soru	Rice, curd and salt	Tamil Nadu	*Streptococcus faecalis, Pediococcus acidilactici*	Ramakrishnan, 1977; 1979
Idli	Rice, black gram dhal, table salt, fenugreek seeds	South India	*Leuconostoc mesenteroides, S. faecalis, P. cerevisiae*	Mukherjee *et al.*, 1965, Steinkraus *et al.*, 1967
Dosa	Rice, black gram dhal (either raw or parboiled rice), table salt	South India	*L. mesenteroides, S. faecalis*	Labana, & Kawatra, 1986, Chavan, & Kadam, 1989; Steinkraus, 1996
Adai Dosa	Boiled rice, Bengal gram, red gram, black gram, green gram	South India	*Pediococcus sp., Streptococcus sp., Leuconostoc sp.*	Chavan, & Kadam, 1989
Kallappam	Boiled or raw rice, coconut toddy	South India	*L. fermentum, L. plantarum*	Satish *et al.*, 2010
Dhokla	Bengal gram dhal, rice and leafy vegetables	North India	*L. fermentum, L. mesenteroides, S. faecalis*	Ramakrishnan *et al.*, 1976; Blandino *et al.*, 2003, Roy *et al.*, 2007
Ambali	*Ragi* (Millet) flour and rice	India	*L. fermentum, L. mesenteroides, S. faecalis*	Ramakrishnan, 1977; 1979
Cereal/pulse and butter milk based fermented food				
Rabdi (*Rabadi*)	Flour of Barley, Pearl millet, corn or soybean and country buttermilk	Rajasthan	*Bacillus sp., Micrococcus sp.*	Blandino *et al.*, 2003
Mor Kuzhambhu	Butter milk, gram flour, vegetables, spices	Tamil Nadu	*Weissella paramesenteroides*	Satish *et al.*, 2010
Cereal based fermented sweets and snacks				
Jilebi	Wheat, sugar and curd	South India	*L. fermentum, S. lactis, L. buchneri, S. faecalis*	Ramakrishnan, 1977, Prakash *et al.*, 2004
Bhaturu or Indigenous bread	Wheat and starter material *Khameer/ Malera*	Himachal Pradesh	*L. plantarum, L. acidophilus, L. mesenteroides, Lactococcus lactis*	Tamang, 1998, Thakur *et al.*, 2004; Kanwar *et*

2.4.4 Traditional fermented food: *dhokla*

Dhokla, a steamed fermented product, is a traditional food popular in Western India, particularly in the States of Gujarat and Maharashtra. The product has a soft and spongy texture which is prepared from a mixture of Bengalgram dhal (*Cicer arietinum*), dehulled Blackgram dhal (*Phaseolus mungo*) and milled rice in the ratio of 2:1:1. The mixture of legumes and cereal, after 6–8 h of soaking in water, is then ground into a grainy consistency. The resulting batter is mixed with curds in 1:1.5 (w/w) and allowed to ferment for 16–18 h. fermented batter is prepared into suitable shapes and steamed. The steamed product is then seasoned with oil, spices and greens (coriander leaves) prior to consumption (Balasubramanyam, B.V., PhD Thesis, Univ. Mysore, Mysore, 1996).

2.5 Nutraceuticals

Nutraceuticals are natural bioactive chemical compounds and have value in health promoting, disease preventing or semi-medicinal properties. They are found as natural products from (a) the food industry, (b) the herbal and dietary supplement, (c) pharmaceutical industry, and (d) the newly emerged bioengineered microorganisms, agroproducts or active biomolecules. It may range from isolated nutrients, herbal products, dietary supplements and diets to genetically engineered "custom" foods and processed products such as cereals, soups and beverages (Dureja, 2003)**.** Chemically the nutraceuticals may be classified as isoprenoid derivatives (terpenoids, carotenoids, saponins, tocotrienols, tocopherols, terpenes), phenolic compounds (couramines, tannins, ligrins, anthrocynins, isoflavones, flavonones, flavanoids), carbohydrate derivatives (ascorbic acid, oligosaccharides, non-starch polysaccharides), fatty acid and structural lipids (n-3 PUFA, CLA, MUFA, sphingolipids, lecithins), amino acid derivatives (amino acids, allyl-S compounds, capsaicnoids, isothiocyanates, indols, folate, choline), microbes (probiotics, prebiotics) and minerals (Ca, Zn, Cu, K, Se) (Malik, 2008).

Some prominent evidences are reported in favor of cancer inhibitory metabolic activity of nutraceuticals in the human body:

1) Nutraceuticals may act as essential amino acid drug like essential nutrients. For example, tryptophan is needed for protein synthesis at low dose in humans. At high dose, it increases brain 5-hydroxytryptamine levels and thus acts as a drug to treat the insomnia (Rishi., 2006)

2) The nutraceutical preparations containing phytosterols are effective in lowering LDL cholesterol and osteoporosis.

3) Bovine milk fat globule acts as anticancer, anticholesterolemic, coronary heart disease (VL., 2005)

4) Vitamin C, vitamin E, β-Carotene, lycopene (carotenoids), lipoic Acid, glutathione(thiols) play role in cancer prevention and inhibition of necrosis; Co-Enzyme Q-10, super oxide dismutase (enzyme), selenium, copper, manganese, zinc (minerals) act as anticancer nutraceuticals in cancer management by delayed apoptosis observed in isolated cancer cells

2.6 Release of bioactive peptides in food as a consequence of proteolysis

2.6.1Proteolytic activity of LAB and cleavage of milk protein

Lactose in milk is the only available carbohydrate for providing energy to LAB that possesses the enzyme lactate dehydrogenase for the synthesis of lactic acid. Nitrogen is a growth-limiting factor for yogurt starter because milk has an inadequate supply of protein breakdown products to support good growth. Although caseins contain all amino acids necessary for the growth of LAB in milk to high cell density, less than 1% of these are actually needed (Kunji et al., 1996). Hence, to obtain nitrogen for their growth, these organisms have to rely on their own enzyme systems.

One important result of the addition of the bacteria necessary for fermentation is the resulting proteolytic activity of yogurt bacteria. Protein degradation by microbial enzymes in yogurt is a desirable process that improves milk digestibility and enhances nutritional quality. Lactic acid bacteria are usually weakly proteolytic; however, they do cause a significant degree of proteolysis in yogurt (Abu-Tarboush, 1995).

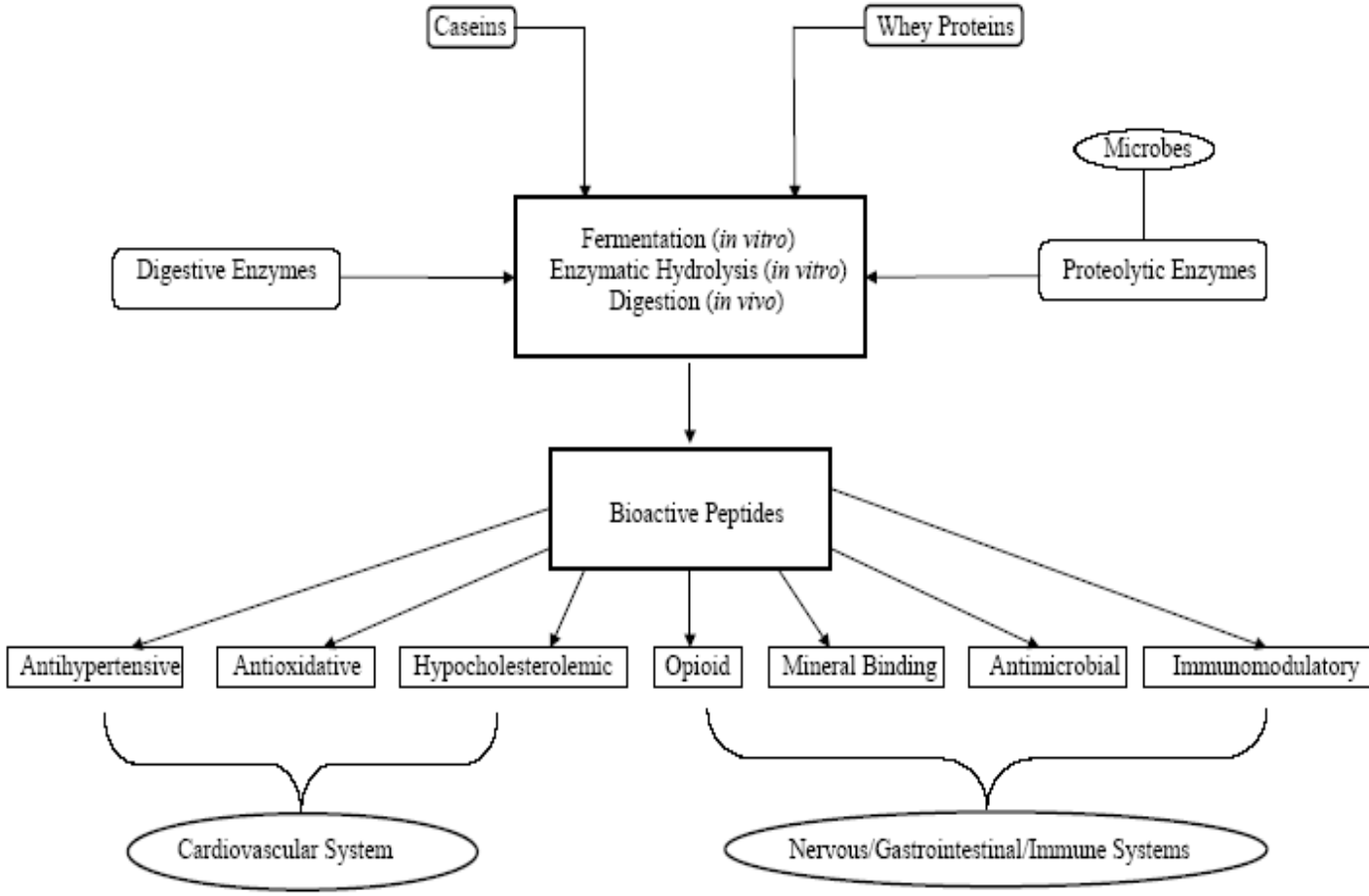
2.6.2 The proteolytic system

The proteolytic enzymes found in different species of LAB show different protease activities and complex system of endo- and exopeptidases, which may differ in nature, specificity and cell location (Kunji et al., 1996). In general, LAB possess: a. Proteases located in the microbial cell envelope that permit the degradation of caseins into oligopeptides b. Peptide transport systems that allow the internalization of the released oligopeptides c. Intracellular peptidases that hydrolyse the oligopeptides into peptides or into amino acids to be used by the cells (Kunji et al., 1996; Juillard et al., 1998). It has been established that degradation of caseins is initiated by a single cell wall-bound extracellular serine-protease. Lactic acid bacteria typically possess only one cell-envelope protease (CEP) but the presence of two CEPs was reported in strains of *L. helveticus* and *L. delbrueckii* ssp. *bulgaricus* (Stefanitsi et al., 1995; Pederson et al., 1999). Cell envelope proteases (PrtP) have a strong preference for hydrophobic caseins. These proteases are critical for growth of LAB in milk because they hydrolyse casein into more than 100 smaller peptide fragments. They have very broad substrate specificity and the biochemical properties of the proteinases of the various LAB are very similar, most of them being serine-proteases of similar size. *Lactococcus* PrtPs are divided into PI- and PIII-type enzymes, distinguished by their substrate specificity for αs1-, β-, and κ-caseins (Kunji et al., 1996). The PI-type primarily degrades β-casein that is cleaved into more than 100 different oligopeptides ranging from 4 to 30 amino acid residues. PI-type enzyme cleaved κ-casein to a lesser extent, whereas the PIII-type is able to cleave αs1-, β-, and κ-caseins equally well. For *Lactobacillus*, CEPs PI-, PIII-, the intermediate PI/PIII-type, and some novel type substrate specificities were reported, whereas CEP exhibiting the intermediate PI/PIII-type specificity was purified from *S. thermophilus* (Savijoki et al., 2006).

