EFFECT OF DRYING PROCESSES ON VIABILITY AND SELECTED FUNCTIONAL PROPERTIES OF PROBIOTIC LACTIC ACID BACTERIA

A Thesis submitted to the

Department of **BIOTECHNOLOGY**
of
UNIVERSITY OF MYSORE

in fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

By

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November, 2007

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PARENTS….

Abstract

Lactic acid bacteria (LAB) are a large group of beneficial bacteria that have similar properties and produce lactic acid, as an end product of the fermentation process and are used in the preparation of traditional fermented foods since many centuries. They are used extensively in fermented milk products like yoghurt, cheese, butter, buttermilk, and kefir all over the world**.** The present research investigation focuses on the isolation of LAB from *Kanjika*, an *Ayruvedic* lactic acid fermented product prepared in our laboratory. Some of the important probiotic properties such as tolerance to low pH, high bile salt concentration up to 1%, antimicrobial activity against food-borne pathogens, β-galactosidase and phytase activity, antibiotic susceptibility and cholesterol lowering capacity were evaluated. Out of the 17 isolates, 6 were found to be potent probiotics and further characterized by 16S rDNA gene sequencing. Total DNA isolation, optimization of PCR conditions for the amplification of the 16S rDNA gene and cloning of PCR product for the sequencing was carried out followed by BLAST analysis and phylogenic study. Three potent probiotic isolates; K3a, K7b and K23c were identified as *Lactobacillus plantarum* CFR 2191, *Lactobacillus fermentum* CFR 2192 and *Pediococcus acidilactici* CFR 2193, respectively. 13 LAB cultures from department culture collection were also evaluated for functional probiotic properties, out of which *L. plantarum* B-4496 and *L. salivarius* CFR-2158 exhibited potent probiotic properties. All these five cultures showed good antibacterial activity against Enterotoxigenic *E. coli* (ETEC), a deadly pathogen which causes traveller's diarrhea.

The effect of spray drying and freeze drying on the viability and retention of key probiotic properties of *Lactobacillus plantarum* CFR 2191, *Pediococcus acidilactici* CFR 2193, *L. salivarius* CFR-2158 were studied. Maltodextrin (MDX) and Non Fat Skimmed Milk (NFSM) were used as carriers in spray drying studies. No significant improvement in viability and retention of probiotic properties were noticed in case of cultures subjected to heat shock treatment. The results highlight that spray drying of LAB cultures with MDX as a carrier is a cost effective way of producing large quantities of probiotic cultures with functional properties for neutraceutical application. Lactose, skimmed milk

and maltodextrin were found to be potent cryoprotectants in freeze drying studies. An increase of 6-8% both in viability and probiotic properties was observed when LAB were subjected to cold shock treatment and freeze dried in comparison with that of those freeze dried without cold shock treatment.

. In depth 'i*n vivo'* studies to evaluate the probiotic properties of the isolated and characterized cultures are further recommended .It is hoped that the work carried out sets the base for future research investigations and would further help in assessing the probiotic properties in animal models This would widen the scope for varied applications on a commercial scale.

ACKNOWLEDGEMENT

The greatest gift you can give another is the purity of your attention. God Almighty has blessed me immensely that I happened to come across people who have been kind enough to give a lot of attention to me during this research work. It gives me great pleasure to express my sincere gratitude to all those who have helped me in this venture.

I am extremely grateful to my guide Dr. (Mrs) S.G.Prapulla, Scientist , Department of Fermentation Technology and Bioengineering (FTBE), Central Food Technology Research Institute (CFTRI), Mysore for her valuable guidance, inspiration and constant support during the course of my work. Her extremely friendly nature, immense patience and freedom given to carry out experiments have helped me a lot to complete this work.

With deep sense of gratitude, I thank Dr. M.C.Misra , Scientist , Department of Fermentation Technology and Bioengineering, CFTRI, Mysore for his guidance and valuable suggestions during my tenure.

I express sincere thanks to Dr. V. Prakash, Director, CFTRI, Mysore for giving me an opportunity to work in this esteemed Institute and allowing me to submit the results of my research work in the form of a thesis and providing facilities for carrying out my research work.

I thank Dr. S. Divakar , Head, FTBE Department, CFTRI, Mysore for permitting to use the infrastructure at FTBE during the course of this research work.

Far too many people to mention individually have assisted in so many ways during my work at CFTRI. They all have my sincere gratitude. I would like to thank Dr. Ghildhyal, Dr. N.G.Karanth, Dr. A. Sattur, Mr. Eugene Raj, Dr. Yogesh, Dr. Somasekhar & Mr. Somanna for their help and support in my work.

I thank Dr. Prakash. M. Halami, Scientist, FM department for valuable suggestions in molecular characterization studies, Dr. Shailaja Dharmesh, Scientist, B&N Department for cell line studies and Mr. Jayaprakash, Food engineering Pilot Plant for assistance in spray drying studies.

I sincerely thank the staff of Central Instrument Facility Services, Food Engineering Pilot Plant, Glass blowing and FOSTIS- library, CFTRI for the facilities and help provided through out my research work.

I also thank my former seniors and friends at CFTRI, Naveen Babu, Mohan, Suresh, Vivek, Sekhar & Sarath who were very helpful. The fun and enthusiasm with the group is never forgettable.

My sincere thanks to Sujith, Raghuraj, Abhijith, Dr. Shabana, Jyothi and other research fellows and project assistants in the FTBE department. A special note of thanks goes to my lab colleagues Mabel, Jolly, Renuka, Ayyappan, Dr. Dina and Sharada Awasthi, Madhu, Gobi and all the dissertation students.

