CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1 Introduction and Review of Literature

The increasing consumer awareness that diet and health are linked is stimulating innovative development of novel products by the food industry. Lactic acid bacteria (LAB) have received much attention over recent decades due to the health-promoting properties of certain strains, called probiotics. The concept probiotics has been redefined over time. Fuller defined it as "A live microbial feed which beneficially affects the host animal by improving intestinal microbial balance" (Fuller, 1989). The probiotic products traditionally incorporate intestinal species of *Lactobacillus* because of their long history of safe use in the dairy industry and their natural presence in the human intestinal tract, which is known to contain a myriad of microbes, collectively called the microbiota. Intestinal LAB in humans is intimately associated with the host's health because they are an important biodefense factor in preventing colonization and subsequent proliferation of pathogenic bacteria in the intestine. In fact, probiotics have been used for as long as people have eaten fermented foods. However, it was Metchnikoff at the turn of the 20^{th}century who first suggested that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract (Metchnikoff, 2004). He hypothesized that lactobacilli were important for human health and longevity, and promoted yogurt and other fermented foods as healthy. Food derived from plants, animals, or their products often contain many types of microbes. These microbes from natural and external sources colonize food by contact, which can occur anytime between production and consumption. Microbial contamination of food (i.e. the colonization by unwanted microorganisms) can have many undesirable consequences ranging from spoilage to food borne illness. However, some microbes possess properties that are beneficial for food production or conversion or storage. These food grade microorganisms are used to produce a variety of fermented foods (with improved storage capability) from raw animal and plant material. Having natural preservatives in mind, LAB and their metabolites are good alternatives. The increasing consumer awareness of the risks derived not only from food-borne

pathogens, but also from the artificial chemical preservatives used to control them (Abee *et al*., 1995), has led to renewed interest in so-called "green technologies" including novel approaches for a minimal processing and exploitation of bacteriocins for biopreservation (Papagianni, 2003). Biopreservation can be explained as the link between fermentation and preservation, and refers to extension of the shelf-life and improvement of the safety of food using microorganisms and/or their metabolites (Kao and Frazier, 1966; Klaenhammer, 1988; Holzapfel *et al.*, 1995). Furthermore, the use of LAB and or their metabolites for food preservation is generally accepted by consumers as something "natural" and "health-promoting" (Montville and Winkowski, 1997). Among LAB, addition of *Lactobacillus* culture to food is an approach to food preservation, it also contributes to taste, texture and also inhibits food spoilage bacteria by producing growth inhibiting substances like bacteriocins, lactic acid etc. Strategies utilized to study incorporation of bio-preservatives into food include: direct use of LAB strains with proven antimicrobial activity as starter cultures or food additives, use of biopreservatives preparation in the form of previously fermented product, or use of partially-purified, purified or chemically synthesized bacteriocins (De Vuyst and Vandamme, 1994).

Food-associated lactic acid bacteria

The first essential step in food fermentation is the catabolism of carbohydrates by the LAB. LAB as a group exhibit an enormous capacity to degrade different carbohydrates and related compounds. LAB are Gram-positive, non-spore forming cocci, coccobacilli or rods and most genera have a DNA base composition of less than 50% G+C, lack catalase, grow under microaerophilic or anaerobic conditions, and typically ferment glucose mainly to lactic acid (homo-fermentative), but can also have lactic acid, CO_2, and ethanol/acetic acid as end products (hetero-fermentative). In nature, species of the LAB are found in gastro-intestinal tract (GIT) of mammals and also in fermented food products (dairy, meat, vegetables, fruits and beverages). LAB associated with foods are generally restricted to the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus*. Orla-Jensen proposed a classification of lactic acid bacteria, which was based on morphology, temperature growth range, nutritional characteristics, carbon sources utilization and agglutination effects. Orla-Jensen differentiated three major groups. The first group contained *Thermobacterium, Streptobacterium* and *Streptococcus,* which were all catalase

negative and produce mainly lactic acid besides traces of other by-products. The second group contained *Betabacterium* and *Betacoccus,* which also lack catalase but as a rule formed detectable amounts of gas and other by-products, besides lactic acid. The third group consisting of *Microbacterium* and *Tetracoccus* show a positive catalase reaction. In 1960, Van den Hamer showed that representative of *Betabacterium* did not possess fructose-1,6-bisphosphate aldolase, in contrast to *Theromobacterium* and *Streptobacterium*. These findings supported the discrimination of the three physiological groups: (i) the obligately homofermentative lactobacilli, lacking both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (*Thermobacterium*), (ii) the facultatively homofermentative lactobacilli having both dehydrogenases but degrading glucose preferably via the Embden-Meyerhof-Parnase pathway (*Streptobacterium*) and (iii) the obligately heterofermentative lactobacilli lacking fructose 1,6-bisphosphatealdolase (*Betabacterium*). *Thermobacterium, Streptobacterium* and *Betabacterium* were considered to be the three subgenera within the genus *Lactobacillus*.