Bioactive peptides usually contain 3 to 20 amino acid residues per molecule. They have been found to have specific activities, such as antihypertensive, antioxidative, antimicrobial, immunomodulatory, opiod or mineral-binding activities. Many milk-derived bioactive peptides reveal multifunctional properties, i.e., specific peptide sequences may exert two or more different biological activities. Due to their physiological and physicochemical versatility, milk-borne bioactive peptides are regarded as important ingredients for health-promoting functional foods (Korhonen and Pihlanto-Leppälä, 2004). The major means for producing biologically active

peptides from milk peptides is shown in Figure 4. Of these, the focus of this review is on the peptides regulating the cardiovascular system, more specifically, those that exhibit angiotensin-I converting enzyme (ACE)-inhibitory and hypocholesterolemic effects.

Figure 4 : Biologically active peptides from major milk proteins



2.6.3 Diabetes and peptides as inhibitors of α-Glucosidase

Diabetes mellitus (DM) is a metabolic disorder characterised by a congenital (DM1) or acquired (DM2) inability to transport glucose from the bloodstream into cells. DM2 and associated cardiovascular diseases and cancer are an increasing problem around the globe, especially in the developed world (Beaglehole, 2003). A worldwide survey reported that diabetes mellitus is affecting nearly 10% of the population every year (Vetrichelvan, 2002). Diet and exercise are the first steps in the treatment of DM2. But if these measures alone fail to suffi- ciently control blood glucose levels, starting oral drug therapy is recommended.

Many efforts have been made searching for effective and safe AGIs from natural materials in order to develop a physiological functional food or lead compounds for antidiabetic (Matsui, 2001).

The findings strongly led us to study antidiabetic compounds from natural resources as an alternative medicinal food. As a continuing part of our screening for AGIs, prospecting Indonesian herbs were investigated to determine the antihyperglycemic effect (Gunawan-Puteri, 2009).

Acarbose, voglibose and migitol are commercial alpha glucosidase inhibitors that are considered as first line of treatment for diabetic individual for post-prandial hyperglycemia. On giving them along with other oral hypoglycemic agents like metformin, sulfonylurea improves glycemic control (reduced HbA1c). However these glucosidase inhibitors have prominent gastrointestinal side effects like flatulence, diarrhoea and abdominal discomfort (Hollander., 1992). This warrants the search for alternate sources that have fewer side effects.

α-glucosidase (EC 3.2.1.20) catalyzes the final step in the digestive process of carbohydrates. Its inhibitors can retard the uptake of dietary carbohydrates and suppress postprandial hyperglycemia and could be useful for treating diabetic and/or obese patients. (Toeller, 1994). Plants have long been used for the treatment of diabetes, particularly in developing countries where most people have limited resources and do not have access to modern treatment. Because of the possible importance of these inhibitors in plant physiology and animal and human nutrition, extensive research has been conducted on their properties and biological effects (Garcia-Olmedo F, 1987).

Other sources include milk. Yogurt is one of the most popular fermented foods and traditionally

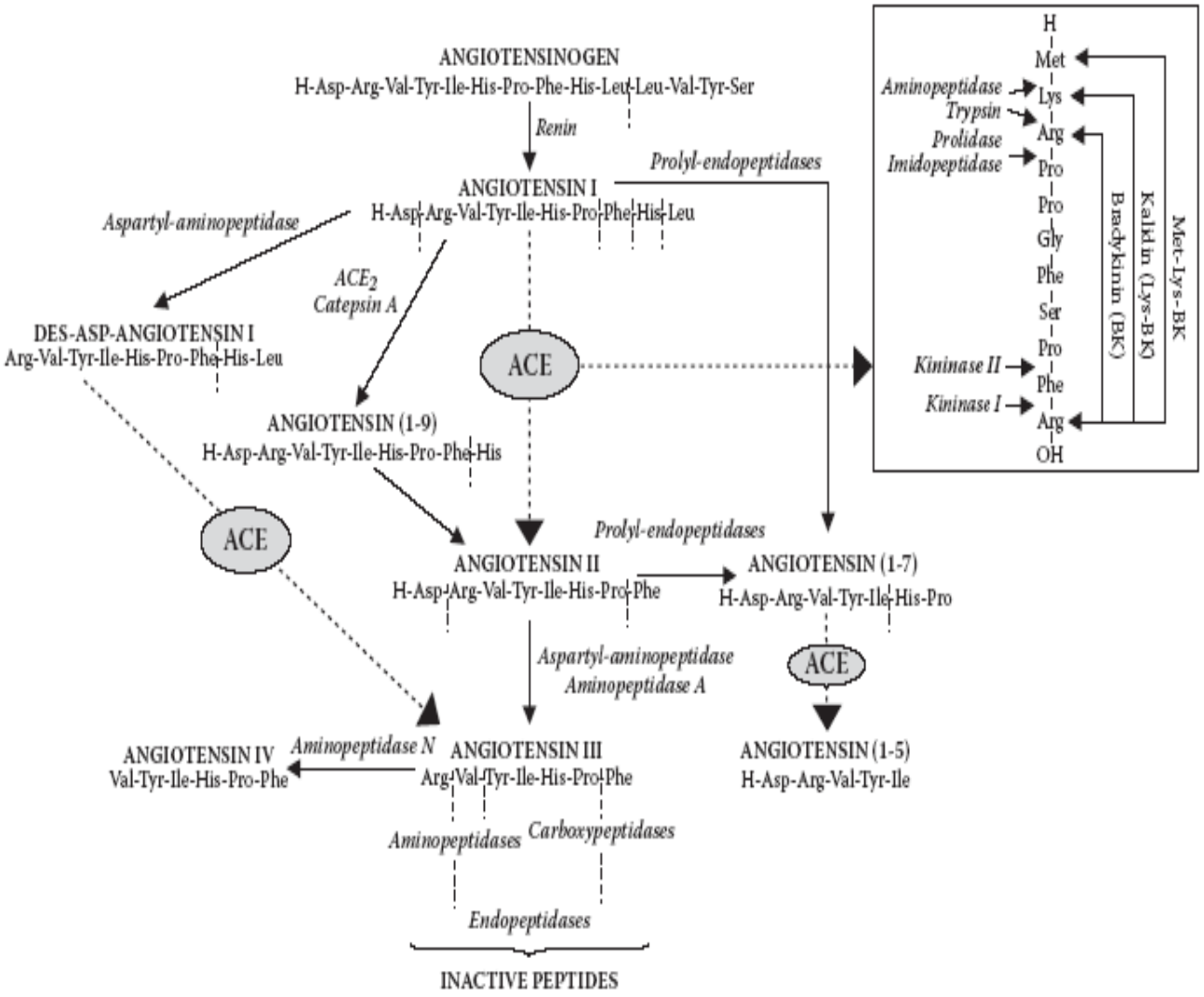
consumed for a long time in many countries (Nakasaki, 2008). It is formed during the slow lactic fermentation of milk lactose by the thermophilic lactic acid bacteria. Most of the of α-glucosidase inhibitors are from botanical sources and few from dairy and other sources**.** There are few reports about development of a food matrix which would deliver inhibitors of α-glucosidase for improving cases of diabetes. This study was thus undertaken to develop a food product which would provide nutraceutical in the form of α-glucosidase and Angiotensin Converting Enzyme (ACE) inhibitors.

2.7 Hypertension and inhibition of Angiotensin Converting Enzyme (ACE)

Hypertension is defined as a sustained increase in blood pressure (BP) and is a controllable risk factor in the development of a number of cardiovascular diseases (CVD) such as stroke and coronary infarction. Uncontrolled high BP increases the risk for CVD, stroke, heart failure and kidney disease. Even a small decrease in BP results in a significant reduction in the risk of CVD and a 5 mm Hg reduction in diastolic BP reduces the risk of heart disease by approximately 16% in hypertensive subjects (Fitzgerald, 2004). Such a reduction in diastolic BP corresponds to a decrease in systolic BP by 9-10 mm Hg. It has been estimated that a decrease in systolic BP by 3 mm Hg would reduce the risk of stroke by about 10-13% (Tuomilehto, 2004). The high cost of and potential adverse side effects associated with pharmacological therapy for hypertension have encouraged individuals to adopt lifestyle modifications such as weight reduction, low-fat dairy products, dietary sodium reduction and regular physical activity to combat hypertension (Miller, 2007b). The blood pressure lowering effects of specific hydrolysates of casein and whey proteins or fermented dairy products provide compelling evidence for a beneficial role of dairy peptides to induce clinically significant reductions in systolic BP and diastolic BP with no reported adverse effects (Huth, 2006)

The main mechanism involved in the antihypertensive effect of food-derived peptides is the inhibition of the angiotensin-converting enzyme (ACE). The activity of this enzyme is decisive for the effectiveness of a system, namely the renin–angiotensin system, which plays a crucial part in the maintenance of arterial blood pressure, and in the organic damage that occurs when this variable is increased. The change in arterial tone caused by the renin–angio-tensin system may in reality be critical in some hypertensive patients.

Figure 5 :Biochemical routes and peptides of the rennin-angiotensin system



The bioactive peptides such as ACE-inhibitory peptides must reach their target organ intact to exert their effects in vivo. Degradation of peptides in the acidic environment of the stomach, alkaline conditions of the small intestines as well as hydrolysis by the brush border peptidases can either activate or deactivate ACE-inhibitory peptides before they reach the portal circulation. Therefore, only those ACE-inhibitors that are not affected by the action of angiotensin-II and gastrointestinal enzymes or those that are converted to stronger ACE-inhibitors exert antihypertensive effects *in vivo* (Korhonen, 2003.) suggested that in case of oral ingestion, purification of the bioactive peptide would be less suitable than using the whole hydrolysate. The release of bioactive peptides could be reinforced after digestion, whereas intake of an in vitro highly active pure peptide would be lost during this in vivo

digestion. Due to the incomplete and often unknown bioavailability of the ACE-inhibitory peptides following oral administration, it is difficult and unreliable to predict the in vivo antihypertensive effect based on inhibitory activity in vitro (Erdmann, 2008.). Although valuable information can be obtained from in vitro model systems regarding the proteolytic/peptideolytic stability and susceptibility to intracellular passage of these peptides, the actual hypotensive effects can be reliably assessed only through in vivo studies. The in vivo effects are tested in spontaneously hypertensive rats (SHR), which constitute an accepted model for human essential hypertension (López-Fandiño, 2006.). Numerous rat studies have been performed to determine the hypotensive effect of milk protein derived ACE-inhibitors.