I deeply treasure the moral support and constant encouragement given by my parents and brother through out my career.

Also my thanks to all the dear friends especially Girish, who have been my strength both in my professional and personal life.

My sincere thanks to CSIR for providing the Senior Research Fellowship to carry out this research work.

Last but not least, deepest gratitude to God almighty for the many ways he has showered his blessings on me throughout my life.

(K.B. Praveen Kumar Reddy)

DECLARATION

I hereby declare that the thesis titled " **Effect of drying processes on viability and selected functional properties of probiotic lactic acid bacteria**" submitted to the University of Mysore, Mysore for the award of the degree of **Doctor of Philosophy** in **Biotechnology**, is the result of research work carried out by me in the department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore under the guidance of **Dr. (Mrs) S.G. Prapulla** during the period **2004-2007**. I further declare that the results of the work have not been previously submitted for any other degree or fellowship.

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CERTIFICATE

This is to certify that the thesis titled "**Effect of drying processes on viability and selected functional properties of probiotic lactic acid bacteria**" submitted to the **University of Mysore, Mysore** by **Mr. Praveen Kumar Reddy K.B**, for the award of degree of **Doctor of Philosophy** in **Biotechnology**, is the result of research work carried out by him in the Department of Fermentation Technology and Bioengineering, **Central Food Technological Research Institute**, Mysore under my guidance during the period **2004-2007.**

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
MRS	DeMann, Rogosa and Sharpe
rRNA	Ribosomal Ribonucleic acid
LAB	Lactic acid bacteria
GRAS	Generally regarded as safe
SCFA	short-chain fatty acids
AAD	Antibiotic Associated Diarrhoea
IBD	Inflammatory Bowel Disease
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
IPTG	iso-propyl-thio-β-D- galactopyranoside
NaCl	Sodium cholride
HCl	Hydrocholoric acid
rpm	Revolution per minute
μL	Micro litre
mL	Milli litre
L	Liter
CFU	Colony forming Unit
NCCS	National center for culture studies
MEM	Eagle's minimal essential medium
BRL	Bethesda Research Laboratories
NB	Nutrient Broth
B	National Center for Agricultural Utilization Research Laboratory
NCIM	National Collection/center for Industrial Microbiology
MTCC	Microbial Type Culture Collection
CFR	Central Food Technological Research Institute Collection Center
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
F	International Public Health Laboratory

Chapter 1

Introduction

1. Introduction

Lactic acid bacteria (LAB) are a large group of beneficial bacteria that have similar properties and produce lactic acid, as an end product of the fermentation process. LAB has been used in the preparation of traditional fermented foods since many centuries. They are used extensively in fermented milk products like yoghurt, cheese, butter, buttermilk, and kefir all over the world. Yoghurt, a well known fermented product contains *Lactobacillus bulgaricus* and *Streptococcus thermophilus,* and may contain *Lactobacillus acidophilus* and *Bifidobacteria*. Acidophilus milk contains *Lactobacillus acidophilus,* kefir contains numerous LAB, including *Lactococcus lactis, Lactococcus cremoris, Lactobacillus kefir, Lactobacillus casei, Lactobacillus acidophilus.* It may also contain *Leuconostoc* species which belong to LAB.

The first reference to the use of biological methods dates back to 6000 to 1000 BC when fermentation was used to produce beer, bread, wine, vinegar, yoghurt, cheese and butter (Soomer, 2002). Some of the earliest evidence of food preservation comes from the post-glacial era, from 15,000 to 10,000 BC. In order to preserve foods, fermentation has been used since centuries throughout the world. Fermented foods have a great economic value & their health benefits are well documented. It has been estimated that 25% of the European diet and 60% of the diet in many Asian and African countries consists of fermented foods (Stiles, 1996). Microorganisms especially LAB have been involved in many food fermentations including non-dairy products. LAB occurs naturally in foods or is added as pure cultures to various food products. LAB have a GRAS status (generally regarded as safe). Use of LAB has led to a considerable growth in fermented food sector world wide.

LAB are well known as starter cultures in the manufacture of dairy products such as acidophilus milk, yoghurt, buttermilk, cottage cheeses, hard cheeses and soft cheeses among others (Carr, *et. al.*, 2002). Lactic acid is produced by the starter to prevent the growth of undesirable microorganisms (Ray and Daeschel, 1992). The coexistence of LAB and fungi is also essential for the success of several biotechnological

applications, e.g. sourdough bread making, where the ratio of LAB/yeast is generally 100:1 (Gobbetti, 1998). The ancient traditions of using LAB in food and feed, combined with recent knowledge on positive health effects caused by the ingestion of probiotic LAB, suggests them as promising alternatives to chemical preservatives (Johan Schnu¨rer, 2005).

With the growing interest in self-care and integrative medicine coupled with health conscious present generation, recognition of the link between diet and health has never been stronger. As a result, the market for functional foods, or foods that promote health beyond providing basic nutrition, is flourishing. Functional foods can be defined as foods which contain significant levels of biologically active components that provide health benefits beyond basic nutrition. Other terms for functional foods include 'Nutraceuticals', 'Pharma Foods', 'Designer Foods', 'Mood Foods' etc.

Some examples of functional foods are:

- oats and barley, which contain beta-glucans and can improve blood sugar control and reduce blood cholesterol.
- cooked tomatoes, which contain the phytochemical lycopene, and may reduce risk of prostrate and cervical cancer.
- yoghurt and other cultured milk products, which contain lactic acid bacteria, and can enhance gastrointestinal system function.

Within the functional foods is the small but rapidly expanding arena of probiotics – live microbial food supplements that beneficially affect an individual by improving intestinal microbial balance.