The genus *Lactobacillus* belongs to the large group of lactic acid bacteria. The genus *Lactobacillus* belongs phylogenetically to the phylum *Firmicutes* (Garrity *et al.,* 2004). The family *Lactobacillaceae* comprises the main family in the order *Lactobacillales* which itself belongs to the class *Bacilli*. Lactobacilli can be found in a variety of ecological niches, such as plants (fruits, vegetables, cereal grains) or plant-derived materials, silage, fermented food (yogurt, cheese, olives, pickles, salami, etc.), as well as in the oral cavities, gastrointestinal tracts (GIT), and vaginas of human and animals. The bacteria that occupy a niche in the GIT are true residents or autochthonous (i.e., found where they are formed). Other bacteria are just "get a lift" through the gut and are allochthonous (i.e., formed in another place). Autochthonous strains have a long-term association with a particular host, and they form stable populations of a characteristic size in a particular region of the gut. It is often difficult to determine whether or not a particular microorganism is truly autochthonous to a particular host (Tannock, 2004).

The role of lactic acid bacteria in the functional food concept

The term "functional food" was first proposed in Japan two decades ago and legally approved there as Food for Specified Health Use (FOSHU). Chow (2002) reported the

notion that food could serve as medicine was first conceived thousands of years ago by the Greek Philosopher and father of medicine, Hippocrates, who wrote 'Let food be thy medicine, and let medicine be thy food'. However, now days the concept of food having medicinal value has been reborn as 'functional foods'. The health benefits ascribed to functional food continues to increase and the gut is an obvious target for the development of functional foods, because it acts as an interface between the diet and all other body functions. One of the most promising areas for the development of functional food components lies in the use of probiotics and prebiotics, which have demonstrated therapeutic evidences. Functional food is food that promotes human health above the provision of basic nutrition. A relatively recently proposed working definition describes functional food as "food that can be satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way relevant to an improved state of health and well-being and/or reduced risk of diseases" (Contor, 2001). Functional foods are also known as designer foods, medicinal foods, nutraceuticals, therapeutic foods, super foods, foodiceuticals, and medifoods (Shah, 2001). Functional food has a significant and growing global market, of which the largest segment in Europe, Japan and Australia comprises food containing probiotics, prebiotics and synbiotics (Stanton *et al.*, 2005).

Probiotics

The idea that LAB prevents intestinal disorders and diseases is nearly as old as the science of microbiology (Molin, 2001). Therefore, in the development of probiotic food intended for human consumption, strains of LAB have most commonly been used. The term "probiotic" (Greek: for life) was first used by Lilly and Stillwell (1965). "Probiotic" was later more widely used and defined by Parker (1974), and further improved by Fuller (1989) with the following definition: "A live microbial food supplement which beneficially affects the host animal by improving its intestinal microbial balance". This definition has later been slightly revised (Schaafsma, 1996; Schrezenme and de Vrese, 2001) to "Foods containing live and defined bacteria, which when given in sufficient numbers, exert beneficial effects by altering the microflora in the host" or as expressed by Salminen *et al*. (1998) "Viable preparation in food or dietary supplements to improve the health of humans and animals". According to these definitions, an impressive number of microbial species and genera

can be considered as probiotics. However, only strains classified as LAB are (due to their traditional use in food) currently considered of importance in regard to food and nutrition.

Prebiotics

Since the viability of the live bacteria in food products and during transit through the GIT may be variable, the "prebiotic" concept has been developed. A prebiotic is defined as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the host health" (Gibson and Ruberfroid, 1995). Thus, selective growth of certain indigenous gut bacteria is improved by the administration of the prebiotic and thereby any viability problems of orally administered bacteria in the upper GIT can be overcome. Some oligosaccharides, due to their chemical structure, are resistant to digestive enzymes and therefore pass into the large intestine where they become available for fermentation by saccharolytic bacteria. Compounds that are either partially degraded or not degraded by the host and are preferentially utilized by probiotic bacteria as a carbon and/or energy source. The criteria which allow classification of a food ingredient as a prebiotic, are defined by Fooks and Gibson (2002), and include the following statements, ex. fructo-oligosaccharides, xylo-oligosaccharides, lactose derivatives such as lactulose, lactitol, galacto-oligosaccharides and soyabean oligosaccharides.

i. It must be neither hydrolysed, nor absorbed in the upper part of the gastrointestinal tract.
ii. It should be selectively fermented by one or a limited number of potentially beneficial bacteria in the colon.
iii. Its presence should alter the colonic microbiota towards a healthier composition.
iv. It should induce effects, which are beneficial to the host's health.

