10

Potential of Probiotic *Lactobacillus* Strains as Food Additives

Sheetal Pithva[1], Padma Ambalam[1],
Jayantilal M. Dave[2] and Bharat Rajiv Vyas[1]
[1]*Department of Biosciences, Saurashtra University, Rajkot*
[2]*201 Shivam, Vrindavan Society, Kalawad Road, Rajkot*
India

1. Introduction

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 are 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 20th century who first suggested that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract (Metchnikoff, 1907). He hypothesized that lactobacilli were important for human health, 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 & 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 & 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 biopreservatives 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 & Vandamme, 1994).

2. 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 (1919) proposed a classification of lactic acid bacteria, which was based on morphology, temperature range of growth, nutritional characteristics, carbon sources utilization and agglutination effects. Orla-Jensen (1919) 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 Hammer showed that representative of *Betabacterium* did not possess fructose-1,6-bisphosphate aldolase, in contrast to *Thermobacterium* and *Streptobacterium*. These findings supported the discrimination of the three physiological groups: (i) the obligately homo-fermentative lactobacilli, lacking both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (*Thermobacterium*), (ii) the facultatively homo-fermentative lactobacilli having both dehydrogenases but degrading glucose preferably via the Embden-Meyerhof-Parnas pathway (*Streptobacterium*) and (iii) the obligately hetero-fermentative 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 foods (yogurt, cheese, olives, pickles, salami, etc.), as well as in the oral cavities, 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).

3. The role of lactic acid bacteria in the functional food concept

3.1 The functional food concept

Functional food is food that promotes human health above the provision of basic nutrition. The term "functional food" was first proposed in Japan two decades ago and legally approved there as Food for Specified Health Use (FOSHU). 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).

3.2 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; Schrezenmeir & 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.

3.3 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 & 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 GIT.
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.

3.4 Synbiotics

A further possibility in microflora management procedures is the use of synbiotics, i.e., the use in combination of probiotics and prebiotics (Gibson & 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 & 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 & Gardner, 2005).

3.5 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). When selecting a preferable probiotic strain, several aspects of functionality have to be considered, as specified below:

i. Strains for human use are preferably of human origin and isolated from a healthy human GIT and non-pathogenic
ii. must survive through upper GIT and arrive alive at its site of action and able to function in the gut environment
iii. adhere to the intestinal epithelium cell lining and colonize the lumen of the intestinal tract
iv. strains should not carry transmissible antibiotic resistance genes

v. must be able to survive GI transit (acid and bile salt tolerant)
vi. must have good technological properties so that it can be manufactured and incorporated into food products without loosing viability and functionality or creating unpleasant flavors or textures
vii. functional aspects include viability and genetic stability

3.5.1 Acid and bile tolerance

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, proteins of cell membranes and disrupt cellular homeostasis. 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).

In our study, we have isolated *Lactobacillus rhamnosus* Fb from healthy human infant feces, is a gram-positive, catalase negative, non-motile and non-spore forming rod-shaped organism (De Man *et al.,* 1960). Its ability to ferment ribose, rhamnose and growth at 15°C and 45°C indicate that it belongs to the group *Streptobacterium* (Orla-Jensen, 1943). The identity of the *L. rhamnosus* Fb was confirmed by 16S rDNA sequence analysis. The primary requirement for potential probiotic organisms is to survive during the passage through the acidic (pH 1-3) environment of stomach. *L. rhamnosus* survives at pH 2 for 2 h, 87% of total cells remain viable, a sufficiently long time for the cells to pass through the stomach and reach their site of action in the intestine. *L. rhamnosus* show high survival which is satisfactory especially as probiotic strains can be buffered by food or other carrier molecules and in fact are not directly exposed to such a low pH in the stomach. After passage through acidic condition cells were exposed to bile salt (0.1% pancreatin, 0.5% bile salt, pH 8) viability increase after 3 h of incubation to 95%. Mimicking gastro-intestinal transit, we observed that the bacterial stress originated by low pH may be overcome after the subsequent treatment in presence of bile (Charteris *et al*., 1998). A bile concentration of 0.3% is usually used for screening of bile tolerant strains, as this is considered as an average intestinal bile concentration of the human GIT (Gilliland *et al.,* 1984). *L. rhamnosus* possesses the ability to grow in the presence of 0.4% phenol and remain viable in 0.6% phenol, a toxic metabolite produced by intestinal bacteria

during putrefaction in the GIT (Khedekar, 1988). *L. rhamnosus* also possesses the ability to grow in the presence of 6% NaCl (Jacobsen *et al.,* 1999). The ability of *L. rhamnosus* cells to survive in the presence of bile, NaCl and phenol can help them to survive, grow, colonize and elicit the beneficial effects to the host.