1.1 LAB as Probiotic

1.1.1 Historical background

The term Probiotic is derived from Greek and means 'for life' and was first used by Lilley and Stillwell (1965) to describe *substances secreted by one microorganism to stimulate the growth of another* - as an antonym for antibiotic. The probiotic concept is not new. In 1885, an attempt to treat tuberculosis was made by Italian physician Cantani, by spraying *Bacterium thermo* into patients lungs. In 1908, the Nobal laureate Elie Metchnikoff has described the potential health-promoting role of beneficial bacteria (Metchnikoff, 1908). Decades later, Tannock introduced the term *bacterial interference* to describe "*interactions between two or more bacteria which leads to the establishment of a non-infected state in the host*". In 1974, Parker defined probiotics "as organisms and substances, which contribute to intestinal microbial balance**"**.

As new knowledge derived from research on microbial interactions has emerged, new definitions for the term probiotics have been proposed. Fuller, in 1991, pointed out the preventive and therapeutic potential of certain microorganisms and defined probiotics as "*live microorganisms which when ingested, may have a positive effect in the prevention or treatment of a specific pathologic condition"*. Shortly after, Havenaar in 1992, described probiotics as "mono or mixed cultures of live microorganisms, which, when applied to animal or men, beneficially affect the host by improving the properties of the indigenous microflora". This definition suggests that probiotic effect may be achieved by the combined action of two or several microorganisms, each working by different mechanisms. Most recently, Saavedra (1995) defined probiotics as "microorganisms capable of modifying the relationship with our immediate microbial environment in ways that may benefit human health".

In healthy animals each part of the intestine is colonized by typical microflora, which is adapted to grow in a beneficial symbiosis with the host. Due to intensive management methods of food today, the farm animals are very susceptible to enteric bacterial imbalance, leading to inefficient digestion and absorption of nutrients, resulting

in retarded growth. To overcome these difficulties diets have usually been supplemented with antibiotics, which have indeed proved to be very effective in decreasing diarrhea and promoting growth (Salminen *et. al.*, 1996). However, the development of resistant strains of harmful bacteria may interfere with the use of veterinary antibiotics and decrease the efficiency of antibiotics. In view of this, research efforts are being directed towards replacing feed antibiotics with more natural feed additives such as Probiotics.

1.1.2 Characteristic properties of probiotic

1.1.2.1 Functional properties of probiotics

An organism can be classified as a probiotic only if it exhibits some of the key functional characteristics (Salminen, *et. al.*, 1996; Vaughan and Mollet, 1999; Table 1.1) To exhibit their beneficial effects, probiotic bacteria need to reach its final destination. Thus, it is necessary for it to be tolerant to acid and bile salts (Chou and Weimer, 1999). Adherence to gastrointestinal cells is important for successful colonisation and thereafter, beneficial effects will last longer in gastrointestinal tract (Ouwehand, *et. al*., 1999). The knowledge of antibiotic susceptibility of potential probiotic strains is necessary for effective application (Charteris, *et. al*., 1998). Production of β-galactosidase in high amounts would aid in lactose digestion (Noh and Gilliland, 1993; Gilliland, 1989), while strains that are able to ferment fructooligosaccharides may be used in symbiotic products (Collins and Gibson, 1999; German *et. al.*, 1999).

Table 1.1: Characteristic functional properties of probiotic bacteria: *

Property	Benefit
Resistance to pancreatic enzymes, acid and bile	Survival of passage through the intestinal tract
Adhesion to the intestinal mucosa	Immune modulation
	Pathogen exclusion
	Enhanced healing of damaged mucosa
	Prolonged transient colonisation (?)
Human origin	Species specific interactions with the host
Documented health effects	Proposed health effects are 'true'
Safe	No health risk to consumer
Good technological properties	Strain stability
	Production at large scale
	Oxygen tolerance

* Arthur, 2002

1.1.2.2 Antimicrobial activity

LAB are capable of preventing the adherence, establishment, replication, and /or pathogenic action of specific enteropathogens (Saavedra, 1995). Several investigations have demonstrated that various species of LAB exert antagonistic action against intestinal and food-borne pathogens (Gibson *et. al.*, 1997). The antagonist property is manifested by

- decreasing the lumenal pH through the production of volatile short-chain fatty acids (SCFA) such as acetic, lactic or propionic acid
- rendering specific nutrients unavailable to pathogens
- decreasing the redox potential of the lumenal environment
- producing hydrogen peroxide under anaerobic conditions
- producing specific inhibitory compounds such as bacteriocins

(Havenaar, *et. al.*, 1992; Sanders, 1993; Kao and Frazier, 1996).

Bacteriocins are antimicrobial proteins and produced by a number of LAB, including *L. reuteri, L. lactis* and *Streptococcus thermophilus* (Silva, *et. al.*, 1987). Nisin, which is produced by some *L. lactis* subsp. *lactis* strains, is the only purified bacteriocin approved for use in products intended for human consumption (Dodd and Gasson, 1994;

Jack *et. al.*, 1995). *L. casei* has been shown to produce an antimicrobial substance with a wide spectrum of inhibitory activity. In an *in vitro* model, inhibitory activity against anaerobic bacteria (*Clostridium*, *Bacteroides* and *Bifidobacterium* spp) and aerobic microorganisms (*Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus* and *Streptococcus* spp) has been reported (Sato, 1984). *L. casei* and *L. acidophilus* were able to inhibit colonization of ingested *Shigella sonnei* resulting in a better gut health (Nader de Macias et, al., 1992). Continuous feeding with *L. casei* (Shirota) could suppress *enterohemorrhagic Escherichia coli* colonization in an infant rabbit model .