Synbiotics

A further possibility in microflora management procedures is the use of synbiotics, i.e., the use in combination of probiotics and prebiotics (Gibson and Roberfroid, 1995). The live microbial additions may be used in conjunction with a specific substrate for growth and the end result should be improved survival of the probiotic, which has a readily available substrate for its fermentation, as well as the individual

advantages that each may offer (Fooks and Gibson, 2002). Many studies suggest that the consumption of synbiotic products has higher beneficial effects on the human health than probiotic or prebiotic products (Gmeiner *et al.,* 2000), leading to improved survival of probiotic bacteria during the storage of the product and during the passage of the intestinal tract. Moreover, the synbiotic product may allow an efficient implantation of probiotic bacteria in colonic microbiota, because the prebiotic has a stimulating effect on the growth and/or activities of both the exogenous and the endogenous bacteria (Champagene and Gardner, 2005).

Important aspects for selection of probiotic strains

When selecting a probiotic strain, a number of aspects should be considered, and the theoretical basis for selection should involve safety, functional as well as technological aspects (Salminen *et al.,* 1998; Adams, 1999; Saarela *et al.,* 2000). The first step in the selection of a probiotic LAB strain is the determination of its taxonomic classification, which may give an indication of the origin, habitat and physiology of the strain. These entire characteristic have important consequences on the selection of the novel strains. An FAO/WHO (2001) expert panel suggested that the specificity of probiotic action is more important than the source of microorganisms. This conclusion was brought forward due to uncertainty of the origin of the human microflora since the infants are born with virtually sterile intestine. However, the panel also underlined a need for improvement of *in vitro* tests to predict the performance of probiotics in humans. Probiotic lactobacilli encounter various environmental conditions upon ingestion by the host and during transit in the GIT. Firstly, they need to survive the harsh conditions of the stomach. Humans secrete approximately 2.5 litres of gastric juice each day, generating a fasting pH of 1.5, increasing to pH 3 to 5 during food intake and that the food transit time through the human stomach is about 90 minutes. The aggregation of cells could possibly be explained by an increased hydrophobicity of the cell surface at low pH. The cell envelope of Gram-positive bacteria consists of an inner plasma membrane and a thick outer layer of peptidoglycan. In contrast to Gram-negative bacteria, cell walls of Gram-positive bacteria contain large amounts of negatively charged teichoic acids (polymers of glycerol or ribitol joined by phosphate groups). Hence, one can assume that the teichoic acids become protonated at low pH, leading to a more hydrophobic surface. Ingested microorganisms must endure numerous environmental extremes to

survive in the human GIT. Bile tolerance is one of the most essential criteria for the selection of a probiotic strain. Bile acids are synthesized in the liver from cholesterol and are secreted from the gall-bladder into the duodenum, where they play an important role in the digestion of fat. Bile acids are conjugated to either glycine or taurine. Bile is a digestive secretion that plays a major role in the emulsification of lipids. Bile acids are surface active, amphipathic molecules with potent antimicrobial activity and act as detergents, disrupting biological membranes. It has the ability to affect the phospholipids and proteins of cell membranes and disrupt cellular homeostatis. Therefore, the ability of pathogens and commensals to tolerate bile is likely to be important for their survival and subsequent colonization in the GIT (Begley *et al.,* 2005).