4. Health benefits of functional probiotic culture

A number of health benefits are claimed in favour of products containing probiotic organisms including antimicrobial activity, 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 & 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).

4.1 Antimicrobial activity of probiotic *Lactobacillus rhamnosus*

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. *Lactobacillus rhamnosus* has shown antimicrobial activity against *Escherichia coli, Enterobacter aerogenes, Salmonella typhi, Shigella sp., Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Helicobacter pylori, Campylobacter jejuni, and Listeria monocytogenes* (Ambalam *et al.,* 2009). *L. rhamnosus* produces other antimicrobial metabolites, as evidenced from the antimicrobial activity of Cell Free Culture filtrate (CFC) even when Extracellular Protein Concentrate (EPC) (independently) was less active. This evidence suggests the multifactorial nature of the antimicrobial activity and possibly a synergistic effect. Roles of other metabolites remain to be identified.

4.2 Classification of bacteriocins from Lactic acid bacteria

Bacteriocins of LAB are a heterogeneous group of bacterial antagonists and several classification criteria have been used to group them. The bacteriocins have been divided into three main categories: Class I Lantibiotics ribosomally synthesized peptides that undergo extensive post-translational modifications for ex. Nisin. Class II Non-lantibiotic peptides

ribosomally synthesized peptides that undergo minimal post-translational modification. They have diverse chemical and genetic characteristics. They have been sub-divided into three main groups: (a) single peptides, often with a characteristic YGNGVXC amino acid motif near the N-terminus (b) two-peptide bacteriocins (c) In Nes' classification bacteriocins produced by the cell's general secretory (sec) pathway and in Klaenhammer's classification, thiol-activated peptides. Class III Non-lantibiotic, heat labile proteins. These are relatively uncommon among the antibacterial compounds of LAB, Class IV A fourth category of complex bacteriocins containing protein and lipid or carbobydrate moieties was included in Klaenhammer's classification (Klaenhammer, 1993) but Nes and coworkers (Nes *et al*., 1996) excluded this category because these compounds have not been purified and evidence for them is based on loss of activity following treatment with carbohydrate-or lipid-hydrolysing enzymes and Class V bacteriocins with circular, unmodified structure ex. enterocin (Eijsink, *et al*., 1998; Guyonnet *et al*., 2000). *L. acidophilus* produces various bacteriocins and antibacterial substances such as Lactocidin, Acidolin, Acidophilin, Lactacium-B and inhibitory protein (Shah, 1999).

4.3 Characteristics of antimicrobial protein(s) of *Lactobacillus rhamnosus*

Antimicrobial activity of Cell Free Culture filtrates (CFC) against the test organisms increase with the culture age of *L. rhamnosus* Fb and became stable when culture reached the stationary phase. A similar antimicrobial spectrum of CFC filtrate was observed against all the test organisms (Fig. 1, b & d). The antimicrobial spectrum of Extracellular Protein Concentrate (EPC) against the test organisms changed with culture age. Antimicrobial activity against *E. coli* was observed in the initial growth phase, activity increases with culture age upto 18 h, it decreases later with increasing culture age (Fig. 1, a & c). Antimicrobial activity against *Ent. aerogenes, B. subtilis, B. megaterium* and *Staph. aureus* appeared after 6 h of growth and increased with culture age became stable in stationary phase. Whereas activity against *Shigella sp., Ps. aeruginosa* and *B. cereus* was observed after 12 h of growth and increased with culture age upto 30 h before decreasing marginally. Antimicrobial activity against *S. typhi* and *P. vulgaris* was observed in stationary phase and it did not change much with the increase in culture age. The antimicrobial extracellular proteins are produced during exponential and stationary phases. Changes in the antimicrobial activity spectrum of the EPC during different growth phases provide evidence that the EPC is a mixture of antimicrobial peptides and its composition changes with the culture age (Ambalam *et al*., 2009). Antimicrobial activity spectrum changes with culture age, indicates that the antimicrobial activity is attributed to the mixture of antimicrobial peptides. Antimicrobial activity of EPC shows activity over broad pH range (2-9) but the activity varies with test organisms. At pH 2 to 5 and 8 mode of inhibition was bactericidal against *E. coli, Ent. aerogenes, S. typhi, Shigella sp., P. vulgaris, Ser. marcescens, Ps. aeruginosa, Staph. aureus, B. megaterium, B. cereus and B. subtilis*. While at pH 6, 7 and 9 the activity was bacteriostatic. Antimicrobial activity of EPC was thermostable (60 min at 100°C), thermostability was evidenced from the bactericidal activity of heat treated EPC, heat treatment caused complete loss of activity against *Ps. aeruginosa* and *Bacillus spp*. Heat stability of antimicrobial proteins has been suggested to be the major feature of low molecular weight bacteriocins and arises from complex pattern of disulphide intramolecular bonds that stabilize secondary structures by reducing the number of possible unfolded structures (Cintas *et al*., 1995). Currently we do not know the reasons for the stability of