Table 3: Functional Products with Antihypertensive Peptides

Produce	Commercial Name	Active Component	Company/Country	Clinical Studies
Fermented milk	"Ámeal Peptide"[a] "Flora Proactive"[b]	IPP, VPP	Calpis Co./Japan[a] Urilever/Spain[b]	Hata et al., 1996; Mizushima et al., 2004; Aihara et al., 2005; Mizuno et al., 2005
Fermented milk	"Evolus"[a] "LH"[b] "KaikuVita"[c] "Emmi-Evolus"[d]	IPP, VPP	Valio Ltd./Finland[a] Mjòlkursamsalan/Iceland[b] Kaiku-Iparlat/Spain[c] Emmi/Switzerland, Portugal, Malta and Italy[d]	Seppo et al., 2002, 2003; Tuomilehto et al., 2004; Jauhiainen et al., 2005
Casein hydrolysate	"Casein DP peptio drink"	FFVAPFEVFGK	Kanebo, Ltd/Japan	Sugai, 1998; Sekiya et al., 1992
Casein hydrolysate	"C12 Peption"	FFVAPFEVFGK	DMV International/Holland	Townsend et al., 2004; Cadee et al., 2007
Whey proteins hydrolysate	"Biozate"	Whey peptides	Davisco/USA	Pins and Keenan, 2002, 2003, 2006
Bonito proteins hydrolysate	"Peptide soup"	LKPNM	NIPPON/Japan	Fujita et al., 1997a,b; Fujita et al., 2001
Sardine proteins hydrolysate	—	VY	Approved by Japanese goverment as FOSHU[4]	Kawasaki et al., 2000, 2002

2.8 Application of functional food to improve conditions of Vitamin deficiency

Vitamin C (ascorbic acid) plays a major role in human metabolism ranging from the synthesis of collagen, carnitine and norepinephrine to a large number of antioxidant activities (Padayatty, 2003). Humans are dependent on dietary sources, mainly fruit and vegetables as they are unable to synthesize vitamin C. Even in high income countries population-based studies have reported blood levels of vitamin C in the range indicating deficiency in around 1 in 5 men and 1 in 9 women in low income groups (Cahill, 2009). There are scarce data on vitamin C deficiency in resource-poor countries where vitamin C deficiency might be expected to be more prevalent. A systematic review found that a third of reproductive age women in resource-poor settings had dietary vitamin C intakes below the Estimated Average Requirements (EAR) rising to nearly 50% in Africa and South East Asia (Torheim, 2010)

Riboflavin is continuously excreted in the urine of healthy individuals. A deficiency of riboflavin can be primary - poor vitamin sources in one's daily diet - or secondary, which may be a result of conditions that affect absorption in the intestine, the body not being able to use the vitamin, or an increase in the excretion of the vitamin from the body (Brody, 1999)

Materials and Methods

3. Materials and Methods

3.1 Microorganisms

Lactococcus lactis subsp. *lactis* used in this study was isolated from pickled yam and has been proved for its probiotic attributes for the production of high levels of ϒ-aminobutyric acid (BHANWAR, 2012). Working cultures of the strains were maintained on de Man, Rogosa and Sharpe (MRS) agar (Himedia, Mumbai) at 4-6 °C and were used to inoculate 100 ml of MRS. Routine cultures were drawn by incubating at 37 °C under shaking conditions.

3.2 Gram staining

Bacterial smear from actively growing cells were spread on a glass slide and heat fixed. Smear was flooded with filtered crystal violet for 10 sec and then washed briefly in water to remove excess crystal violet. Later, it was flooded with Gram's iodine for ten sec and washed briefly in water. Smear was decolourised with acetone until the moving dye front had passed the lower edge of the section and washed immediately in tap water. Counterstaining was carried out with safranin for fifteen sec and washed with water to remove the excessive stain. Finally, samples were visualized under microscope at different magnification. The morphology of strains was studied upto 100 x magnifications under microscope (Nikon, 200 N, Japan)

3.3 Growth Kinetics Study

Hundred microlitres of routine cultures were used to inoculate 100 ml of MRS media. Samples were analysed at intervals of 2 hrs where density of viable cells was measured by noting Absorbance at 660 nm in a spectrophotometer (Hitachi, Japan).

3.4 Preparation of *Lactococcus lactis* yoghurt

Following overnight incubation at 37 °C, the culture was centrifuged at 8000 rpm for 10 min, 4 °C) **(**Hitachi, Japan**)** with successive washing of pellets washed with 0.85 % saline. Double toned milk (Verka) was purchased from a local supermarket (Patiala, Punjab) and was heated to 80 °C in a water bath for 10 minutes and cooled to room temperature. Three hundred millilitres of the milk was inoculated with the cell pellets (1 %) in order to ferment milk to

form yoghurt (*Lactis* yoghurt) which would provide starter to ferment the food batter. The milk was fermented for 10-15 hrs to allow cells to reach exponential growth.

3.5 Fermented food formulation and sensory evaluation

Commercially available gram flour (chickpea flour from *Shakti bhog besan*), dehusked Blackgram (V*igna mungo*) flour was purchased from a local grocery store in Patiala, Punjab, India. Preparation protocal of the snack food followed traditional recipe of *Dhokla* (**Balasubramanyam, 1996, Mysore)**. *V.mungo* flour was added as a source of glutamate. Chickpea flour, V*igna mungo* flour and *Lactis* yoghurt was mixed in the ratio of 1.5: 1.5: 1 to form a batter which was left to ferment at ambient temperature. The process of fermentation was confirmed with the leavening of batter and drop in pH. The fermented batter was mixed with spoonful of sodium bicarbonate poured into moulds and steamed for 20-30 minutes or until cooked. Protocol for control product was exactly the same but without the addition of *lactis* yoghurt, replacing it with sterile water. The steamed product was seasoned with a mixture containing fried mustard seeds, chillies and lemon juice with a pinch of salt and sugar, serving size being approximately 100 grams.

3.6 Sensory Evaluation and Storage

Sensory qualities of the formulated food fermented by probiotic cultures were evaluated by a panel of judges by grading for colour, smell, consistency, texture and overall acceptability score on a 9-point hedonic scale (Moretti VM, 2004). Food samples were stored in sealed aseptic containers at 4 °C.

3.7 Extraction of putative peptides

3.7.1 Preparation of yoghurt extract (Trichloroacetic acid filtrate)

Trichloroacetic acid filtrate was prepared by mixing 5 ml sample with 1 mLMilliQ water and 10mL of 0.75 N TCA followed by centrifugation at 4000 × *g* for 30 min at 4 °C. The supernatant thus obtained was passed through a 0.45-μm membrane filter and stored at −20 °C until assayed (SHAH, 2007)

3.7.2 Preparation of food extract

Sample of the prepared food was dried and ground into powder; 10 grams of the sample were extracted with 50 ml of phosphate buffer (PB) pH 7 at 80 °C in a water bath for 2 hours. The sample extract was centrifuged at 12000 rpm for 10 minutes at 27 °C. The resultant supernatant was filtered and the filtrate was stored at -20 °C. The extract was equilibrated to room temperature before any assay performed on it.

3.8 Enzyme assays

3.8.1 Preparation of Acarbose stock

Glucobay 50 tablets, labelled to contain 50 mg acarbose under the licence of Bayer (India) Ltd. were purchased from commercial source in local market. Stock solutions of acarbose (1mg/ml) were prepared by dissolving 50 mg in 50 ml distilled water. These solutions are found to be stable for almost three days when refrigerated (F. A. Ibrahim, 2007)

3.8.2 Assay for α-glucosidase inhibitory activity

The assay for α-glucosidase inhibitory activity was performed according to the chromogenic method as described by Watnabe *et al.* 1997 (Jun Watanabe, 1997). α-glucosidase enzyme from *Saccharomyces cerevisiae* was obtained from Sigma chemicals and was dissolved in 100 mM of phosphate buffer (pH 7.0) containing 2 g/litre bovine serum albumin and 0.2 g/litre Sodium azide and was called enzyme solution. 5 mM Para-Nitrophenyl-alpha-D-glucopyranoside (PNPG), obtained from Sigma chemicals, was dissolved in the same buffer (pH 7.0) and was used as a substrate solution. 500 µl of the enzyme solution and 100 µl of the food extract were mixed and incubated for 5 minutes in 2 ml Eppendorf tubes at room temperature. 500 µl of substrate PNPG solution was then added to the reaction mixture and incubated for another 5 minutes at room temperature. For positive control Control ,
100 µl of the food extract was replaced with same volume of Acarbose stock (1mg/ml) and for negative control C^- , phosphate buffer was used in place of food extract. The absorbance at 405 nm was measured with a spectrophotometer (Hitachi,Japan). Inhibitory activity was expressed as 100 minus relative absorbance difference (%) of test compounds to absorbance of the control (C^-) where test solution was replaced by phosphate buffer.

3.8.3 Assay for Angiotensin Converting Enzyme (ACE) inhibitory activity

Spectrophotometric stop rate determination protocol for ACE inhibitory activity was according to the quality control test procedure of provider of ACE, Sigma chemicals. Briefly, 200 µl of substrate Hippuryl-L-Histidyl-L-Leucine (HHL) Solution (0.3% w/v) in HEPES buffer (50 mM) was incubated with 250 µl of food extract. In case of positive control, equal volume of 1M Hydrochloric acid replaced the food extract. The mixture was equilibrated at 37 °C for 15 minutes before addition of 50 µl of ACE solution. For negative control, the substrate was directly incubated with 50 µl of the enzyme solution thus allowing uninhibited reaction to proceed. For a reaction volume of 250 µl enzyme concentration was 0.016 U/ml. Enzyme and substrate were allowed to react for 15 min before terminating with 250 µl of 1M Hydrochloric acid. Amount of hippuric acid released from HHL was extracted with ethyl acetate. 2 ml of ethyl acetate was added to the reaction mixture and centrifuged at 10,000 rpm for 2 minutes at 25 °C. 1 ml of the clear upper layer from each vials were transferred to fresh vials and kept in boiling water bath for 15 minutes or until ethyl acetate has evaporated. 3 ml of deionized water were added to all the vials and mixed by inversion. Absorbance A_{228nm} was measured using a spectrophotometer (Hitachi,Japan). Inhibitory activity was expressed as 100 minus relative absorbance difference (%) of test compounds to absorbance of negative control.

3.8.4 Measurement of Peptide Content

The peptide content of hydrolysate was measured by method of Church et al. (Church FC, 1983) with some modifications. Fifty milliliters of reagent was prepared by mixing 25 ml of 100 mMborax, 2.5 ml of 20% (w/w) sodium dodecyl sulfate, 40 mg of o-phthaldialdehyde solution (dissolved in 1 ml of methanol) and 100 ml of b-mercaptoethanol and then adjusted the volume to 50 ml with deionized water. Fifty microliters of this permeate was mixed with 2 ml of reagent. The reaction mixture was incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured with spectrophotometer (LABINDIA). The peptide content was quantified using enzyme hydrolysate of casein (Himedia, Mumbai) as standard

3.9 Evaluation of functional properties of the novel formulated food

3.9.1 Estimation of Ascorbic Acid

The method of redox titration was followed using iodine solution (British Pharmacopoeia 2003; Page 163,164). The reaction proceeded with the oxidation of ascorbic acid by iodine to dehydroascorbic acid and conversion of iodine to iodide. Once all the ascorbic acid has been oxidised, the excess iodine is free to react with the starch indicator, forming the blue-black starch-iodine complex. This is the endpoint of the titration. To 10 ml aliquots of sample food extracts in 250 ml conical flasks, 4-5 drops of 1 % starch indicator solution was added. Titration was performed with 0.05 M iodine solution till the end point (blue-black colour, due to starch-iodine complex) was reached. The concentration of ascorbic acid present in the food extract was estimated from a standard plot where L-ascorbic acid was used in varying concentrations.