Selection criteria for screening of novel probiotic strains

Aspects	**Properties**
Functional	Tolerance to gastric acids and digestive enzymes
	Adhesion and colonization of gut epithelium
	Antimicrobial activity
	Immunomodulation
	Influence on metabolic activities
	a. lactase activity, b. cholesterol assimilation, c. vitamin production
Safety	Healthy human origin
	Non-pathogenic, non-haemolytic, DNase negative
	Should not carry any transmissible antibiotic resistance genes
Technological	Genetically stable
	Should withstand production conditions
	Desired viability in the final formulation of the food products
	Good sensory properties
Other benefits	Antimutagenic and anticarcinogenic activities
	Anti-*Helicobacter pylori* activity
	Antifungal activity

Health benefits of functional probiotic culture

A number of health benefits are claimed in favour of products containing probiotic organisms including antimicrobial activity and gastrointestinal infections, improvement in lactose metabolism, antimutagenic properties, anticarcinogenic properties, reduction in serum cholesterol, anti-diarrhoeal properties, immune system stimulation, improvement in inflammatory bowel disease and suppression of *Helicobacter pylori* infection (Ambalam *et al.,* 2009; 2011; Kurmann and Rasic, 1991; Shah, 2007). Some of the health benefits are well established, while other benefits have shown promising results in animal models. However, additional studies are required in humans to substantiate these claims. Health benefits imparted by probiotic bacteria are strain specific, and not species- or genus-specific. It is important to note that no strain will provide all proposed benefits, not even strains of the same species, and not all strains of the same species will be effective against defined health conditions. The strains of *Lactobacillus* and *Bifidobacterium* are able to restore the normal balance of microbial populations in the intestine and most commonly used as probiotics (Shah, 2006).

Antimicrobial activity against food-borne and gastrointestinal pathogens

Many mechanisms have been postulated by which Lactobacilli could produce antimicrobial activity. In addition to their competitive inhibition of the epithelial and mucosal adherence of pathogens and inhibition of epithelial invasion by pathogens, lactobacilli and bifidobacteria show antimicrobial activity by producing antimicrobial substances and/or stimulating mucosal immunity (Servin, 2004) Probiotic bacteria produce organic acids, hydrogen peroxide and bacteriocins as antimicrobial substances that suppress the multiplication of pathogenic and putrefying bacteria. Lactic and acetic acids account for over 90% of the organic acids produced. Lowering of pH due to lactic acid or acetic acid produced by these bacteria in the gut has a bacteriocidal or bacteriostatic effect. Lactic acid produced by lactobacilli acts as a permeabilizer of the Gram-negative bacterial outer membrane, allowing other antimicrobial substances produced by the host to penetrate and thereby increasing the susceptibility of pathogens to these antimicrobial molecules (Alokomi *et al*., 2000). Production of H_2O_2 by *Lactobacillus spp*. may be a non-specific antimicrobial defense mechanism of the normal vaginal ecosystem (Reid, 2002; Reid and Burton, 2002). Hydrogen peroxide inhibits both Gram-positive and Gram-negative organisms.

Production of bacteriocins, recent reports have revealed that some intestinal lactobacilli and bifidobacteria produce antimicrobial substances that are active against these enteropathogens. Bacteriocins are ribosomally synthesized antimicrobial peptides and bactericidal proteinaceous molecule produced by bacteria. The term "bacteriocins" was originally coined by Jacob *et al*. (1953), specifically to define protein antibiotics of the colicin type, but it is now accepted to include peptide inhibitors from any bacteria. Tagg *et al*. (1991) proposed the term "bacteriocins-like inhibitory substance" for designating the antimicrobial protein from Gram-positive microorganisms, to tell them apart from colicins which is produced by *E. coli*. Today, however, most antimicrobial peptides are named "bacteriocin', irrespective of Gram-positive or Gram-negative origin.

The bacteriocin family includes a wide variety of peptides and proteins in terms of their size, microbial targets, and mechanism of action and immunity (Graneau *et al*., 2002). Bacteriocins are divided into four main categories as described in table 1 (Klaenhammer, 1993; Belkum *et al*., 2000). Although bacteriocin may enhance survival of LAB in complex ecological system, interest has focused on prevention of growth of harmful bacteria in the fermentation, preservation of dairy products and as anti-infective drug. It is therefore more interesting with respect to probiotics that individual strains may inhibit growth or adhesion of pathogenic microorganism by secreted products like bacteriocin and not merely an effect of acidic pH. There are many evidences reporting secretory antibacterial components produced by LAB having broad range of activity against Gram-positive and Gram-negative organisms (Nomoto, 2005), which are independent of lactic acid and hydrogen peroxide. However, the overall antimicrobial activity of LAB may be due to a synergistic action of lactic acid and proteinaceous substances. Many lactic acid bacteria produce antibacterial peptides, bacteriocins; including lactacin B and acidocin from *Lactobacillus acidophilus,* plantaricin from *Lactobacillus plantarum* and nisin from *Lactococcus lactis,* have a narrow spectrum of activity acting only against closely related bacteria (Bierbaum and Sahl 2009). Antimicrobial peptides produced by *L. rhamnosus* are distinct from bacteriocins produced by other *Lactobacillus spp*., as they exhibit a broad spectrum of activity against Gram-positive and Gram-negative organisms and may belong to least characterized fourth class of complex bacteriocins (Ambalam *et al*., 2009; Pithva *et al*., 2011, 2012).