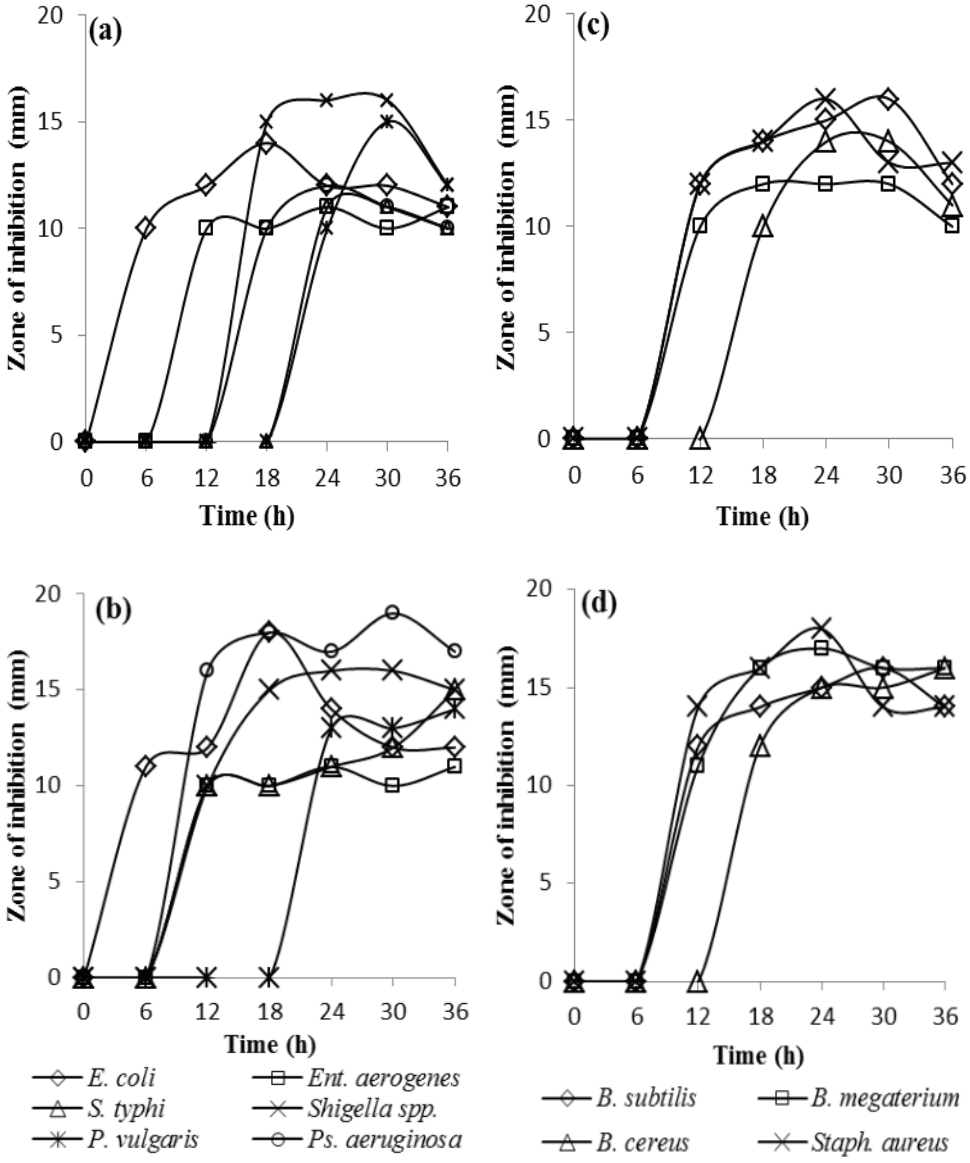


Fig. 1. Association of antimicrobial spectrum of EPC produced *by L. rhamnosus* Fb culture during logarithmic and idiophasic growth. (a) & (c) Antimicrobial activity of Extracellular Protein Concentrate (EPC) and (b & d) Cell Free Culture Filtrate (CFC) of *Lactobacillus rhamnosus* produced at the different growth phases (6, 12, 18, 24, 30 and 36 h) determined by well-diffusion assay against *E. coli, Ent. aerogenes, S. typhi, Shigella sp., P. vulgaris, Ps. aeruginosa, B. subtilis, B. megaterium, B. cereus* and *Staph. aureus*

antimicrobial peptides but the work is in progress to further characterize the structure and functions of EPC. Sensitivity of EPC to proteolytic enzymes like Proteinase K, Trypsin and Pepsin shows strain specificity, it varies with test organism which also further indicates the proteinaceous nature of the active agent. EPC treated with Proteinase K cause reduction in antimicrobial activity against *B. megaterium* (80%), *Staph. aureus* (79%), *B. cereus* (71%), *P. vulgaris* (60%), *Ser. marcescens* (53%), *S. typhi* (46%), *E. coli* (41%), *Ent. aerogenes, Shigella sp.* and *Ps. aeruginosa* marginally (<10%). Proteinase K exhibits broad substrate specificity. Proteinase K degrades many proteins in the native state even in the presence of detergent. The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids which block alpha amino group. It is commonly used for its broad specificity (Ebeling *et al.,* 1974). Trypsin cleaves peptide chains mainly at the carboxyl site of the amino acids lysine or arginine, except when either is followed by proline. Antimicrobial activity of EPC treated with trypsin was completely lost against *Ps. aeruginosa*, *Staph. aureus* and *Bacillus spp.* indicates the presence of the active site for antimicrobial action may be present at the carboxyl site of protein. Pepsin is most efficient in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Dunn, 2001). EPC treated with pepsin results in complete loss of antimicrobial activity against *S. typhi* and *Staph. aureus* while partially reduced against *Bacillus spp.