3.9.2 Estimation of Riboflavin (Vitamin B2)

Estimation of riboflavin was determined colorimetrically, 0.8 ml of the sample was added to 0.2 ml of 1 M Sodium Hydroxide. To 0.4 ml of the resulting solution 1 ml of 0.1 M Potassium phosphate buffer (pH 6) was added to neutralize. Absorbance at 444 nm was measured using a spectrophotometer. Concentration of riboflavin in the food samples was estimated with riboflavin as standard.

3.9.3 Qualitative determination of ϒ-aminobutyric acid (GABA)

GABA in the food extract was presumptively detected using thin-layer chromatography (TLC) with some modifications as in the method described by Cho *et al.* (Cho, 2007). Silica gel (30-40 %) in distilled water was layered on clean glass plate using TLC applicator. Spotting was performed using 2 µl of GABA standard (GNC, USA) along with 20 µl of the food extract on the silica plate. The elution solvent for TLC analysis was n-butanol, acetic acid, water in the ratio 4:1:1 which was allowed to run in a sealed glass chamber for 40 minutes. The solvent was evaporated in a hot air oven at 60 °C. The TLC plate was stained with ninhydrin solution and was incubated at 110 °C in a hot air oven for development of red-violet spots. The retention value, $R_{f,}$ of the sample was compared with R_f of GABA which was 0.06.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Since peptides are generally not elaborated in media and a non dairy food product was required as dairy products were mostly investigated we choose fermented traditional product preferably as a snack food or as a side dish

Lactococcus lactis subsp. lactis which was previously isolated (from pickled yam) and has been thoroughly characterized for some of its functional properties. For instance β-galactosidase, lactic acid and GABA was elucidated in a recent study (Bhanwar, 2012). It was pertinent to explain the culture for its attributes specially for α-glucosidase and ACE inhibition. Working culture was revived from glycerol stock and colonies were maintained on MRS agar plates after continuous streaking on them.

4.1 Gram Staining and Growth Kinetics

Gram staining revealed their morphology as gram positive cocci which appeared in pairs and short chains.

Figure 6: Gram Staining of *L. lactis*

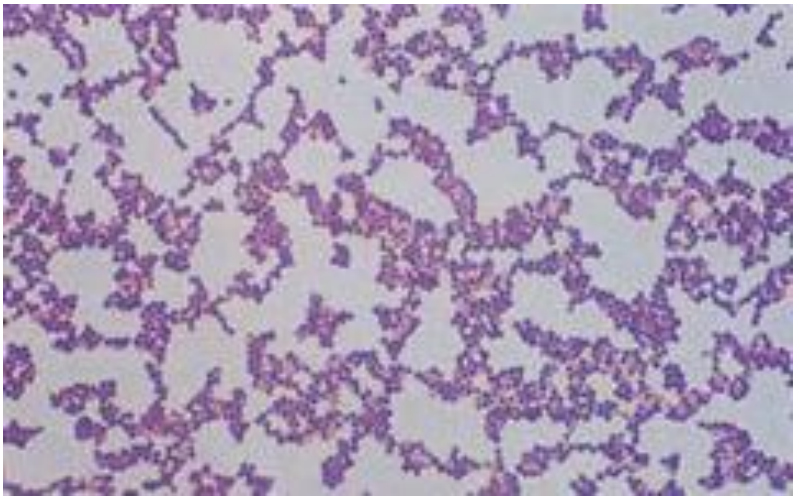
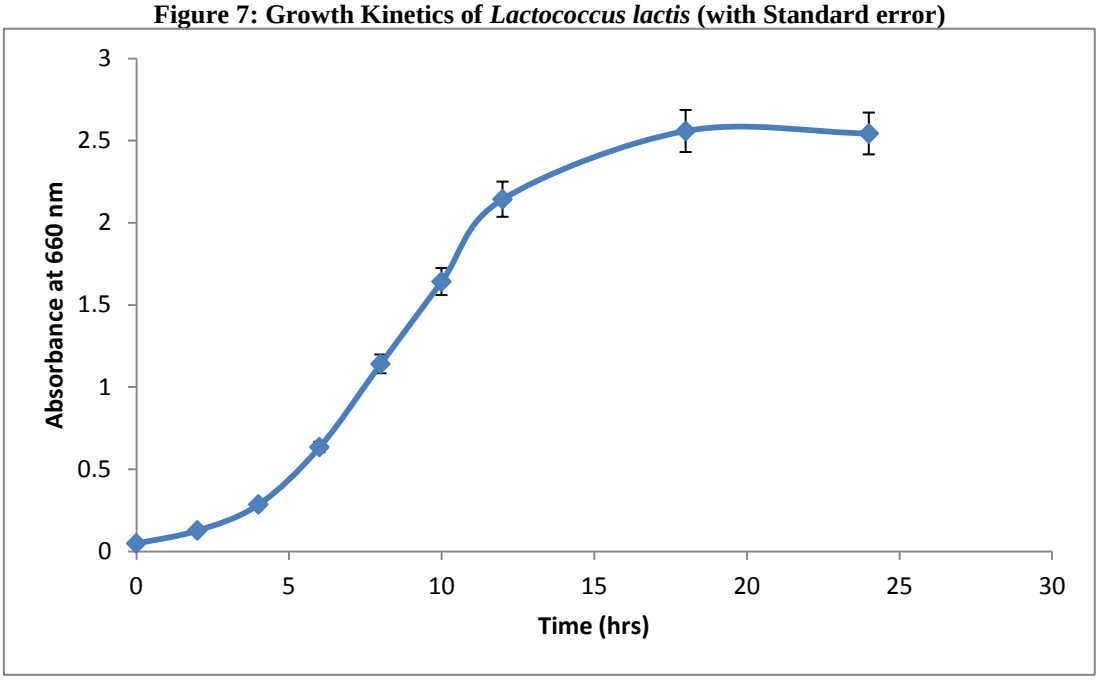


Figure 7: Growth Kinetics of *Lactococcus lactis* (with Standard error)



L. lactis exhibited typical bacterial growth pattern initially from lag phase to exponential phase beginning from 4th to 5th hour where it starts actively growing and maintaining the same to almost 18 hours after which it experiences a deceleration in its growth. The highest nutritional requirement is thus in the exponential phase (Specific growth rate μ = 0.25/hr), promoting the bacteria to utilize nutrients from broth and slowly depleting it. Increase in Absorbance value depicts a high cell density till the end of the exponential phase. Reports suggested that the consumption of sugars during the exponential phase (10–12 h) resulted in the accumulation of lactic acid and acetic acid which decreased the pH (Charalampopoulos, 2002). LAB are fastidious organisms who require complex nutritional source and high specificity of its requirement is evident from its elaborate proteolytic system.

Inoculation of milk for the production of *lactis* yoghurt was done from cells of the exponential phase. 0.5 McFarland adjusted culture (approximately 1.5x10^{8} cfu/ml) was used to achieve inoculum size.

Different inoculum sizes were used (1 %, 2 % and 3 %) for yoghurt preparation. Higher sizes (2 % and 3 %) yielded yoghurt which had poor acceptability due to high acidity. Optimized size of 1 % was thus proceeded with.

Figure 7: Fermented food product ‘dhokla’ seasoned with mustard seeds, green chillies

4.2 Evaluation of sensory attributes

Table 4: Sensory evaluation of freshly prepared food product with varying fermentation time

FRESHLY PREPARED (0^{th} hr)	FERMENTATION TIME (HOURS)		
Attributes	4	16	24
Smell (9)	7.8±0.07	7.6±0.03	6.8±0.02
Consistency (9)	7.5±0.02	7.4±0.05	6.5±0.04
Texture (9)	7.7±0.03	7.3±0.05	6.6±0.03
Colour and appearance (9)	7.3±0.02	7.3±0.03	6.4±0.02
Overall Acceptability (9)	7.6±0.02	7.4±0.07	6.6±0.05

Date represented as Mean±SD, n=10

The sensory scores revealed that the overall acceptability remained favourable for a fermentation time of 4 hours. Scores for other attributes gradually became low when fermented for a longer time (16 and 24 hrs). This is supported by the observed fact that during fermentation the population levels of LAB and yeast increased during 0–18 h of fermentation period accompanied with changes in pH, titratable acidity and volatile fatty acids which contributed to the sensory properties. The lactic acid bacteria produce lactic acid and acetoin, imparting a sour taste and a pleasant flavour (Joshi, 1989)

Table 5: Sensory evaluation of food product after 48 hrs of storage at 4 °C

AFTER 48 HRS STORAGE AT 4 °C	FERMENTATION TIME (HOURS)		
Attributes	4	16	24
Smell (9)	7.8±0.05	7.3±0.04	6.6±0.03
Consistency (9)	7.4±0.02	7.4±0.05	6.5±0.05
Texture (9)	7.6±0.04	7.3±0.03	6.5±0.02
Color and appearance (9)	7.4±0.02	7.2±0.02	6.4±0.03
Overall Accepetibility (9)	7.5±0.04	7.2±0.02	6.3±0.04

Date represented as Mean±SD, n=10

Sensory attributes after storage of 48 hrs in normal refrigerated condition produced unfavourable results as the scores for 24 hrs fermentation drastically reduced notably rendering it unacceptable for consumption. But the overall acceptability of food product having fermentation time of 4 hrs remained similar even after 2 days of storage with slight

deterioration in colour and appearance. Keeping general acceptability in mind the 4 hrs fermented food product was analyzed further.

4.3 Peptide content of food sample

Peptide content of the food sample extract (4 hrs fermentation, extract concentration 200 mg/ml) was determined from the standard plot of enzyme hydrolysate of casein and was found to be 1.056± 0.01 mg/ml (n=3). The peptide content was difficult to interpret directly because of the lack of a standard that reflects the real distribution of peptidic molecular weights of the peptides liberated by the action of bacterial Proteinases. The peptide content was determined to measure the Inhibitory Concentration (IC_{50}). Calculation of IC in milligrams per milliliter yields a number that is characteristic of the peptide pool formed and is thus an indicator of the amount of peptide pool necessary to inhibit α- Glucosidase and ACE by 50% under the present assay conditions. (Fuglsang, 2002). 4 hrs fermented control food product showed poor results in terms of peptide concentration, value being less than 0.2 mg/ml of the food extract.