Table 1. Classification of bacteriocins from Lactic acid bacteria

I. Lantibiotics	Ribosomally produced peptides that undergo extensive post-translational modification
	Small (<5 kDa) peptides containing lanthionine and methyl lanthionine
	Ia. Flexible molecules compared to Ib
	Ib. Globular peptides with no net charge or net negative charge
II. Nonlantibiotics	Low-molecular-weight (<10 kDa), Heat stable peptides
	Formed exclusively by unmodified amino acids
	Ribosomally synthesized as inactive peptides that get activated by post-translational cleavage of the N-terminal leader peptide
	IIa. Anti-listerial single peptides that contain YGNGGVXC amino acid motif near their N termini
	IIb. Two peptide bacteriocins
	IIc. Bacteriocin produced by the cell's general sec-pathway
III. Nonlantibiotics	High-molecular-weight (>30 kDa), heat labile proteins
IV	Complex bacteriocins carrying lipid or carbohydrate moieties, which appear to be required for activity
	Such bacteriocins are relatively hydrophobic and heat stable

Mode of action of bacteriocins

Bacteriocins may possess a bactericidal or bacteriostatic mode of action on sensitive cells, this distinction being greatly influenced by several factors such as bacteriocins dose and degree of purification, physiological state of the indicator cells (growth phase) and experimental conditions (eg., temperature, pH, other antimicrobial compounds). Most bacteriocins exert bactericidal mode of action against the sensitive microorganisms, although some have been shown to act in a bacteriostatic manner.

The majority of bacteriocins kill susceptible bacteria by membrane permeabilization or by interference with essential enzymes; Nisin forms a complex with ultimate cell wall precursor lipid II, thereby inhibiting the cell wall biosynthesis. Subsequently the complex aggregates and incorporates further peptides to form a pore in the bacterial membrane. Several theories have been proposed to explain the exact mechanism by which antimicrobial peptides kill bacteria. The 'barrel-stave' mechanism describes the formation of transmembrane channels/pores by bundles of peptides. Progressive recruitment of additional peptide monomers leads to steadily increasing pore size. Leakage of intracellular components through these pores subsequently causes cell death. The 'carpet-like' peptide first bind on to the surface of the target microbial cell membrane and subsequently the membrane is covered by 'carpet-like' clusters of peptides and cause membrane permeation, leads to lysis of the microbial cell (Deraz *et al*., 2005).

Present approaches and future prospects for bacteriocins in food application

The increasing demand for high-quality 'safe' foods that are not extensively processed has created a niche for natural food preservatives. The ideal natural food preservative should fulfil the following criteria (Hill *et al.,* 2002), acceptably low toxicity, stability to processing and storage, efficacy at low concentration, economic viability, no medical use, and no deleterious effect on the food. While most bacteriocins fulfil all these criteria, to date nisin is the only bacteriocin to be commercially exploited on a large scale, having gained Food and Drug Administration (FDA) approval in the USA in 1988, although it had been in use in Europe for some time (the WHO approved the use of nisin in 1969). Its success has stimulated further research targeted towards identifying new bacteriocins from LAB which potentially could be used in a similar manner. Many bacteriocins have now been characterized that exhibit antibacterial activity against a range of pathogenic and food spoilage bacteria. It is to be expected that bacteriocins and bacteriocin-producing LAB (used as starters or protective cultures) will find many roles in both fermented and nonfermented foods as a means of improving food quality, naturalness and safety. Three approaches are commonly used in the application of bacteriocins for biopreservation of foods (Schillinger *et al.,* 1996) Inoculation of food with LAB that produce bacteriocin in the products. The ability of the LAB to grow and produce bacteriocin in the products is crucial for its successful use, Addition of purified or

semi-purified bacteriocins as food preservatives, use of a product previously fermented with a bacteriocin producing strain as an ingredient in food processing.