* Variable sensitivity of antimicrobial activity of EPC against test organisms imply the presence of a more than one antimicrobial peptides active against different test organisms. Gel permeation chromatography (Sephadex G-25) of EPC provides additional evidence of inhibitory protein is low molecular protein. Gel electrophoresis (Tricine SDS PAGE) of EPC shows that proteins present in EPC resolved into three bands, one diffuse band representing low molecular weight peptides (4 kDa) and the other marking the presence of higher molecular weight proteins (Schagger & Von Jagow, 1987). Gel over-layer confirmed that inhibition of the test organisms was due to the diffuse band of low molecular weight protein(s). EPC is a dynamic mixture of antimicrobial peptides, since the antimicrobial spectrum of the EPC is intimately associated with the growth phase. The following experimental evidences related to heat stability, sensitivity to proteolytic enzymes, and gel permeation chromatography further implicate the presence and involvement of more than one antimicrobial peptides in the EPC. Purification and characterization of antimicrobial peptides of *Lactobacillus rhamnosus* Fb strain provides novel approach as anti-infective drug, as it shows wide spectrum of antimicrobial action against human pathogens and food spoilage organisms. It has also potential for food additives, treatment of antibiotic resistant organisms. Probiotic formulation derived from this culture can be used to treat gastrointestinal problems including various forms of dysbacteriosis. However further studies are necessary to investigate the possibility of using this novel antimicrobial peptides as an anti-infective agent and *in vivo* study.

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

4.5 Antimutagenic properties

Humans are continually exposed to a variety of natural and artificial mutagens generated by industrial and environmental activities (Vorobjeva *et al.*, 2002). 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 *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. Similar results were also observed by Sreekumar & Hosono (1998) instantaneous binding of the mutagen Trp-P-1 by *Lactobacillus gasseri*. 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. Probiotic bacteria are reported to reduce faecal enzymatic activities including β-glucuronidase, azoreductase, and nitroreductase, which are involved in activation of mutagens (Goldin & Gorbach, 1984).