4.4 *In vitro* inhibition of α- glucosidase enzyme

Table 6: α- Glucosidase inhibitory activity of test and control food product (freshly prepared)

α- GLUCOSIDASE INHIBITION FOR FRESHLY PREPARED FOOD PRODUCT								
Fermentation time	**%inhibition**			**Mean**	**Std. deviation**	**Peptide conc. (mg/ml)**	**IC_{50} (mg/ml)**	**IC_{50} (ml)**
4	13.95	14.2	13.49	13.88	0.360139	1.056	3.804	0.360
16	11.5	11.63	11.19	11.44	0.226053	ND	ND	0.437
24	11.1	10.24	10.45	10.59667	0.448367	ND	ND	0.439
α- GLUCOSIDASE INHIBITION FOR FRESHLY PREPARED CONTROL FOOD PRODUCT								
Control	**%inhibition**			**Mean**	**Std. deviation**	**Peptide conc. (mg/ml)**	**IC_{50} (mg/ml)**	**IC_{50} (ml)**
4	0.64	0.72	0.62	0.66	0.052915	0.198	15.469	7.81
16	1.01	0.96	1.18	1.05	0.115326	ND	ND	4.95
24	1.74	1.74	1.79	1.756667	0.028868	ND	ND	2.87

ND: Not Determined

Figure 9: α-glucosidase inhibition for freshly prepared food product with Control

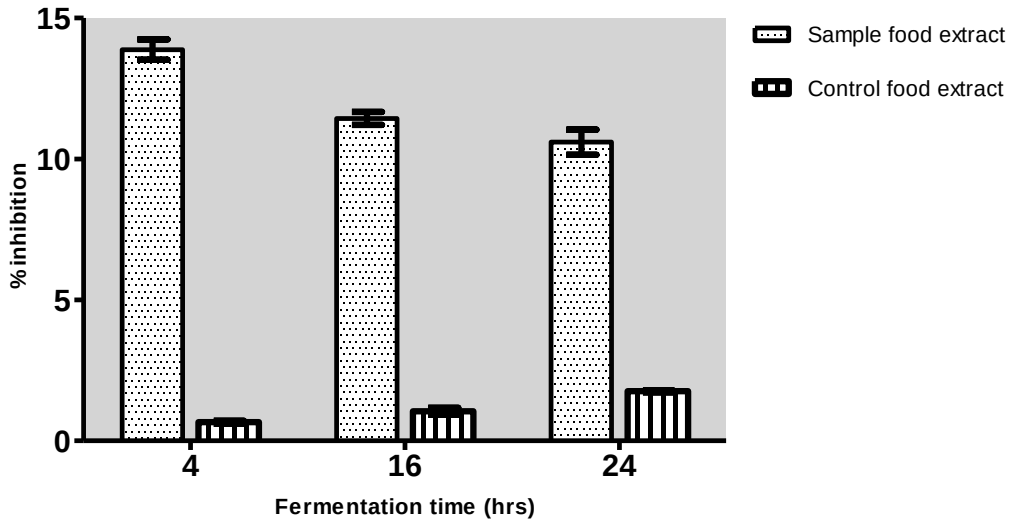
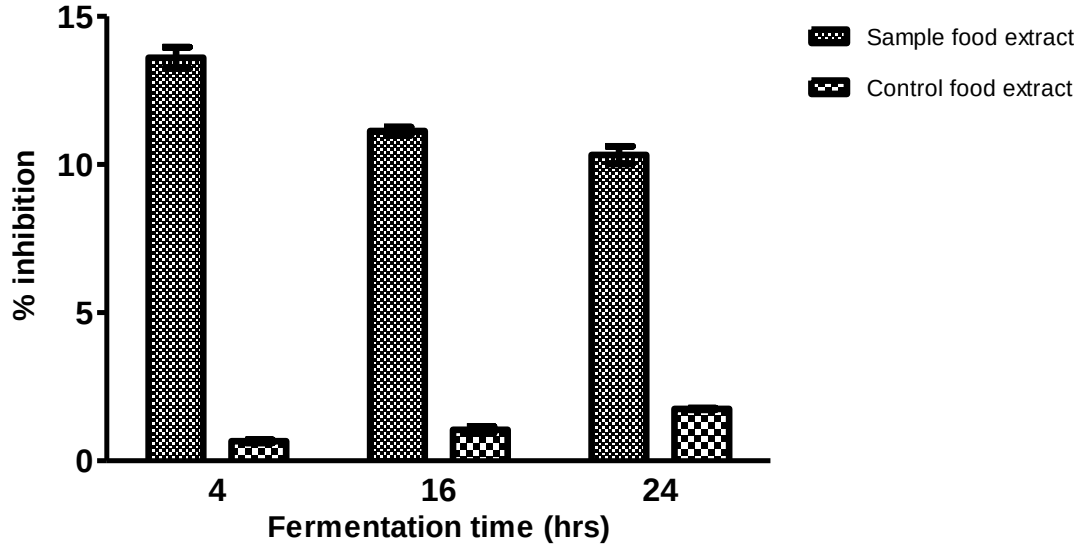


Table 7: α- Glucosidase inhibitory activity of test and control food product (After 48 hrs of storage)

α- GLUCOSIDASE INHIBITION OF FOOD PRODUCT AFTER 48 HRS OF STORAGE								
Fermentation time	**%inhibition**			**Mean**	**Std. deviation**	**Peptide conc. (mg/ml)**	**IC_{50} (mg/ml)**	**IC_{50} (ml)**
4	13.62	13.95	13.23	13.60	0.360416	1.048	3.850	0.378
16	10.98	11.24	11.19	11.13	0.137961	ND	ND	0.449
24	10.67	10.13	10.18	10.32	0.298385	ND	ND	0.484
α- GLUCOSIDASE INHIBITION OF CONTROL FOOD PRODUCT AFTER 48 HRS OF STORAGE AT 4 °C								
Control	**%inhibition**			**Mean**	**Std. deviation**	**Peptide conc. (mg/ml)**	**IC_{50} (mg/ml)**	**IC_{50} (ml)**
4	0.56	0.62	0.62	0.6	0.034641	0.204	15.454	7.57
16	1.01	0.96	1.18	1.05	0.115326	ND	ND	4.76
24	1.74	1.74	1.79	1.756667	0.028868	ND	ND	2.84

ND: Not Determined

Figure 10: *α*-glucosidase inhibition of food product after 48 hrs of storage with Control



4.5 *In vitro* inhibition of Angiotensin Converting Enzyme (ACE)

Table 8: ACE inhibitory activity of sample fermented food

INHIBITION OF ACE BY TEST FOOD EXTRACT AND CONTROL							
Fermentation time	**%inhibition**		**Mean**	**Std. deviation**	**Peptide conc. (mg/ml)**	**IC_{50} (mg/ml)**	**IC_{50} (ml)**
4	25.67	28.03	26.85	1.166	1.056	1.966	0.466
Control	**%inhibition**		**Mean**	**Std. deviation**	**Peptide conc. (mg/ml)**	**IC_{50} (mg/ml)**	**IC_{50} (ml)**
4	3.86	4.01	3.93	0.106066	ND	ND	3.18

ND: Not Determined

IC_{50} value in mg/ml is the measure of peptide concentration from the peptide pool required to inhibit 50 % of α- glucosidase enzyme.

IC_{50} value in millilitres signifies the volume required from food extract (extract conc. of 200 mg/ml) to inhibit 50 % of the enzyme where total reaction volume is 1.1 ml.

Maximum α- glucosidase inhibition was exhibited for a fermentation time of 4 hrs. Results exhibited almost 21 times increase in the percent inhibition with respect to Control food product with a 5 fold increase in the peptide content of the test sample. IC_{50} (mg/ml) also showed a significant difference ($p<0.05$) between the test food product and control thus showing increased potency of the food to inhibit the enzyme as lesser the IC_{50} value more is the potential for inhibition of enzyme. Commercial α-glucosidase inhibitor *Acarbose* (Stock conc. 1 mg/ml) exhibited inhibitory activity of 33.9 ± 3.395 %. ACE inhibitory activity was on the higher side and compared to the control, 6.83 times activity was observed in the test food product.

Though there has been a limited study on the microbial and biochemical changes occurring in the composite food batter (chickpea and *V. mungo* flour) batter during fermentation it was established that the population levels of LAB and yeast increased during 0–18 h of fermentation period. The microflora usually comprised *Lb. fermentum*, *Leu. mesenteroides*

and *Hansenula silvicola* (Joshi, 1989). The increase in microbial counts was accompanied with the changes in pH, titratable acidity and volatile fatty acids. Studies with the use of antagonistic isolates of *Lactobacillus* spp. in the preparation of *dhokla* batter revealed that spoilage bacterial species, for example, *B. laterosporus*, *B. licheniformis,* and *B. subtilis* occurring as preprocessing contaminants were unable to increase in their population levels. Reports on the use of antagonistic cultures of *Lactobacillus spp*. to prepare *dhokla* batter resulted in the growth inhibition of food borne pathogenic species such as *S. aureus* and *B. cereus*. In an approach similar to *kadhi*, *dhokla* batters prepared with the antagonistic cultures of *Lactobacillus* spp. and heat sealed in laminate pouches revealed acceptable sensory attributes in a storage period of 7 days at 25–30°C and 14 d at 6°C. The stored batters were free from spoilage and pathogenic bacteria also due to the drop in pH from 5.2 to 4.5. According to Nielsen, (Nielsen et al. 2009) found that the pH at the end of fermentation influences the ACE-inhibitory activity of fermented milk which varies with the strain of LAB used and concluded that proteolysis should not be too extensive.

Studies on fermented camel milk supplemented with *Allium sativum* have seen an increase in the potential to inhibit α-glucosidase than plain cow and camel milk yoghurt. Plain milk as control exhibited a decrease in the inhibitory activity from 11.3 % to 5.5 % during a storage period of seven days (Shori, 2011). In the present study, storage under refrigerated condition (4 °C) for 2 days revealed a negligible drop in activity from 13.88 % to 13.66%.

The α-glucosidase inhibitory activity of 50 types of fruits and vegetables commonly consumed in Korea was evaluated using both whole juice (WJ) samples and ethanol extracts (1 gram//ml extract concentration). Results indicated variation in activity ranging from 5 % to 30 % for various vegetables, ethanol extracts being higher (Park, 2012). Compared to yoghurt and fermented food extracts the values are higher as plants continue to be a good source of anti-diabetic compounds although in some cases the inhibitory effect of the fermented food extract (200 mg/ml extract concentration) was higher.

An interesting observation was the α-glucosidase inhibitory activity of the control fermented food. Slight increase in the activity was observed when fermentation time increased. This could be the result of action of proteolytic enzymes present in food components (legumes and pulses).

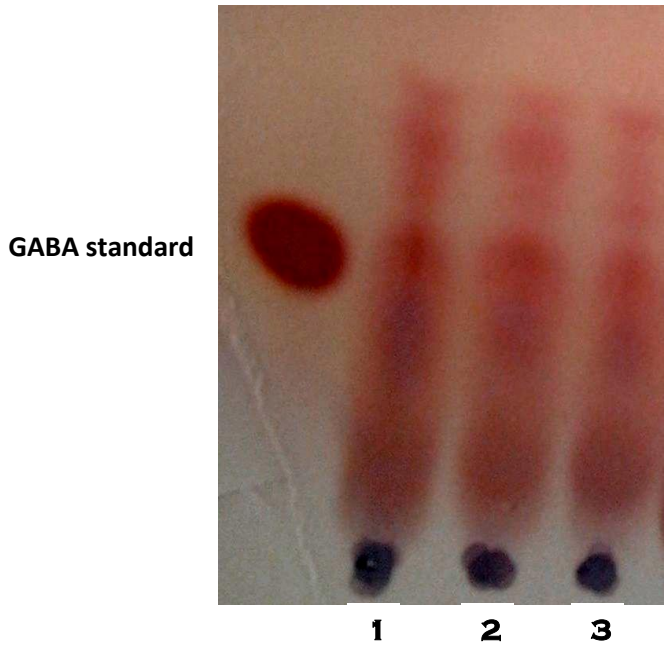
Reports on the nutraceutical aspects of the traditional *dhokla* have not been favourable to establish the food as a high value product. Bio accessibility of Zinc, Iron, Phytase and tannin reduction have been on the lower side as compared to other cereal fermented food e.g. *idli*

and *dosa* (Ramakrishnan *et al*., 1976). Hence developing the '*dhokla*' having high value nutraceuticals showing hypoglycaemic and hypotensive properties was the main motivation to carry out this investigation. Enrichment with Vitamin C and B2 was also a part of this study.