1.1.2.3 Competitive adherence

The interest in probiotics as a remedy for a number of gastrointestinal and other infectious diseases has gained wide interest over the last few years. But little is known about their underlying mechanism of action. Lactobacillus can adhere to the epithelium in the intestinal tract and may thus prevent the adherence of pathogenic microorganisms. Adherence indicates the capacity of the strain to colonise the mucosa. Traditionally, adherence to mucosa has been considered to be a prerequiste for the therapeutic potentials of probiotics (Salminen, *et. al*., 1996). Attachment to mucosa prolongs the time for probiotics to influence the gastrointestinal immune system and microbiota of the host. Thus the ability to adhere to intestinal surfaces is considered to be an indicator of the efficacy of the probiotic strain. The antibody titres detected from the serum of people treated with probiotic bacteria has been shown to be directly correlated with the adherence ability of the used LAB strain (O'Halloran, *et. al.*, 1997). Bacterial adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesion (usually proteins) and complementary receptors (Beachey, 1981). Studying bacterial adhesion *in vivo* is difficult and hence *in vitro* models with intestinal cell lines are widely accepted methods assceing the ability (Lehto, and Salminen, 1997). The mucus covering the epithelial cells is the initial surface that the ingested micro-organisms confront in the human gut and is considered an important site for bacterial adhesion and colonisation (Mikelsaar, 1998). Mucus is continually subjected to degradation; conversely new mucin glycoproteins (the major

components of mucus) are constantly secreted. Thus, bacteria adhered to mucus may not reach the epithelial cells and might get dislodged from the mucosal surface, with the degraded mucin, which gets washed away with the luminal contents. This may partly explain the transient pattern of colonisation characteristic for most probiotic bacteria. On the basis of these remarks, an *in vitro* evaluation of the bacterial adhesion to human intestinal mucus provides a good additional model for studying the ability of probiotics to adhere to intestinal surfaces.

Adhesion of pathogenic bacteria to mucosal surfaces is considered to be the first step of intestinal infections (Finlay and Falkow, 1997). The adhesion of pathogens is mediated by bacterial adhesins, which recognises specific mucosal receptors. Inhibition of adhesion may prevent colonisation of the intestine by the pathogen and thereby prevent the infection. Adhesion may be inhibited by blocking the receptor with specific adhesion analogues or by stearic hindrance. Some probiotic bacteria with beneficial health effects have been found to adhere to the intestinal mucosa. Therefore, adhesive probiotics could prevent the subsequent attachment of pathogens, referred to as competitive exclusion.

Successful probiotic bacteria are usually able to colonize the intestine, at least temporarily, by adhering to the intestinal mucosa (Tamura, 1983; Benno, 1992). Studies have also suggested that adhesive probiotic bacteria could prevent the attachment of pathogens, such as coliform bacteria and clostridia, and stimulate their removal from the infected intestinal tract (Benno, 1992; Saxelin, 1995). Laboratory models using human intestinal cell lines such as Caco-2 (Chauviere, 1992; Greene, 1994; Tuomola, 1998;) and intestinal mucus (Ouwehand, 1999) have been developed to study the adhesion of probiotic LAB and their competitive exclusion of pathogenic bacteria.

1.1.3 **Preservation of probiotics**

Probiotic preparations vary in the way in which they are presented; they may be in the form of powder, tablets, pastes or sprays with different excipients to maintain the preparation in the required condition. The type of preparation employed is determined by

the way in which the probiotic is intended to be used. For example, pastes are used for individual dosing of calves and pigs, whereas sprays may be used to treat day-old chicks. Preservation of frozen or freeze-dried biomolecules and cells is a complex topic which affects a number of pharmaceutical, biotechnology, and food industries. Because of its broad applicability, this is a highly active research area. However, because of its complexity, many questions remain unanswered. Microscopic studies indicate that death after freezing and thawing is correlated well with membrane damage (i.e., rapture & leakage) (Pringle and Chapman, 1981). Cell membranes are involved in solute transport, cellular attachment, and many other essential functions. It therefore follows that stabilization of the membrane and its integral proteins is key to cell recovery after freezing and thawing (Hoekstra, *et. al.*, 1997). The literature has shown that disaccharides, in particular, can stabilize cell membranes during both freezing and drying (Crowe, 1998). α,α-Trehalose, a naturally occurring disaccharide of glucose, has been found to be especially effective in freeze drying the biomolecules (Miller, *et. al.*, 1998). Cryo- (freezing) and Lyo (freeze drying) protectants are known to function at several levels. From a kinetic perspective, cryoprotectants such as trehalose promote the formation of amorphous or "glassy" solids and reduce ice formation, which can be damaging to proteins and cells (Green and Angell, 1989).

However, there are many disadvantages associated with this approach; freeze drying is time-consuming and expensive, there are high transport and storage costs associated with frozen concentrated cultures, and in addition, freeze-thaw process results in loss of culture viability. In comparison, spray drying, one of the predominant processing tools used in the dairy industry, can be used to produce large amounts of dairy ingredients relatively inexpensively; it has been estimated that the cost of spray drying is six times lower per kilogram of water removed than the cost of freeze drying (Knorr, 1998). Spray dried powders can be transported at a low cost and can be stored in a stable form for prolonged periods. However, there are obvious challenges associated in using spray drying to produce viable cultures, including the requirement that the

microorganisms survive the relatively high temperatures used (Daemen and vander Stege, 1982). While freeze drying is more suitable than spray drying for some cultures (Johnson and Etzel, 1995), researchers have observed that there is no difference in microbial viability between these methods (Teixeira, *et. al.,* 1995).