4.6 Anticarcinogenic properties

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. Certain strains of *L. acidophilus* and *Bifidobacterium* spp. are reported to decrease the levels of enzymes such as β-glucuronidase, azoreductase, and nitroreductase responsible for activation of procarcinogens and consequently decrease the risk of tumour development (Yoon *et al*., 2000). Short chain fatty acids produced by *L. acidophilus* and *Bifidobacterium, L. plantarum* and *L. rhamnosus* are reported to inhibit the generation of carcinogenic products by reducing enzyme activities (Cenci *et al.,* 2002). 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. Orally administered *Bifidobacterium* is also reported to play a role in increasing production of IgA antibodies and functions of Peyer's patch cells (Singh *et al.,* 1997).

4.7 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 & 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 & Meer, 1993).

5. Conclusions

Lactobacillus strains play an important role in food fermentation processes. Modern concepts or perspectives of the application of *Lactobacillus* strains include the following selections; the best adapted and safe for human application as it is an important biodefense factor in human intestinal tract, non-pathogenic, with probiotic effects and/or health-promoting effects and food protective activities. From our study we conclude that *L. rhamnosus* is a potential candidate for probiotic product preparation or as food additives as evidenced by its ability to acid-bile tolerance, salt, antimicrobial activity against human pathogens and food spoilage organisms. The antimicrobial extracellular low molecular weight proteins are produced during exponential and stationary phases. Changes in the antimicrobial activity spectrum of the EPC during different phases of growth provide evidence that the EPC is a mixture of antimicrobial peptides and its composition changes with the culture age. Strain-specific thermostability and sensitivity to proteolytic enzymes of antimicrobial peptides provide further evidence that the antimicrobial activity of EPC is due to mixture of peptides that are heat sensitive and/or resistant. Thermostability of antimicrobial peptides confers additional advantage which can survive the thermal processing cycle of foods and can also work over a broad pH range and could therefore be used in acidic food condition. These

broad antimicrobial spectra produced by *L. rhamnosus* are potentially valuable in topical treatment, bio-control, food additives, and other applications that aim at eradicating gram-positive and gram-negative pathogens or non-pathogenic contaminants in the targeted environment. Further work on the purification, characterization of these antimicrobial peptides and mode of action is in progress.

6. Acknowledgements

Meritorious fellowship of University Grants Commission to Sheetal Pithva is gratefully acknowledged

7. References

Abee, T.; Krockel, L. & Hill, C. (1995). Bacteriocins: mode of action and potential in food preservation and control of food poisoning. *International Journal of Food Microbiology*, vol no. 28, pp.169-185

Adams, M.R. (1999). Safety of industrial lactic acid bacteria. *Journal of Biotechnology, vol no.* 68, pp. 171-178

Ambalam, P.; Prajapati, J.B.; Dave, J.M.; Nair, B.M.; Ljungh, A. & Vyas, B.R.M. (2009). Isolation and Characterization of Antimicrobial Proteins Produced by a Potential Probiotics strain of Human *Lactobacillus rhamnosus* 231 and Its Effect on Selected Human Pathogens and Food Spoilage Organisms. *Microbial Ecology in Health and Disease,* vol no. 21, pp. 211-220

Ambalam, P; Dave J. M.; Nair, B.M. & Vyas B.R.M. (2011*). In vitro* Mutagen Binding and Antimutagenic Activity of Human *Lactobacillus rhamnosus* 231. *Anaerobe,* vol no. 17, pp. 217-222

Barefoot, S.F & Klaenhammer T.R. (1984). Purification and characterization of the *L. acidophilus* bacteriocin lactacin B. *Antimicrobial Agents & Chemotherapy,* vol no. 26, pp. 328-334

Begley, M.; Gahan, C. G. & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Review,* vol no. 4, pp. 625-51

Cenci, G.; Rossi, J.; Throtta, F. & Caldini, G. (2002). Lactic acid bacteria isolated from dairy products inhibit genotoxic effect of 4-nitroquinoline-1-oxide in SOS-chromotest. *Systematic and Applied Microbiology*, vol no. 25, pp. 483–490

Champagne, C.P. & Gardner, N.J. (2005). Challenges in the addition of probiotic cultures to foods. *Critical Reviews in Food Science and Nutrition,* vol no. 45, pp. 61-84

Charteris, P. W.; Kelly, M. P.; Morelli, L. & Collins, K. J. (1998). Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology*, vol no. 84, pp. 759-768

Cintas, L.M.; Rodriguez, J.M.; Fernandez, M.F.; Sletten, K.; Nes, I. F.; Hernandez, P.E. & Holo, H., (1995). Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidiliactici* with a broad inhibitory spectrum. *Applied & Environmental Microbiology,* vol no. 61, pp. 2643-2648