An important modification of the protocol for '*dhokla*' preparation was addition of *Vigna mungo* (urad dal) in place of rice in the food batter; *V. mungo* is a rich source of glutamate which has been utilized for sour dough fermentation using *Lactococcus lactis* subsp. *lactis* for enhanced ϒ-aminobutyric acid production (Bhanwar *et al.* 2012)

4.6 Qualitative determination of ϒ-aminobutyric acid (GABA)

Fig 11: TLC plate showing the presence of GABA with standard



1: Sample food extract (spotting volume 20 µl)
2: Sample food extract (spotting volume 15 µl)
3: Control food extract (spotting volume 20 µl)

For the presence of GABA, inclusion of *V. mungo* as a good source of glutamate rendered it completely natural which LAB could utilize for GABA production though there are reports on the use of Monosodium glutamate which enhanced GABA production by food grade Lactic Acid Bacteria (Cho, 2007).

4.7 Estimation of ascorbic acid and riboflavin

Table 9: Vitamin C content of sample and control food product with increasing storage time

Storage time (hours)	Vitamin C content of sample 'dhokla' (mg) for serving size of 100 grams	Vitamin C content of control 'dhokla' (mg) for serving size of 100 grams	RDA* for adult and elderly
0	115.64	109.48	90-120 mg/day
24	115.37	109.34	
48	115.72	107.21	

*RDA: Recommended Dietary Allowance Source: Food and Nutrition Board, Institute of Medicine, National Academies

Table 10: Vitamin B_2 content of sample and control food product with increasing storage time

Storage time (hours)	Vitamin B_2 content of sample 'dhokla' (mg) for serving size of 100 grams	Vitamin B_2 content of control 'dhokla' (mg) for serving size of 100 grams	RDA* for adult and elderly
0	54.76	34.76	1.2-1.6 mg/day
24	54.24	33.91	
48	53.67	33.67	

*RDA: Recommended Dietary Allowance Source: Food and Nutrition Board, Institute of Medicine, National Academies

Comparative study of Vitamin C content in sample fermented '*dhokla*' and that of control revealed slight increase in the concentration of Ascorbic acid in the test sample and a considerable improvement in riboflavin content from the control. Storage of the samples at 4 °C did not significantly alter the amount of Vitamin C. Recent studies concluded that LAB are increasingly gaining attention for elaborate production micronutrients such as vitamins (Russo, 2012). The proper selection and exploitation of nutraceutical-producing LAB is an interesting strategy to produce novel fermented foods with increased nutritional and⁄or health-promoting properties (Hugenholtz, 2002). Many industrially important LAB such as

Lactococcus lactis and *Streptococcus thermophilus* have the ability to synthesize folate (vitamin B11) and folate biosynthesis by yogurt starter cultures can increase the "natural" folate levels in this product (LeBlanc, 2010).

5. Conclusions

The main objective of the study was to develop a traditional fermented food similar to '*dhokla*' consumed in India with a wide range of bioactive compounds. *Lactococcus lactis* subsp. *lactis* was used in this study this strain was previously characterized for high GABA production. Incorporation of GABA, having tranquillizing and hypotensive properties, in diet has been an established remedy for stress, a major risk factor in coronary heart disease. Various other causes of hypertension have been extensively studied. Hyperglycaemia, a major health concern, is on the rise at an alarming rate was also taken into consideration. Thus investigations on *L. lactis* for the production of hypotensive and anti-diabetic compounds were carried out.

In the present study optimization and analysis of the potency of the strain to inhibit α-glucosidase enzyme and Angiotensin Converting Enzyme (ACE) under *in vitro* within a frame work of a complex food matrix was carried out maintaining adequate sensorial attributes which would render the food as a marketable product.

The strain exhibited good inhibition of ACE and α-glucosidase. ACE inhibition was higher than that of α-glucosidase making the stain reputed for its 'stress-busting' capabilities. The effect of fermentation by *L. lactis* also enhanced the content of ascorbic acid and riboflavin which are considered as essential micronutrients in the food matrix. Results from this study suggests the novel food product to contain many folds increase in inhibitory activity as compared to the traditional '*dhokla*'.

The exact identity of bioactive peptides which elicited such inhibitory effects remain to be elucidated. Most of the studies have revealed milk as the source of such peptides but the combination of milk and leguminous protein together in a complex food matrix have not been studied before. In this regard, this work provides valuable insights in further developing novel traditional food formulations with therapeutic potential.

Although *in vitro* studies reveal the ability of the strain (*L. lactis* subsp. *lactis*) to inhibit ACE and α-glucosidase, the results need to be validated under *in vivo* conditions. Therefore an elaborate study on such effects *in vivo* in small animals is essential along with clinical studies prior to application of the food.

6. Participation in Conference

Awarded 2nd prize in oral presentation at DST, ICMR and DBT sponsored International Conference on Technological Advances in Super Foods for Health Care ICTASH-2013 organized by IIFANS, New Delhi and BGCW, Puducherry, Pondicherry, May2013

Abstract

This study aimed to develop a fermented snack food having α-glucosidase inhibiting bioactive peptides and other functional properties. First, the snack food which comprised of a mixture of flour of *Vigna mungo* and chickpea were made into a batter, fermented with a *Lactococcus lactis* strain (previously characterized in the laboratory for probiotic attributes) and then steamed. Under optimized conditions of inoculum size (1% v/v) and fermentation time (4 hours), one serving size of approximately 100 grams of the food provided with almost 54.76 mg of riboflavin and about 115 mg of ascorbic acid. Extract concentration of 200mg/ml exhibited 13.6 % α-glucosidase inhibition. Sensorial analysis based on 9 point hedonic scale scored adequately for general acceptability both for adults and elderly. Both sensory and nutraceuticals produced were retained over 48 hours at 4°C. The results of this study suggest a potential of applicability of this functional snack food for both adults and the elderly. However stability of these peptides in acidic environment (gut) and clinical studies are of utmost importance prior to application of this functional food.

Keywords: Nutraceutical, fermented snack food, α-glucosidase inhibition, riboflavin, ascorbic acid, bioactive peptides

References

7. References

Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., & Itoh, T. "Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion." *Journal of Dairy Science* 81 (1998): 3131–3138.

Agarwal KN, Bhasin SK, Faridi MM, Mathur M, Gupta S. "Lactobacillus casei in the control of acute diarrhea--a pilot study." *Indian Pediatr* 38, no. 8 (2001): 905-10.

Amal Bakr Shori, Ahmad S. Baba. "Comparative antioxidant activity, proteolysis and in vitro a-amylase and a-glucosidase inhibition of Allium sativum-yogurts made from cow and camel milk." *Journal of Saudi Chemical Society*, 2011: 1-8.

Anders Fuglsang, Dan Nilsson, and Niels C. B. Nyborg. "Cardiovascular Effects of Fermented Milk Containing Angiotensin-Converting Enzyme Inhibitors Evaluated inPermanently Catheterized, Spontaneously Hypertensive Rats." *Appl Environ Microbiol* 68, no. 7 (2002): 3566–3569.

Atkinson, A.B., and J.I.S. Robertson. "Captopril in the treatment of clinical hypertension and cardiac failure." *Lancet* 2 (1979): 836–839.

Beaglehole, R., & Yach, D. "Globalisation and the prevention and control of non-communicable disease: The neglected chronic diseases of adults." *Lancet* 362 (2003): 903–908.

Braun, C., Brayer, G. D., & Withers, S. G. "Mechanism-based inhibition of yeast a-glucosidase and human pancreatic a-amylase by a new class of inhibitors. 2-Deoxy-2,2-difluoro-a-glycosides." *The Journal of Biological Chemistry,* 270 (1995): 26778–26781.

Brody, Tom. *Nutritional Biochemistry.* San Diego: Academic Press, 1999.

Cahill L, Corey PN, El-Sohemy A. "Vitamin C Deficiency in a Population of Young Canadian Adults." *Am J Epidemiol* 170 (2009): 464–471.

Casalta E, Montel MC. *Int J Food Microbiol*, 2008.

Cho YR, Chang JY, Chang HC. "Production of g-aminobutyric acid (GABA) by Lactobacillus buchneri isolated from Kimchi and its neuroprotective effect on neuronal cells." *J Microbiol Biotech* 17, no. 1 (2007): 104–109.

Church FC, Swaisgood HE, Porter DH, Catignani GL. "Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins." *J Dairy Sci* 66 (1983): 1219–27.

D. Charalampopoulos, S.S. Pandiella, C. Webb. "Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates." *Journal of Applied Microbiology* 92, no. 5 (2002): 851-59.

De Roos, N.M. and Katan, M.B. "Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenosis: A review of papers published between 1988 and 1998." *American Journal of Clinical Nutrition* 71 (2000): 405–411.

Donkor, O.N., Henriksson, A., Singh, T.K., Vasiljevic, T. and N. P. Shah. "ACE-inhibitory activity of probiotic yoghurt." *International Dairy Journal* 17 (2007): 1321-1331.

Dunne C., Murphy L., Flynn S., O'MahonyL., O'Halloran S., Feeney M., Morrissey D., Thornton G., Fitzgerald G., Daly C., Kiely B., Quigley E.M.M., O'Sullivan G.C., Shanahan F. and Kevin J. "Probiotics: from myth to reality. Demostration of functionality in animal models of diseases and in human clinical trials." 76 (1999): 279-292.

Dureja D, Kaushik D, Kumar V. "Developments in nutraceuticals." *Indian J Pharmacol* 35 (2003): 363-72.

Erdmann, K., Cheung, B. W. Y., and Schröder, H. " The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. ." *The Journal of Nutritional Biochemistry.* 19 (2008.): :643-654.

F. A. Ibrahim, F. A. Ali, S. M. Ahmed, M. M. Tolba. "Kinetic Determination of Acarbose and Miglitol in Bulk." *InternatIonal journal of BIomedIcal scIence* 3 (2007): 20-30.

Fenley J, Bray F, Pisani DMe. World Health Organization. GLOBOCAN 2000, Cancer incidence mortality and prevalence worldwide. Lyon, France: IARC Press: 2001.

Fitzgerald, R. J., Murray, B. A., and Walsh, D. J. 134: 980S-988S. "Hypotensive peptides from milk proteins." *The Journal of Nutrition.* 134 (2004): 980S-988S.

Fonseca, V. "Clinical significance of targeting post-prandial and fasting hyperglycemia in managing type 2 diabetes mellitus." *Curr Med Res Opin.* 19 (2003): 635-41.

Fuller, Gibson G.R and. "Aspect in in viro and in vivo research approaches directed toward identifying probiotics and probiotics for human use." *Journal of Nutrition* 130 (2000): 391S-395S.

Fuller, R.,. "Probiotics in man and animals: A review." *J. Appl. Bacteriol.* 66 (1989): 365-378.

Garcia-Olmedo F, Salcedo G, Sanchez-Monge R, Gomez L, Royo J, Carbonero P,. *Cell Biol* 4 (1987): 275.

Gobbetti M, Ferranti P, Smacchi E, Goffredi F, Addeo F. 2000. "Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermentedmilk started by Lactobacillus delbrueckii subsp. bulgaricus SS1 and Lactococcus actis subsp. cremoris." *Appl Environ Microbiol* 66 (2000): 3898-3904.

Gomes, A.M.P. and Malcata, F.X. "Bifidobacterium spp. and Lactobacillus acidophilus: Biological, biochemical, technological and therapeutical properties relevant for use as probiotics." *Trends in Food Science and Technology* 10 (1999): 139–157.

Guarner F, Schaafsma GJ. "Probiotics." *Int J Food Microbiol* 39, no. 3 (1998): 237-8.

Gunawan-Puteri., Bhandari, M. R., & Kawabata, J. "Indonesian medicinal plants and their anti-diabetic potencies." *Functional foods for chronic diseases* 4 (2009): 110–123.

Gunawan-Puteri, M., & Kawabata, J. "Novel alpha-glucosidase inhibitors from Macaranga tanarius leaves." *Food Chemistry* 123, no. 2 (2010): 384–389.