Antimutagenic activity

Human gastrointestinal tract, from small intestine to colon, harbors a variety of bacterial species approximately containing 10^7- 10^{12} cells per gram of the intestinal content (Guarner and Malagelada 2003; Eckburg *et al*., 2005). New born have a sterile intestinal tract that gets swarmed with favorable and unfavorable microbes along with the first feed; and following the childhood, the intestinal microflora remain fairly constant until the alterations are brought by the environmental factors, life style and modified genetic set-up. Human gut microbes are broadly categorized as symbionts, commensals and pathobionts (Round and Mazmanian, 2009). Gut microbiota performs vital functions of the host including, immune and nutritional status, thus assist in health maintenance (Cerf-Bensussan and Gaboriau-Routhiau, 2010). Equilibrium among various gut bacterial strains and host immunity decide the occurrence of physiological (regulates the presence of resident gut microbiota) and pathological inflammation (depends on the number and virulence of the invading pathogens). Besides these, chronic inflammation profoundly triggers local immune response leading to the release of reactive oxygen species (ROS) and nitric oxide that induce DNA damage and consequently altering tissue homeostatis (Terzi *et al*., 2010). Cytokines produced during this process play a major role in tissue homeostatis. TNF-α, IL-6, IL-1 and chemokines induce tumor growth by promoting angiogenesis and suppressing immune-mediated tumor elimination; and IL-10 and TGF-α acts as inhibitor in cancer establishment (Terzi *et al*., 2010). Thus, altered gut microbiota promote pathogenesis through chronic inflammation, immune evasion and suppression. Significant elevation of Bacteroides/Prevotella population were reported in cancer patients and were correlated with the elevated levels of IL-17 producing cells in the mucosa. A conspicuous difference in the microbial colonization patterns between the tumorous tissue and adjacent non-malignant mucosa suggests that colorectal-associated physiological and metabolic changes recruit tumor-foraging commensal-like bacteria *Clostridium* spp. (Marchesi *et al.,* 2011). In the recent years, a great deal of research has been dedicated in understanding the role of specific microbes/microbial community/microbial molecules that confer health benefits under patho-physiological conditions. These microbes may have an apparent competitive

advantage in the tumor microenvironment, in replacing pathogenic bacteria in colorectal cancer etiology. The dynamic interplay between intestinal microbial ecology (balance between favorable and unfavorable bacteria) and sporadic colorectal cancer was investigated by Marchesi *et al*. (2011) which might be an important lead toward the novel microbiome-related diagnostic tools and therapeutic interventions. With the fact that in colonic environment, microflora and diet are closely involved in the etiology of colorectal cancer, an intense interest has been shown towards the use of probiotics, prebiotics and synbiotics in modulating gut microbiota, host metabolism and thereby aiding in cancer prevention (Geier *et al*., 2006; Liong, 2008). Hence, the concept of probiotics, prebiotics and synbiotics having a myriad of health-promoting effects is becoming a revolution.

Antimutagenic and anticarcinogenic activities of probiotics are manifested by the following possible mechanisms:

1. Mutagen binding, biotransformation and degradation by probiotics

One of the possible ways of the lowering of mutation pressure on animals and human is the increasing antimutagens levels and of the antimutagenic activity of bacteria, predominantly those inhabiting the intestine of mammals being the ingredients of probiotic, used in food processing and ensilage. Probiotic organisms are reported to bind mutagens to the cell surface (Orrhage *et al.,* 1994). Probiotic bacteria along with dietary ingredient help in detoxification and biotransformation of procarcinogens and carcinogens into less toxic metabolites, thus preventing tumor formation (Pool-Zobel *et al*., 2005). Biotransformation of mutagens/carcinogens occur in the gut with the help of phase I and phase II enzymes and regulate the toxic, mutagenic and neoplastic effects of environmental carcinogens. These enzymes, in turn, are modulated by dietary agents (Nowak and Libudzisz, 2009). Phase I enzyme causes bio-activation and phase II enzymes causes the inactivation of mutagen/carcinogen. *Lactobacillus* strains from different commercial dairy products exhibited >80% antigenotoxicity against 4-NQO (Cenci *et al*., 2002). Antigenotoxicity of *Lactobacillus* strains against 4-NQO and MNNG was strain-specific. Antigenotoxic activities were correlated with the spectral modifications observed upon co-incubation with probiotic bacteria and biotransformation has been hypothesized however, biotransformed products were not identified (Caldini *et al.,* 2005). *L. casei* DN 114001 (Actimel) adsorb and metabolized heterocyclic aromatic amines (Nowak and Libudzisz, 2009). Probiotic *L.*