Contor, L. (2001). Functional food science in Europe. *Nutritional Metabolites and Cardiovascular Diseases,* vol no. 11, pp. 20-23

De Man, J.C.; Rogosa, M. & Sharpe, M.E. (1960). A medium for the cultivation of Lactobacilli. *Journal of Applied Bacteriology,* vol no. 23,pp. 130–5

De Vuyst, L. & Vandamme, E. J. (1994). Antimicrobial potential of lactic acid bacteria. In bacteriocins of Lactic acid bacteria, Microbiology, Genetics and Applications (ed.) De Vuyst, L., and Vandamme, E. J. pp. 99-141. Glasgow, UK: Blackie Academic and Professional

Dunn B. M. (2001). "Overview of pepsin-like aspartic peptidases". *Current Protocols in Protein Science*, Chapter 21, Unit 21.3.

Ebeling, W., *et al.* (1974). Proteinase K from *Tritirachium album* Linder. *European Journal of Biochemistry*, vol no. 47, pp. 91

Eijsink, V.; Skeie, M.; Middelhoven, P.; Brurberg, M. & Nes, I. (1998). Comparative studies of class IIa bacteriocins of lactic acid bacteria. *Applied & Environmental Microbiology,* vol no. 64, pp. 3275–3281

Fooks, L.J. & Gibson, G.R. (2002). Probiotics as modulators of the gut flora. *British Journal of Nutrition,* vol no. 88, S39-S49

Fuller, R. J. (1989). Probiotics in man and animals. *Applied Biotechnology,* vol no. 66, pp. 365-378

Garrity, G.M; Bell, J.A. & Lilburn, T.G. (2004). Taxonomic outline of Prokaryotes. Bergey's Manual of Systematic Bacteriology, 2nd edition Release 5, Springer-verlag New York: 1-399

Gibson, G.R. & Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *Journal of Nutrition,* vol no. 125, pp. 1402-1412

Gilliland, S.E.; Stanley, E. T. & Bush, J. L. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *Journal of Dairy Science*, vol no. 67, pp. 3045-3051

Gmeiner, M.; Kneifel, W.; Kulbe, K.D.; Wouters, R.; De Boever, P.; Nollet, L. & Verstraete, W. (2000). Influence of a synbiotic mixture consisting of *Lactobacillus acidophilus* 74-2 and a fructooligosaccharide preparation on the microbial ecology sustained in a simulation of the human intestinal microbial ecosystem. *Applied Microbiology and Biotechnology,* vol no. 53, pp. 219-223

Goldin, B. R. & Gorbach, S. L. (1984). The effect of milk and *Lactobacillus* feeding on human intestinal bacterial enzyme activity. *American Journal of Clinical Nutrition*, vol no. 39, 756–761

Guyonnet, D.; Fremaux, C.; Cenatiempo, Y. & Berjeaut, J. (2000). Method for rapid purification of class IIa bacteriocins and comparison of their activities. *Applied & Environmental Microbiology*, vol no. 66, pp. 1744–1748

Hill, C.; Keeffe, T. & Ross, R.P. (2002). Bacteriocins-antimicrobial agents with potential in food preservation and food safety. In: *Encyclopaedia of Dairy Science*. Academic Press, London, UK

Holzapfel, W. H.; Geisen, R. & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food grade enzymes. *International Journal of Food Microbiology,* vol no. 24, pp. 343-362

Jacobsen, C.N.; Rosenfeldt Nielsen, V.; Hayford, A.E.; Moller, P.L.; Michaelsen, K.F.; Paerrgaard, A. et al. (1999) Screening of probiotic activities of 47 strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of

5 selected strains in humans. *Applied & Environmental Microbiology*, vol no. 65, pp. 4949–56

Kao, C. T. & Frazier, W. C. (1966). Effect of lactic acid bacteria on growth of *Staphylococcus aureus. Applied & Environmental Microbiology,* vol no. 14, pp. 251-255

Khedekar, C.D. (1988). Characterization of human strains of *Lactobacillus acidophilus* isolates for their suitability in preparation of milk beverage and their antibacterial cum therapeutic ability. MSc (Dairy Microbiology) thesis, Gujarat Agriculture University, Anand

Klaenhammer, T. R. (1988). Bacteriocins of lactic acid bacteria. *Biochemie,* vol no. 70, pp. 337-349

Klaenhammer, T. R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews,* vol no. 12, pp. 39-85