Hollander P. "safety profile of acarbose, an alphaglucosidase inhibitor." *Drugs* 44, no. Supp 3 (1992): 47-53.

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U., Huis in't Veld, J.H.J. " Overview of gut flora and probiotics." *Intl. J. Food Microbiol* 41 (1998): 85–101.

Hugenholtz J, Smid EJ. "Nutraceutical production with foodgrade microorganisms." *Curr Opin Biotechnol* 13 (2002): 497–507.

Huth, P. J., DiRienzo, D. B., and Miller, G. D. "Major scientific advances with dairy foods in nutrition and health." *Journal of Dairy Science.* 89 (2006): 1207-1221.

J´erˆome T, Laurent M, Jean-Luc G. "Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysates of bovine α2-casein." *FEBS Lett* 531 (2002): 369–374.

Jae-Hee Park, Rae-Young Kim, and Eunju Park. "Antidiabetic Activity of Fruits and Vegetables Commonly Consumed in Korea: Inhibitory Potential against α-Glucosidase and Insulin-like Action in vitro." *Food Sci. Biotechnol.*, 2012: 1187-1193.

JEYARAM, K., ROMI, W., SINGH, T. A., ADEWUMI, G. A., BASANTI, K., OGUNTOYINBO, F. A. "Distinct differentiation of closely related species of Bacillus subtilis group with industrial importance." *Journal of Microbiological Methods* 87, no. 2 (2011): 161-164.

Jun Watanabe, Jun Kawabata, Hideyuki Kurihara,and Ryoya Niki. "Isolation and Identification of alpha-glucosidase Inhibitors from Tochu-cha(Eucommia ulmoides)." *Biosci. Biotech. Biochem.* 61, no. 1 (1997): 177-178.

Jussawalla DJ, Gangadharam P. Cancer of the colon: 32 years of experience in Bombay, India. J Surg Oncol. 1977:9:607-22.

Kalra, Ekta K. "Nutraceutical - Definition and Introduction." *AAPS PharmSci* 5, no. 3 (2003): Article 25.

Khalil, A.H. and Mansour, E.H. "Alginate encapsulated bifidobacteria survival in mayonnaise." *Journal of Food Science* 63 (1998): 702–705.

Knai C, Suhrcke M, Lobstein T (2007). Obesity in Eastern.

Knai C, Suhrcke M, Lobstein T. *Obesity in Eastern Europe: an overview of its health and economic implications.* Econ Hum Biol, 5, 392–408., 2007.

Korhonen, H., and Pihlanto, A. " Food derived bioactive peptides – Opportunities for designing future foods." *Current Pharmaceutical Design.* 9: (2003.): 1297-1308.

Kunji, E. R. S., I. Mierau, A. Hagting, B. Poolman, and N. Konings. "The proteolytic system of lactic acid bacteria." *Antonie Leeuwenhoek* 70 (1996): 187–221.

"Lactic acid bacteria in fish: a review." *Aquaculture* 160 (1998): 177-203.

LeBlanc JG, Taranto MP, Molina V, Sesma F. "B-group vitamins production by probiotic lactic acid bacteria." In *Biotechnology of lactic acid bacteria: novel applications*, 211–232. Ames: Wiley-Blackwell, 2010.

Lin, M.Y. andYen, C.L. "Antioxidative ability of lactic acid bacteria." *Journal of Agricultural and Food Chemistry* 47 (1999): 1460–1466.

López-Fandiño, R., Otte, J., and van Camp, J. " Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity." *International Dairy Journal.* 16: (2006.): 1277-1293.

Malik A, Kumar P, Kaushik N, Singh A. The potential of nutraceuticals. "The potential of nutraceuticals." *Indian J Pharmacol* 6, no. 2 (2008).

Matsui, T., Ueda, T., Oki, T., Sugita, K., Terahara, N., & Matsumoto, K. "alpha-Glucosidase inhibitory action of natural acylated anthocyanins. 1. Survey of atural pigments with potent inhibitory activity." *Journal of Agricultural and Food Chemistry* 49 (2001): 1948–1951.

Meisel H, Goepfert A, Gunther S. "ACE-inhibitory activities in milk products." *Milchwissenschaft* 52 (1997): 307–11.

Micanel, N., Haynes, I.N., and Playne, M.J. Kamaly, K.M. "Viability of probiotic cultures in commercial Australian yogurts. Bifidobacteria fermentation of soybean milk." *Australian Journal of Dairy Technology. Food Research International* 52. 30, no. 9 (1997): 24–27. 675–682.

Miller, G. D., Jarvis, J. K., and McBean, L. D. "Dairy foods and hypertension." In *In Handbook of Dairy Foods and Nutrition (3rd edn)*, pp 99-139. Florida: CRC Press, 2007b.

Mine, Y. and Shahidi F. *Nutraceutical proteins and peptides in health and disease.* Boca Raton: FL: CRC Press., 2006.

Mooradian, A.D. and Thurman, J.E.,. "Drug therapy of postprandial hyperglycemia." *Drugs* 57 (1999): 19-29.

Moretti VM, Madonia G, Diaferia C, Men-Tasti T, Paleari MA, Panseri S, Pirone G, Gandini G. "Chemical and micro biological parameters and sensory attributes of a typical Sicilian salami ripened in different conditions." *Meat Sci.* 66 (2004): 845-854.

Moretti VM, Madonia G, Diaferia C, Men-Tasti T, Paleari MA,Panseri S, Pirone G, Gandini G. "Chemical and micro biological parameters and sensory attributes of a typical Sicilian salami ripened in different conditions." *Meat Sci.* 66 (2004): 845-854.

Mukesh Kumar Singh, Richu Singla, Arashdeep Singh, Moushumi Ghosh, and Abhijit Ganguli. "Survival of Probiotic Strains in Non-dairy Indian Spice Condiment." *Food Sci. Biotechnol.* 21, no. 5 (2012): 1309-1315.

Nakasaki, K., Yanagisawa, M., Kobayashi, K. "Microbiological quality of fermented milk produced by repeated-batch culture." *J.Biosci. Bioeng* 105, no. 1 (2008): 73–76.

Neeta Joshi, S.H. Godbole, P. Kanekar. "Microbial and biochemical changes during dhokla fermentation with special reference to flavour compounds." *J. Fd. Sci. Technol* 26 (1989): 113–115.

Ondetti, M.A. "Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents." *Science* 196 (1977): 441–444.

Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, et al. "Vitamin C as an antioxidant: evaluation of its role in disease prevention." *J Am Coll Nutr* 22 (2003): 18–35.

Parkin et al., 2002).

Prajapati, J.B., Shah, R.K., and Dave, J.M. "urvival of Lactobacillus acidophilus in blended-spray dried acidophilus preparations." *Australian Journal of Dairy Technology* 42 (1987): 17–21.

Prashant Chauhan, M Ratna Sudha, Kalpana Dixit. "Probiotics as complementary therapy for hypercholesterolemia." *Biology and Medicine* 1, no. 4 (2009).

Puhan, Z. "Effect of probiotic fermented dairy products in human nutrition." *Industria Latte* 35, no. 3–4 (1999): 3–11.

R.K., Tamime A.Y. and Robinson. "Yogurt science and technology." Edited by Cambridge and CRC Press, p. 619. Boca Raton: Woodhead Publishing, 1999.

Rastogi T, Devesa S, Mangtani P, et al. Cancer incidence rates among South Asians in four geographic regions: India, Singapore, UK and US. Int J Epidemiol. 2008, and 37:147-60.

Ring E., Gatesoupe F.J. "Lactic acid bacteria in fish: a review." *Aquaculture* 160 (1998): 177-203.

RK Rishi. "Nutraceuticals: Borderline between food and drug?" *Pharmacol Rev* 1 (2006): 51-3.

Rokka, Hannu J. Korhonen and Susanna. "Properties and Applications of Antimicrobial Proteins and Peptides from Milk and Eggs." In *BIOACTIVE FOOD PROTEINS ANDPEPTIDES APPLICATIONS IN HUMAN HEALTH*, 49. Taylor & Francis Group, LLCCRC, 2011.

Rolfe, R.D. "The role of probiotic cultures in the control of gastrointestinal health." *Journal of Nutrition* 130 (2000): 396S–402S.

Russo P, López P, Capozzi V, Palencia P, Dueñas MT, Spano G, Fiocco D. "Beta-glucans improve growth, viability and colonization of probiotic microorganisms." *Int J Mol Sci* 13 (2012): 6026–6039.

Sakai, K., Mishima, C., Tachiki, T., Kumagai, H., and Tochikura, T. "Mortality of bifidobacteria in boiled yogurt." *Journal of Fermentation Technology* 65 (1987): 215–220.

Salminen, S., Ouwehand, A., Benno, Y., and Lee, Y.K. "Probiotics:How should they be defined?" *Trends in Food Science and Technology* 10 (1999): 107–110.

Schrezenmeir, J. and de Vrese, M.,. "Probiotics, prebiotics, and synbiotics: approaching a definition." *Am. J. Clin. Nutr.* 73 (2001): 361S-364S.

SEEMA BHANWAR, MEENAKSHI BAMNIA, MOUSHUMI GHOSH, & ABHIJIT GANGULI. "Use of Lactococcus lactis to enrich sourdough bread with g-aminobutyric acid." *International Journal of Food Sciences and Nutrition*. Early Online: (2012): 1–5.

Sekar, S. and Mariappan, S. "Usage of traditional fermented products by Indian rural folks and IPR." *Indian Journal of Traditional Knowledge* 6, no. 1 (2007): 111-120.

Senesi, S., Celandroni, F., Tavanti, A. and Ghelardi, E. "Molecular characterization and identification of Bacillus clausii strains marketed for use in oral bacteriotherapy." *Appl. Environm. Microbiol* 67 (2001): 834-839.

Sgouras, D., Maragkoudakis, P., Petraki, K., Martinez-Gonzales, B., Eriotrou, E.,and Mentis, A.,. "In vitro and in vivo inhibition of Helicobacter pylori by Lactobacillus casei strain shirota." *Appl. Environ. Micriobiol.* 70, no. 1 (2004): 518-526.

SHAH, L. RAMCHANDRAN AND N.P. "Proteolytic Profiles and Angiotensin-I Converting Enzyme and α-Glucosidase Inhibitory Activities of Selected Lactic Acid Bacteria." *Food Microbiology and Safety*, 2007: M75-M82.

Shah, N.P. "some beneficial effects of probiotic bacteria." *Bioscience Microflora* 19 (2000a): 99–106.

Shin, J.W., Lee, Y.W., Lee, H.S., Shin, K.S., Choi, C.S., Kwon, G.S. "Isolation and characterization of lactobacillus brevis AML15 producing γ-aminobutyric acid." *Journal of Life Science* 17 (2007): 970–975.

Steinkraus, K. *Handbook of Indigenous Fermented Foods 9.* Hardcover, 1983.

Swennen, K., C.M. Courtin, and J.A. Delcour. "Non-digestible oligosaccharides with prebiotic properties." *Critical Reviews in Food Science and Nutrition* 46 (2006): 459–471.

Takahashi, M., Taguchi, H., Yamaguchi, H., Osaki, T.,Komatsu, A. and Kamiya, S. "The effect of probiotic treatment with Clostridium butyricum on enterhemorrhagic Escherichia coli O157:H7 infection in mice." *FEMS Immunol. Med. Microbiol* 41 (2004): 219–226.