The product quality is assessed only by the viability of cultures. To the best of our knowledge, no reports are available which discusses the probiotic property of spray dried and freeze dried powder. Spray dried and freeze dried probiotic cultures are available for commercial use. But they are not quoting the probiotic properties of the microorganism. This may mislead the consumer.

1.1.4 Application of probiotics in Food /Pharma industries

1.1.4.1 Probiotics in Dairy industries

An increasing commercial interest in the addition of probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus casei*, and bifidobacteria) to fermented dairy products has been the recent trend. Formulated probiotic foods offer consumers, a low cost dietary product that has the potential to promote health in a variety of ways (Goldin, 1998). In the fermentative dairy industry, the current trend is to add cultures composed of defined single strains to fermented milks (Gilliland, 1998) and cheeses (Stanton *et. al.,* 1998;Vinderola *et. al.,* 2000).A wide variety of LAB and probiotic LAB are commercially available for use in diary industries. Different combinations of starter lactic and probiotic cultures allow the production of fermented dairy products with defined technological characteristics, potential nutritional and health benefits (Juillard *et. al.,* 1987). However, microbial interactions, either beneficial (protocooperation) or unfavorable (antagonism) among these cultures may generate undesirable changes in the composition of the bacterial flora during the manufacture and cold storage of fermented dairy products (Bellengier *et. al.,* 1997). For lactic acid bacteria, it has been found that some rod/coccus culture combinations were inhibitory, stimulatory, or neutral with regard to the rate of lactic acid production compared with single-strain cultures. Although a synbiotic relationship between *Streptococcus thermophilus* and *Lactobacillus delbrueckii*

subsp. *bulgaricus* is generally assumed, not all strains are actually compatible, and growth imbalance in fermentations with mixed cultures may occur (Radke-Mitchell and Sandine, 1984). There is little information regarding the possible interactions among lactic acid starter and probiotic bacteria. It was established that interaction among species is a factor affecting the viability of *L. acidophilus* and bifidobacteria in yoghurt (Kailasapathy and Rybka, 1997; Vinderola *et. al.*, 1999). Regarding interactions among probiotic bacteria strains added to fermented dairy products, there is no information available, with the exception of some cases such as the synergistic growth-promoting effects observed between *L. acidophilus* and *B. bifidum* strains (Kneifel *et. al.,* 1993) and the growth inhibition among probiotic species due to bacteriocin production (Yang, 1998; Yildirim and Johnson, 1998). Besides, in Argentina, the current practice is to add *Lactococcus* cultures to probiotic LAB (Vinderola *et. al.,* 2000) or traditional cheeses manufactured by thermophilic technologies, without knowledge of the compatibility with the other genera used (*Streptococcus/Lactobacillus*). Further studies on interactions among strains is needed (Juillard *et. al.*, 1987;Rajagopal and Sandine, 1990) because these bacteria must reach alive to the intestinal tract to perform their probiotic role (Kailasapathy and Rybka, 1997).

1.1.4.2 Probiotics in Pharma industries

A number of studies indicate that the consumption of probiotic is useful in the treatment of many types of diarrhea, including antibiotic-associated diarrhea in adults, travellers' diarrhea, and diarrheal diseases in young children caused by rotaviruses (Siitonen, 1990;Arthur, 2002; Table 1.2). Diarrhea is a major cause of infant death worldwide and can be incapacitating in adults. The widespread use of probiotics could be an important, non-invasive means to prevent and treat these diseases, particularly in developing countries. Probiotic bacteria have also been shown to preserve intestinal integrity and mediate the effects of inflammatory bowel diseases, irritable bowel syndrome, colitis, and alcoholic liver disease (Gade and Thorn, 1989; Nanji, *et. al.*, 1994; Kruis, *et. al.*, 1997). *Lactobacillus GG*, *L. casei*, *B. bifidum* and *S. thermophilus* are some of the widely studied species for pharma application. Fermentation of food with LAB has

been shown to increase folic acid content of yoghurt, bifidus milk and kefir and to increase niacin and riboflavin levels in yoghurt, vitamin B12 in cottage cheese and vitamin B6 in Cheddar cheese (Shahani and Chandan, 1979; Alm, 1982) and are generally used for treating the above diseases.

Evidence from *in vitro* systems, animal models and humans suggests that probiotics can enhance both the specific and nonspecific immune response, possibly by activating macrophages, increasing the levels of cytokines, natural killer cell activity, and/or increasing the levels of immunoglobulins (Sanders, 1999). In spite of limited testing in humans, these results may be particularly important to the elderly, who could benefit from an enhanced immune response. Several lines of evidence show that the appropriate strains of lactic acid bacteria, such as *S. thermophilus, L. bulgaricus* and other lactobacilli in fermented milk products, can alleviate the symptoms of lactose intolerance by providing bacterial lactase to the intestine and stomach. This is an important finding as lactose intolerance affects almost 70% of the worldwide population, and consumption of these products may be a good way to incorporate dairy products and their accompanying nutrients into the diets of lactose intolerant individuals.

1.1.5 Scope of the work

The market for functional products, such as probiotics is undoubtedly the fastest growing area in the development of newer food products. Lactic acid bacteria (LAB) are drawing an increasing amount of attention from both medical and nutritional scientists. The health promoting effects of some of the LAB are well documented. Probiotics, prebiotics and synbiotics aimed at improving 'gut' health currently represent the largest segment of functional food market. In addition, the benefits of foods with added live microbes/ probiotics on human health, in particular of milk/milk based dairy products on children and other high risk populations are being increasingly promoted by health professionals.