Klaver, F. M. & Meer, R. V. D. (1993). The assumed assimilation of cholesterol by lactobacilli and Bifidobacterium bifidum is due to their bile salt deconjugating activity. *Applied & Environmental Microbiology,* vol no. 59, pp. 1120–1124

Kurmann, J. A. & Rasic, J. L. (1991). The health potential of products containing bifidobacteria. In R. K. Robinson (Ed.), Therapeutic properties of fermented milks (pp. 117–157). London, UK: Elsevier Applied Science Publishers

Lankaputhra, W. E. V. & Shah, N. P. (1998). Antimutagenic properties of probiotic bacteria and of organic acids. *Mutation Research,* vol no. 39,pp. 169–182

Lilly, D.M. & Stillwell, R.H. (1965). Probiotics, growth promoting factors produced by microorganisms. *Science,* vol no. 147, pp. 747-748

Liong, M. T. & Shah, N. P. (2006). Effects of *Lactobacillus casei* synbiotic on serum lipoprotein, intestinal microflora and organic acids in rats. *Journal of Dairy Science*, vol no. 89, pp. 1390–1399

Metchnikoff, I.I. (1907). The prolongation of life: Optimistic studies. Springer Published Comp. New York

Molin, G. (2001). Probiotics in foods not containing milk or milk constituents, with special reference to *Lactobacillus plantarum* 299v. *American Journal of Clinical Nutrition,* vol no. 73, pp. 380S-385S

Montville, T. J. & Winkowski, K. (1997). Biologically based preservation systems and probiotic bacteria. In: Doyle, M. P., Beuchat, L. R., Montville, T. J. (Eds.), Food Microbiology. Fundamentals and Frontiers. ASM press, USA, pp. 557-576.

Nes, I.F.; Bao Diep, D.; Havarstein, L.S.; Brurberg, M.B.; Eijsink, V. & Holo, H. (1996). Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leeuwenhoek,* vol no. 70, pp. 113-128

Orla-Jensen, S. (1919). The Lactic Acid Bacteria. Copenhagen: Andr. Fred Host and Son. Mem. Acad. Roy. Sci., *Danemark, Sect. Sci.* 8, 5, 81-197

Orla-Jensen, S.(1943). The lactic acid bacteria. Copenhagen: Munksgaard. Ergaenzungsband. Mem. Acad. Roy. Sci. *Danemark, Sect. Sci. Biol*. Vol no. 2, 3, pp. 1-145

Orrhage, K.; Sillerstro¨ m, E.; Gustafsson, J. A.; Nord, C. E. & Rafter, J. (1994). Binding of mutagenic heterocyclic amines by intestinal and lactic acid bacteria. *Mutation Research*, vol no. 311, pp. 239–248

Parker, R.B. (1974). Probiotics, the other half of the antibiotic story. *Animal Nutrition and Health,* vol no. 29, pp. 4-8

Papagianni, M. (2003). Ribosomally synthesized peptides with antimicrobial properties: biosynthesis structure, function and application. *Biotechnology Advances*, vol no. 21, pp. 465-499

Saarela, M.; Mogensen, G.; Fonden, R.; Matto, J. & Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology,* vol no. 84, pp. 197-215

Salminen, S.; Deighton, M.A.; Benno, Y. & Gorbach S.L. (1998). Lactic acid bacteria in health and disease. In: Salminen S, Von Wright A, eds. Lactic acid bacteria: microbiology and functional aspects, 2nd ed. New York: Marcel Dekker Inc, 211-253

Schaafsma, G. (1996). State of the art concerning probiotic strains in milk products. *IDF Nutritional News Letters,* vol no. 5, 23-24

Schagger, H.; Von Jagow, G. (1987). Tricine-SDS-PAGE for separation of 1-100 kDa proteins. *Analytical Biochemistry,* vol no. 166, pp. 368–79

Schillinger, U.; Schillinger G. R. & Holzapfel, W.H. (1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends in Food Science and Technology,* vol no. 7, pp.158-164

Schrezenmeir, J. & de Vrese, M. (2001). Probiotic, prebiotic and symbiotic-approaching a definition. *American Journal of Clinical Nutrition,* vol no. 73, pp.S361-S364

Servin, A. L. (2004). Antagonistic activities of lactobacilli and Bifidobacteria against microbial pathogens. *FEMS microbiology Reviews,* vol no. 28, pp. 405-440