rhamnosus 231 cells has ability to bind, biotransform and detoxify different mutagens like acridine orange (AO), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 2-amino-3,8-dimethylimidazo-[4,5-*f*]-quinoxaline (MeIQx) (Ambalam *et al.,* 2011). Binding of AO by Lr 231 is due to adsorption, thereby leading to removal of mutagen in solution and is instantaneous, pH- and concentration-dependent. Whereas, binding of MNNG and MeIQx by Lr 231 results into biotransformation leading to detoxification with subsequent loss of mutagenicity as determined by spectral analysis, thin layer chromatography and Ames test. Lr 231 exhibits ability to bind and detoxify potent mutagens, and this property can be useful in formulating fermented foods for removal of potent mutagens. Lankaputhra and Shah (1998) studied the antimutagenic activity of organic acids produced by probiotic bacteria against several mutagens and promutagens. In their study, butyric acid showed a broad-spectrum antimutagenic activity against all mutagens or promutagens studied and live bacterial cells showed higher antimutagenicity than killed cells. Elucidation of the mechanisms involved in biotransformation of mutagens/carcinogens by the probiotic bacteria may offer new ways for the management of mutagen or carcinogen-induced colorectal cancer.

2. Inhibition of procarcinogen conversion to carcinogens

Probiotic lactobacilli and bifidobacteria decreased the expression of xenobiotic-metabolizing enzymes compared with *Bacteroides*, *Clostridia* and enteropathogens that mediate carcinogenesis through various enzymes such as β-glucuronidase, azoreductase and nitroreductase (Wollowski, 2001). *L. acidophilus* and *Bifidobacterium* spp. lowered the activity of these enzymes and reduced the risk of tumor development (Goldin and Gorbach, 1984; Yoon *et al*., 2000; Ouwehand *et al*., 2002). Modulation of conversion of procarcinogens to carcinogens by beneficial bacteria is yet another exciting area that needs further detailed investigation at cellular and molecular level.

3. Production of short chain fatty acids

Probiotic bacteria produce lactic acid and other short chain fatty acids (SCFA) as metabolic products from non-digestible carbohydrate fermentation in the gut. These SCFA decreases the load of pathobionts, helps in maintaining homeostatis and lower the intestinal pH (Wollowski, 2001). It also assists in lowering solubility of bile acids and ammonia absorption and increases mineral absorption (Roy *et al*., 2006)

4. Immunomodulation

The immune system is a complex cascade, acting and reacting locally at systemic level. Recently, research is focused on understanding the regulation of immune system and the interaction within and between the components of inflammatory cascades (Medzhitov, 2007). Different markers are used to explore these cascades and they are integrated to cope up with the microbial challenges from the environment and to manage common or severe infections (Albers *et al*., 2005). Dendritic cells (DCs) and natural killer (NK) cells play a critical role in early defense against cancer (Fernandez *et al*., 1999; Takeuchi *et al*., 2001). Desmutagenic activities of different LAB strains were reported to regulate myeloid DCs maturation, polarizing the subsequent T-cell activity toward Th1, Th2 or even T-reg responses (Christensen *et al*., 2002). Probiotic-induced immune suppression of carcinogenesis is associated with lower natural killer cell activity. Oral administration of *L. casei* strain Shirota (LcS) to methylanthracene-induced tumor in mice, displaying enhanced host innate immunity by stimulating the spleenic NK cell activity; thus leading to delayed onset of tumor development (Zeuthen *et al*., 2006). Intrapleural injection of an inactivated strain of LcS in mice improved the immune system against tumor (Takagi *et al*., 2001). LAB modulated DCs, which in turn are potent activators of NK cells. DCs encountering probiotics undergo maturation, stimulating NK cells. This desmutagenic potential of probiotics has been recently addressed, and now it's evident that human monocyte-derived DCs, blood DCs, mouse splenic and lymph node DCs were maturated by IL-12 produced upon LAB induction. Later this molecule IL-12 activates NK cells to produce IFN-γ production in NK cells (Rizzello *et al*., 2011). Probiotic might also exert beneficial effect by macrophage activation, cytochrome p450 blocking, reduction of carcinogen generation, downregulation of Ras-p21 expression, increase of cell differentiation, inhibition of cyclooxygenase-2 upregulation and inhibition of nitric oxide synthetase (Liong, 2008).