The key/ specific issues to be addressed, in making probiotics for a commercially viable venture

1. Screening and selection of probiotic strains
2. Assessment of their probiotic property
3. Probiotic product specification.
4. Shelf stability and quality assurance.

Safety, regulation issues; and study and establishment of beneficial health effects supported with clinical data are also essential.

It is very important these probiotic cultures/products are made widely available for relief work and population of high risk. In order to develop shelf stable starter cultures spray drying, freeze drying of LAB with stabilizer is viewed as one of the most viable technology. Development of such stable form of starter cultures with high viability and also retention of functional properties would make these LAB widely available for use in pharma and nutraceutical industries.

Inspite of the wide application of probiotics there is still a lacuna. It is the storage of stable probiotic formulations/ cultures which can be easily transported and hence the present research work focuses on the development and validation of the existing methods for the preparation of shelf stable starter cultures for use in various industries. Protection of viability and functional properties of probiotic LAB was the main criteria for the selection of the drying medium. An attempt was made to improve the protection of properties by subjecting the culture to stress (Heat shock & cold shock). The present research work mainly focuses on the selection of LAB from well known ayruvedic lactic acid fermented product and characterization of viability and functional properties and also development of a suitable technology for the preparation of shelf stable LAB.

The research work carried out is presented in eight chapters

Chapter 1: An introduction to LAB its history, as probiotic, functional characteristics, preservation and its applications is presented.

Chapter 2 : Details the literature survey relating to area of probiotics.

Chapter 3: Isolation and evaluation of LAB, from *kanjika*, an ayruvedic fermented product for potent probiotics.

Chapter 4: A detailed study on the physiological, biochemical and molecular characterization of the selected potent probiotic LAB isolates is presented.

Chapter 5 : An *in vitro* evaluation of probiotic properties of the LAB procured from the cultural collection centers is detailed.

Chapter 6: Deals with the spray drying of the probiotic lactic acid bacteria and evaluation of the probiotic properties after spray drying.

Chapter 7: Freeze drying of probiotic lactic acid bacteria with different combinations of carbohydrates and evaluation of the probiotic properties after freeze drying is detailed.

Chapter 8 : Highlights the summary of the present research work.

Chapter 2

Literature review

2.1 Lactic acid bacteria

Fermentation has been used for many centuries throughout the world. Microorganisms, especially lactic acid bacteria (LAB), have been involved in many food fermentations including non-dairy products. LAB were used in various fermented foods since antiquity. The preservation and health benefits of such traditional foods have been recognized for thousands of years and accordingly lactic acid fermentation played an important role in the early years of microbiology. After Louis Pasteur advocated his germ theory for the fermentation changes in 1857, Joseph Lister attempted to prove the microbial nature of lactic acid fermentation. Using boiled milk as a nutrient medium, he isolated by chance the first pure bacterial culture, described it as '*Bacterium' lactis* (Lister;1873). After decades of isolation and description of this new bacteria, the genus *Lactobacillus* was proposed by Beijernick (1901), which still remains a genus containing 64 valid species.

Metchnikoff (1907) proposed the health benefits related to the regular consumption of fermented milk, based on his research findings with 'Bulgarian bacillus', an organism closely related to *lactobacillus delbreuckii* ssp. *bulgaricus*, a common LAB starter of yoghurt. Although there are little scientific reports about effect of the non-dairy fermented foods, large amounts of investigations were performed about various benefits of consuming fermented milk since Metchinikoff's suggestion in 1907 .

A wide array of microorganisms colonizes the human gastrointestinal tract, some being beneficial and others detrimental. Tissier (1905) isolated one such beneficial bacterium from the gut of breastfed infants and designated the organism as *Bacillus bifidus*. The use of Bifidobacteria as probiotic emerged from this finding. LAB is considered as probiotic microorganisms due to the safety and GRAS status; use in fermentation food products since ages and also due to its innumerable beneficial health effects that are well documented. The first use of another genus of bacteria, *Streptococcus*, as probiotic was in the form of sour milk and yoghurt (Fuller,1993).

Probiotic is a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract. Where as probiotic – active substance is a cellular complex of LAB that has a capacity to interact with the host mucosa and may beneficially modulate the immune system independent of LAB's viability

LABs with probiotic activity are generally enteric flora, believed to play a beneficial role in the ecosystem of the human gastrointestinal tract. The probiotic spectrum of activity can be divided into nutritional, physiological and antimicrobial effects. These observation have led to the development of a variety of foods and feeds containing LAB cells for probiotic use in man and animals. LAB are also potential adjutants. And their oral administration trigger(s) both mucosal and systemic immune responses (Gerritse *et. al.,* 1990). Some of the nutritional and therapeutic effects ascribed to LAB are summarized as follows:

1. Improvement in nutritional quality of food and feed
2. Metabolic stimuli of vitamin synthesis and enzyme production
3. Stabilization of gut microflora and competitive exclusion of enteric pathogens
4. Enhance innate host defenses by production of antimicrobial substances
5. Reduction of serum cholesterol by assimilation mechanisms
6. Decreased risk of colon cancer by detoxification of carcinogens
7. Tumor suppression by modulation of cell mediated immunity

Furthermore, LAB possess several properties of economic importance such as lactose utilization, proteinase activity, bacteriophage defense mechanisms, and bacteriocin production. Many of these probiotic activities are offen genetically regulated by unstable and naturally transferable plasmids (McKay, 1985). Therefore , research is now being focused on the improvement and stabilization of such industrially important characteristics. In recent year's, recombinant DNA technology such as gene cloning, gene transfer and gene expressions have been applied for the development of improved and tailor-made starter cultures.