Shah, N. P. (1999). Probiotic bacteria: Antimicrobial and antimutagenic properties. *Probiotica*, vol no. 6, pp. 1–3

Shah, N. P. (2001). Functional foods, probiotics and prebiotics. *Food Technology*, vol no. 55, pp. 46–53

Shah, N. P. (2006). Health benefits of yogurt and fermented milks. In R. C. Chandan (Ed.), Manufacturing yogurt and fermented milks (pp. 327–340). Iowa, USA: Blackwell Publishing Professional

Shah, N. P. (2007). Functional cultures and health benefits. *International Dairy Journal*, vol no. 17,pp. 1262–1277

Singh, J.; Rivenson, A.;Tomita, M.; Shimamura, S.; Ishibashi, N. & Reddy, B. S. (1997). *Bifidobacterium longum* and *Lactobacillus acidophilus* producing intestinal bacteria inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. *Carcinogenesis*, vol no. 18, pp. 833–841

Sreekumar, O. & Hosono, A. (1998). Antimutagenicity and the influence of physical factors in binding *Lactobacillus gasseri* and *Bifidobacterium longum* cells to amino acid pyrolysates. *Journal of Dairy Science,* vol no.81, pp. 1508–1516

Stanton, C.; Paul Ros, R.; Fitzgerald, G.F. & Sindern, D.V. (2005). Fermented functional foods based on probiotics and their biogenic metabolites. *Current Opinion in Biotechnology,* vol no. 16, pp. 198-203

Tannock, G. W. (2004). A Special Fondness for Lactobacilli. *Applied & Environmetal Microbiology*, Vol. 70, No. 6 pp. 3189–3194

Van der Hammer, E. (1960). The carbohydrate metabolism of the lactic acid bacteria. Thesis, University of Utrecht, The Netherlands

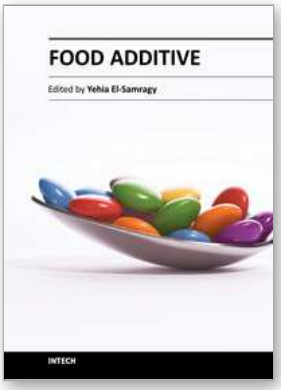
Vandamme, P.; Pot, B.; Gillis, M.; de Vos, P.; Kersters, K. & Swings, J. (1996). Polyphasis taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews,* vol no. 60, pp. 407-438

Vorobjeva, L. I. & Abilev, S. K. (2002). Antimutagenic Properties of Bacteria: Review. *Applied Biochemistry and Microbiology,* Vol. 38, No. 2, pp. 97–107

WHO (World Health Organization) (1969). Specification for identity and purity of some antibiotics. *World Health Organization/Food Add,* 69, 34, 53-67

U.S. Food & Drug Administration (1988). Nisin preparation: Affirmation of GRAS status as direct human food ingredient. *Federal Register,53,* April 6

Yoon, H.; Benamouzig, R.; Little, J.; Francois-Collange, M. & Tome, D. (2000). Systematic review of epidemiological studies on meat, dairy products, and egg consumption and risk of colorectal adenomas. *European Journal of Cancer Prevention,* vol. 9, No. 3, pp. 151–164



Food Additive

Edited by Prof. Yehia El-Samragy

ISBN 978-953-51-0067-6
Hard cover, 256 pages
Publisher InTech
Published online 22, February, 2012
Published in print edition February, 2012

A food additive is defined as a substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value. Food additives are natural or manufactured substances, which are added to food to restore colors lost during processing. They provide sweetness, prevent deterioration during storage and guard against food poisoning (preservatives). This book provides a review of traditional and non-traditional food preservation approaches and ingredients used as food additives. It also provides detailed knowledge for the evaluation of the agro-industrial wastes based on their great potential for the production of industrially relevant food additives. Furthermore the assessment of potential reproductive and developmental toxicity perspectives of some newly synthesized food additives on market has been covered. Finally, the identification of the areas relevant for future research has been pointed out indicating that there is more and more information needed to explore the possibility of the implementation of some other materials to be used as food additives.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Sheetal Pithva, Padma Ambalam, Jayantilal M. Dave and Bharat Rajiv Vyas (2012). Potential of Probiotic Lactobacillus Strains as Food Additives, Food Additive, Prof. Yehia El-Samragy (Ed.), ISBN: 978-953-51-0067-6, InTech, Available from: http://www.intechopen.com/books/food-additive/potential-of-probiotic-lactobacillus-strains-as-food-additives

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821