Anticarcinogenic activity

Currently, there is a much interest in the microbial inhibition of DNA-reacting compounds. In particular, antigenotoxicity is now frequently included among the functional properties characterizing probiotic bacteria (Burns and Rowland 2004; Commane *et al*., 2005). Epidemiological and experimental studies have shown that functional foods and bacteria acting as probiotics reduce the risk of intestinal

carcinogenesis from environmental factors, such as dietary and endogenous xenobiotics (Knasmuller *et al*., 2001; Commane *et al*., 2005). The ability of bacteria to interact with xenobiotic is also directly involved in the deactivation of harmful compounds in the gastrointestinal tract (Zsivkovits *et al*., 2003, Oberreuther-Moschner *et al*., 2004). Genotoxic inhibition depends on cell-genotoxin interactions, and therefore different mechanisms have been suggested, the most important of which are: binding to bacterial cell components (peptidoglycan complex, polysaccharides), reaction with bacterial metabolites (peptides, polyamines), genotoxin conjugation with bacterial metabolites and bioconversion by bacterial enzymes to nonreactive moieties (Orrhage *et al*., 2002; Cenci *et al*., 2005). Many studies have described the interactions between the most common probiotics (lactobacilli and bifidobacteria) and food-related mutagens i.e. heterocyclic aromatic amines, protein pyrolysates, nitrosamines, polycyclic aromatic hydrocarbons and mycotoxins as well as other DNA-reacting compounds such as nitroarenes, alkylating agents, antimicrobial agents, etc. (Haskard *et al*., 2001; Tavan *et al*., 2002). Bacteria and metabolic products such as genotoxic compounds (nitrosamine, heterocyclic amines, phenolic compounds, and ammonia) are responsible for colorectal cancer. The consumption of cooked red meat, especially barbequed meat, and low consumption of fibre are reported to play a major role in causing colorectal cancer. The colonic flora is also reported to cause carcinogenesis mediated by microbial enzymes such as β-glucuronidase, azoreductase, and nitroreductase, which convert procarcinogens into carcinogens. The anticarcinogenic effect of probiotic bacteria reported is due to the result of removal of sources of procarcinogens (or the enzymes that lead to their formation) improvement in the balance of intestinal microflora, normalized intestinal permeability (leading to prevention or delaying of toxin absorption), strengthening of intestinal barrier mechanisms, and activation of non-specific cellular factors (such as macrophages and natural killer cells) via regulation of γ-interferon production.

Other health benefits of probiotics

The significant involvement of the gut microbiota in human health and disease suggests that manipulation of commensal microbial composition through combinations of antibiotics, probiotics and prebiotics could be a novel therapeutic approach (Jia *et al*., 2008).

Gut microbiota-targeted therapies involves two strategies (i) direct elimination or modification of a well-define, specific gut microbes, or certain species of bacteria, as disease target(s) using antibiotics. An alternative method for such a strategy is vaccination using an antigenic epitope of a bacterium or toxin of interest to elicit an immune response capable of interacting with the gut flora upon microbe proliferation in the gut. (ii) different structural patterns of the gut microbiota that are associated with different clinical manifestation are identified and categorized using profiling methods such as metabonomics and metagenomics. The metabolic or genomic profiling of the gut microbiota from plasma, urinary or faecal samples could allow a stratification of certain gut microbiota structures in association with disease phenotypes. Examples of gut microbiota-related diseases and therapeutic strategies given in Table 2. Combinations of antibiotics, probiotics, prebiotics could be used to manipulate the gut microbiota to achieve a therapeutically effective regimen and eventually, restore the homeostatis of the gut ecology in the host. Moreover lactobacilli also have shown significant influence of cholesterol level.

Reduction in serum cholesterol

The level of serum cholesterol is a major factor for coronary heart disease, and elevated levels of serum cholesterol, particularly LDL-cholesterol, have been linked to an increased risk (Liong and Shah, 2006). There is a high correlation between dietary saturated fat or cholesterol intake and serum cholesterol level. Feeding of fermented milk containing very large numbers of probiotic bacteria to hypercholesterolaemic human subjects has resulted in lowering cholesterol from 3.0 to 1.5 g/L. Probiotic bacteria are reported to de-conjugate bile salts: deconjugated bile acid does not absorb lipid as readily as its conjugated counterpart, leading to a reduction in cholesterol level. *L. acidophilus* is also reported to take up cholesterol during growth and this makes it unavailable for absorption into the blood stream (Klaver and Meer, 1993).

In this context, the present study focused to investigate the following aspects

1. Isolation, identification and screening of probiotic lactobacilli of human origin
2. Production and characterization of extracellular antimicrobial peptides of probiotic lactobacilli
3. Evaluation of antigenotoxic and antimutagenic activities of *Lactobacillus* strains against direct acting mutagens
4. Binding of promutagens by *Lactobacillus* strains
5. Evaluation of survival during lyophilization process and shelf-life of probiotic formulation