Recent global marketing trends of probiotics are based on expectations of a prophylactic effect and in many cases as an alternative to more conventional pharmaceutical preparations. Although used in humans and animals for generations, only recently, probiotics have been subjected to clinical research. The most common use of probiotics is as food in the form of fermented milk products. The list of probiotic effects and health claims with the use of LAB is expanding. This literature review is an attempt to outline various documented *in vitro* and *in vivo* findings on the probiotic spectrum of LAB

2.2 Survival during journey in gastrointestinal tract

Bacteria from food and the environment enter the mouth and are washed with saliva into the stomach. Most of the bacteria are destroyed in the stomach by gastric acid. Among many mechanisms operating in the gastrointestinal tract, gastric acid is a major host defense mechanism against infection from ingested pathogenic microorganisms. Gastric acid is also important in maintaining a sparse bacterial population in the upper small bowel because, only the most acid resistant organisms survive transit through the stomach. The small intestine constitutes a zone of transition between the sparsely populated stomach and the luxuriant bacterial flora of the colon. Intestinal motility and the inhibitory effects of bile salts are major host factors in preventing bacterial overgrowth in the small bowel.

Probiotic bacteria that are delivered through food systems, have to firstly survive during the transit through the upper gastrointestinal tract, and then persist in the gut to provide beneficial effects for the host (Chou and Weimer, 1999). Apart from this, successful probiotic bacteria should be able to survive gastric conditions and colonize the intestine, at least temporarily, by adhering to intestinal epithelium (Lee and Salminen 1995). Such probiotic microorganisms appear to be the promising candidates for the treatment of intestinal disorders occurring due to abnormal gut microflora.

2.2.1 Acid tolerance property

The low pH of the stomach and the antimicrobial action of pepsin are known to provide an effective barrier against entry of bacteria into the intestinal tract (Holzapfel *et. al.*, 1998). The pH of the stomach could be as low as 1.5 (Lankaputhra and Shah, 1995), or as high as 6.0 or above, after food intake (Johnson, 1977), but generally ranges from pH 2.5 to pH 3.5 (Holzapfel *et. al.*, 1998). The nature of food in the stomach affects the transit time through the stomach. Normally, food remains in the stomach between 2 and 4 h (Smith, 1995), however, liquids empty from the stomach faster than solids, and only take about 20 min to pass through the stomach (Gastro Net Australia, 2001). There are no agreed rules for the screening of acid tolerance of potential probiotic strains. A range of pH values, from pH 1.0 to pH 5.0, has been used to screen *in vitro* the acid tolerance of *Lactobacillus, Bifidobacterium* and some dairy *propionibacterial* strains (Conway *et. al.*, 1987; Lankaputhra and Shah, 1995; Charteris *et. al.*, 1998; Chou and Weimer, 1999; Chung *et. al.*, 1999; Zarate *et. al.*, 2000).

Most lactic acid bacteria encountered in dairy products are regarded as neutralophiles, but their intra-cellular pH (pHi) is not as tightly regulated as that of *E. coli* (Kashket,1987). *Lactococci* (Kashket, and Kashket,1985; Ten Brink, and Konings, 1982) and *Lactobacilli* (McDonald, *et. al.*, 1990), as well as other fermentative bacteria, such as *Clostridia* (Baronofsky, *et. al.*, 1985), allow their pHi to decrease as the extracellular pH (pHo) decreases due to the build up of acidic end products. Induced acid tolerance defines a condition where in bacteria acquire the ability to survive lethal acid concentrations on exposure to mildly acidic conditions,. This inducible mechanism is referred to as the acid tolerance response (ATR) and has been observed in a variety of LAB. It was first reported in 1989, when Goodson and Rowbury (Goodson, and Rowbury, 1989) demonstrated habituation of *E. coli* to normally lethal acidity by prior growth at sublethal pH. Since then, it has also been observed in *Leuconostoc mesenteroides*, *Lactobacillus plantarum* (McDonald, *et. al.*, 1990), *Listeria monocytogenes* (Kroll, and Patchett,1992; O'Driscoll, *et. al.*, 1996)

2.2.2 Bile tolerance property

Another barrier, probiotic bacteria must survive is the small intestine. The adverse conditions of the small intestine include the presence of bile salts and pancreatin (Floch *et. al.,* 1972; Le Vay, 1988). The transit time of food through the small intestine is generally between 1 and 4 h (Smith, 1995). The pH of the small intestine is around 8.0 (Keele and Neil, 1965). Bile salt resistant lactic acid bacteria can be selected by testing their survivability in the presence of bile salt and their growth in selective medium containing various levels of bile salts (Gilliland *et. al.,* 1984; Ibrahim, and Bezkorovainy, 1993; Clark and Martin, 1994; Chung *et. al.,*1999). A concentration of 0.15–0.3% of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Goldin and Gorbach, 1992). Gilliland et al. (1984) reported that when a diet supplemented with a more bile resistant strain of *L. acidophilus* was fed to newborn dairy calves, greater numbers of facultative *lactobacilli* were observed in the upper small intestines than when a strain with lower bile resistance was used. Gilliland and Speck (1977) reported that the bile tolerance of *L. acidophilus* varied among strains.