Tamang, J.P. Tamang, B., Schillinger, U., Guigas, C. and Holzapfel, W.H. "Functional properties of lactic acid bacteria isolated from ethnic fermented vegetables of the Himalayas." *International Journal of Food Microbiology* 135 (2009): 28-33.

Toeller, M. "α-Glucosidase inhibitors in diabetes: efficacy in NIDDM subjects." *Eur. J. Clin. Invest.* 24(Suppl. 3) (1994): 3l-35.

Tontisirin K, Nantel G, Bhattacharjee L. "Food-based strategies strategies to meet the challenges of micronutrient malnutrition in the." *Proc Nutr Soc* 61 (2002): 243–250.

Torheim LE, Ferguson EL, Penrose K, Arimond M. "Women in resource-poor settings are at risk of inadequate intakes of multiple micronutrients." *J Nutr* 140 ((2010)): 2051S–2058S.

Tuomilehto, J., Lindström, J., Hyyrynen, J., Korpela, R., Karhunen, M-L., Mikkola, L., Jauhiainen, T., Seppo, L. and Nissinen, A. " Effect of ingesting sour milk fermented using Lactobacillus helveticus

bacteria producing tripeptides on blood pressure in subjects with milk hypertension." *Journal of Human Hypertension.* 18 (2004): 795-802.

Varadaraj, M.C., B.V. Balasubramanyam, R. Joseph. "Antibacterial effect of Lactobacillus spp. on spoilage bacteria in selected Indian fermented foods." Conference on Traditional Foods, Central Food Technological Research Institute, Mysore, 2000.

Varadaraj, Roopashri AN and. "Molecular characterization of native isolates of lactic acid bacteria, bifidobacteria and yeasts for beneficial attributes." *Appl Microbiol Biotechnol* 83 (2009): 1115 –1126.

Vasiljevic, T. and N.P. Shah. "Probiotics – from Metchnikoff to bioactives." *International Dairy Journal* 18 (2008): 714-728.

Verna EC, Lucak Sv. "Use of probiotics in gastrointestinal disorders: what to recommend?" *Therap Adv Gastroenterol* 5 (2010): 307-19.

Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W. "Probiotic bacteria as biological control agents in aquaculture." *Microbiology and Molecular Biology Review* 64 (2000a): 655–671.

Vetrichelvan, T., Jagadeesan, M., & Uma Devi, B. A. "Antidiabetic activity of alcohol extract of Celosia argentea Linn. seeds in rats." *Biological and Pharmaceutical Bulletin* 25 (2002): 526–528.

VL., Spitberg. "Bovine milk fat globule membrane as a potent nutraceutical." *J Dairy Sci* 88 (2005): 2289-94.

Wadström, Åsa Ljungh* and Torkel. "Lactic Acid Bacteria as Probiotics." *Curr. Issues Intestinal Microbiol.* 7: 73–90.

Watanabe, J., Kawabata, J., Kurihara, H., and Niki, R.,. *Isolation and identification of alpha glucosidase inhibitors from Tochu-cha (Eucommia ulmoides).* 61. Biosci. Biotechnol. Biochem.

WHO, Global Strategy on Diet. "Physical Activity and Health, Annual Report." Geneva, Switzerland, 2006.

Yamamoto, Naoyuki. *Antihypertensive Peptides Derived from Food Proteins.* John Wiley & Sons, Inc., 1997.

APPENDIX

MRS (De Man, Rogosa and Sharpe) broth

Ingredients	Quantity (g/L)
Peptone from casein	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Dipotassium hydrogen phosphate	2.0
Tween 80	1.0
Triammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.2
Manganese sulphate	0.04

pH adjusted with 10N NaOH to pH 7.0 ± 0.1 before autoclaving. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

MRS agar: MRS broth containing 15.0 g/l agar

BUFFERS AND SOLUTIONS

1. ***0.1M Phosphate buffer pH 7***

   Monobasic sodium phosphate, monohydrate (1 M) 61.5 ml
   Dibasic sodium phosphate, monohydrate (1 M) 38.5 ml
   Dilute to 1 L with distilled water

2. ***HEPES buffer (50 mM)***

   300 mM NaCl
   HEPES sodium salt
   Deionized Water
   pH adjusted to 8.3 at 37 °C

3. ***Hippuryl-L-Histidyl-L-Leucine Solution (HHL)***

   0.3 % (w/v) Hippuryl-His-Leu, Free Base, Sigma,
   in HEPES buffer (50 mM)

4. ***Angiotensin Converting Enzyme stock***

   Stock: 1ml of cold deionised water to 50 μg enzyme in vial
   Stock concentration: 0.1 U/ml

5. α-glucosidase enzyme stock

1mg of enzyme in 1 ml in Phosphate Buffer pH 6.5
Stock conc. 14 U/ml

6. para-Nitrophenyl-alpha-D-glucopyranoside (PNPG) stock

24 mg in 2 ml Deionised water.
Stock conc. 40 mM

7. Iodine solution (0.05 M)

0.332 g of Iodine and 0.507 gm Potassium Iodate are dissolved in 200 ml distilled water.

8. Starch indicator solution (1 %)

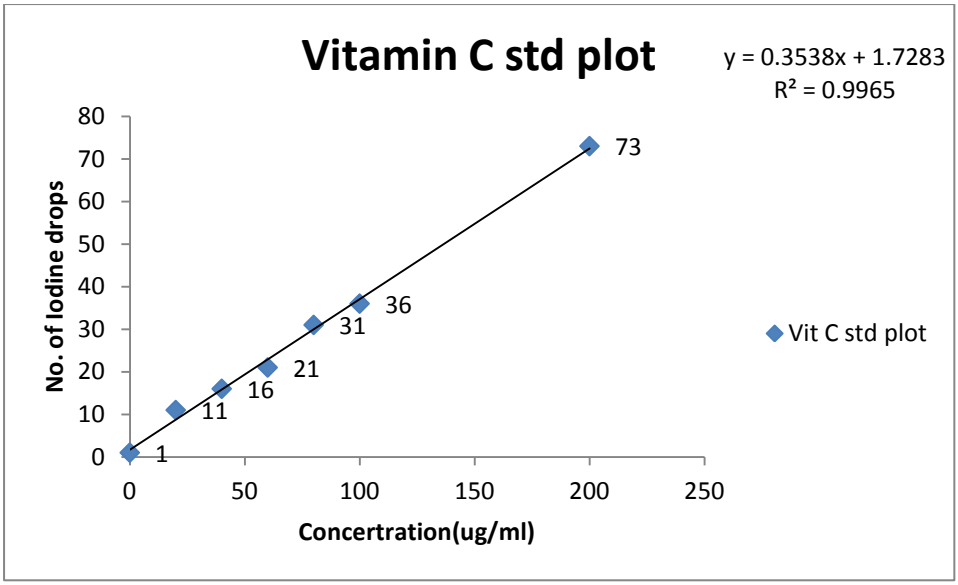
0.5 g of soluble starch added to 50 ml of near boiling water. Stirred to dissolve and cooled before use.

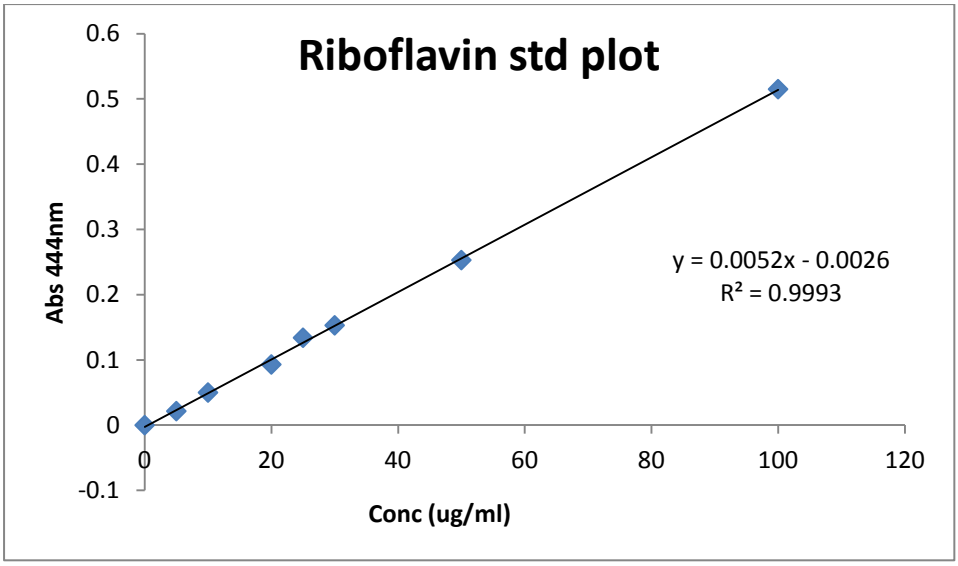
9. Ninhydrin Solution

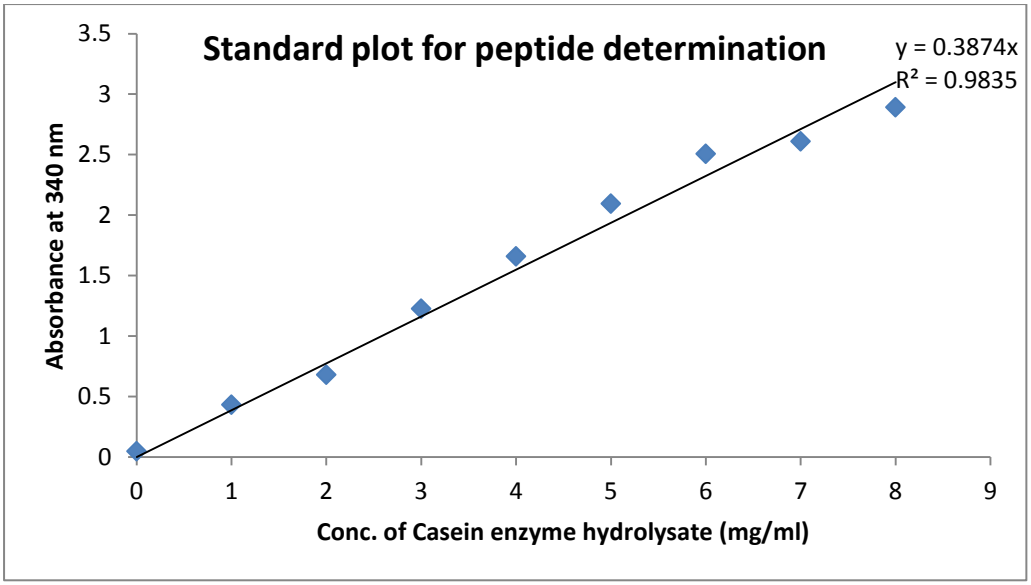
20g of ninhydrin in 600mL of ethanol

10. o-phthaldialdehyde solution

40 mg of o-phthalaldehyde dissolved in 1 ml of methanol


Vitamin C std plot
y = 0.3538x + 1.7283
R² = 0.9965
No. of Iodine drops
Concertration(ug/ml)
Vit C std plot
1
11
16
21
31
36
73
0
10
20
30
40
50
60
70
80
0
50
100
150
200
250


Riboflavin std plot
y = 0.0052x - 0.0026
R² = 0.9993
Abs 444nm
Conc (ug/ml)
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0
20
40
60
80
100
120


Standard plot for peptide determination
y = 0.3874x
R² = 0.9835
Absorbance at 340 nm
Conc. of Casein enzyme hydrolysate (mg/ml)
0
0.5
1
1.5
2
2.5
3
3.5
0
1
2
3
4
5
6
7
8
9