Indigenous microbiota of the intestinal tract is exposed to bile acids, which are products of cholesterol metabolism in the liver and play an important role in the digestive process due to their amphipathic nature. When selecting lactic acid bacteria for use as dietary adjuncts, a number of factors should be considered. While the functionality of probiotics depends on their ability to survive and colonize the gastrointestinal tract, resistance of cells to bile acids is strictly a necessary property. Tolerance to bile salts has generally been associated with the presence of bile salt hydrolase activity [De Smet, *et. al.,* 1994; Moser, and Savage, 2001]. However, different research studies have shown that, at least in lactobacilli, bile salt resistance could not be correlated to this enzyme [Gilliland, and Speck, 1977; Moser, and Savage, 2001; Schmidt, *et. al.,* 2001]. Furthermore, conjugated bile acids are less inhibitory than free bile acids (cholic and deoxycholic) towards intestinal aerobic and anaerobic bacteria [Floch, 1972]. Significant variations in bile tolerance have been reported among lactobacillus species and strains

[Chateau, *et. al.*, 1993; De Boever, *et. al.*, 2000; Gilliland and Speck, 1977]. In Gram-positive bacteria, the toxicity pattern of bile acids resembles that of detergents such as SDS [Begley, *et. al.*, 2001; Flahaut, *et. al.*, 1996; Schmidt, *et. al.*, 2001]; however, the actual effects of bile acids on the bacterial cell, and consequently the mechanisms of tolerance/resistance, have not been clearly established.

2.2.3 Adherence property

One of the main criteria for selecting probiotic strains is their ability to adhere to intestinal surfaces. Attachment to mucosa prolongs the time probiotics can influence the gastrointestinal immune system and microbiota of the host. Thus the ability to adhere to intestinal surfaces is thought to correspond to the efficacy of the probiotic strain. The antibody titers detected from the serum of people treated with probiotic bacteria has been shown to be directly correlated with the adherence ability of the used strain [O'Halloran, *et. al.*, 1997]. Bacterial adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesions (usually proteins) and complementary receptors [Beachey, 1981]. Studying bacterial adhesion *in vivo* is difficult and *in vitro* models with intestinal cell lines are widely adapted methods for this assessment [Lehto, and Salminen, 1997]. The mucus covering the epithelial cells is the initial surface that ingested microorganisms confront in the human gut and is considered to be an important site for bacterial adhesion and colonisation [Mikelsaar, *et. al.*, 1998]. Mucus is continually subjected to degradation; conversely new mucin glycoproteins (the major components of mucus) are constantly secreted. Adherence of probiotic strains has also been investigated using immobilised human intestinal mucus glycoproteins extracted from faeces (Kirjavainen *et. al.*, 1998; Ouwehand *et. al.*, 1998) or isolated from ileostomy effluent (Tuomola *et. al.*, 1999). The strains tested showed considerable variation in their degree of adhesion to intestinal mucus glycoproteins. However, the mechanisms involved in probiotic attachment to mucus glycoproteins are poorly known. The attachment of lactobacilli to intestinal cell lines is dependent on different bacterial surface properties. Bacterial protein structures have been proven to be involved in adhesion of some strains, as treatment with proteases was found to decrease

the adhesion ability (Chauviere *et. al.,* 1992; Coconnier *et. al.,* 1992; Bernet *et. al.,* 1993; Greene and Klaenhammer, 1994;Adlerberth *et. al.,* 1996).

Thus, bacteria that are able to adhere to mucus but unable to reach the epithelial cells might be dislodged from the mucosal surface with the degraded mucin and washed away with the luminal contents. This may partly explain the transient pattern of colonisation characteristic for most probiotic bacteria. On the basis of these remarks, an *in vitro* evaluation of the bacterial adhesion to human intestinal mucus provides a good additional model for studying the ability of probiotics to adhere to intestinal surfaces.

2.3 Antimicrobial effects

Several investigations have demonstrated that various species of LAB exert antagonistic action against intestinal and food borne pathogens (Gibson, *et. al.,* 1997). LAB are capable of preventing the adherence, establishment, replication and/or pathogenic action of specific enteropathogens (Saavedra, 1995). These antagonist properties may be manifested by

1. Decreasing the luminal pH through the production of volatile short chain fatty acid (SCFA) such as acetic, lactic, or proprionic acid.
2. Renedering specific nutrients available to pathogens
3. Decreasing the redox potential of the luminal environment
4. Producing hydrogen peroxide under anaerobic condition
5. Producing specific inhibitory compounds such as bacteriocins.

(Havenaar, *et. al.,* 1992; Sanders, 1993)

2.3.1 Lactic acid and volatile acids

Fermentation involving LAB results in accumulation of organic acids, primarily lactic acid as a major end product of carbohydrate metabolism, generated from pyruvate by lactic acid dehydrogenase. The accumulation of lactic acid and the concomitant reduction in pH of the milieu results in a broad-spectrum inhibitory activity against gram-positive and gram-negative bacteria. The acidic pH, dissociation constant (pK value), and

mole concentration are the factors that determine the inhibitory activity of lactic acid and acetic acid in the milieu (Ingram, *et. al.*, 1956). Because of the high pK value, acetic acid (pK 4.75) has more antimicrobial activity than the lactic acid (pK 3.86) (Rasic, and Kurmann, 1983) . Lipophilic acids such as lactic acid and acetic acid in undissociated form penetrate the microbial cell membrane, and at higher intracellular pH dissociate to produce hydrogen ions that interfere with essential metabolic functions such as substrate translocation and oxidative phosphorylation (Baird-Parker, 1980).

2.3.2 Hydrogen peroxide

In the presence of oxygen, LAB produces hydrogen peroxide (H_2O_2) through electron transport *via* flavin enzymes, and in the presence of H_2O_2, produces superoxide anions from destructive hydroxyl radicals. This process may lead to per-oxidation of membrane lipids (Morris, 1979), and increased membrane permeability (Kong, and Davison, 1980). The resulting bactericidal effect of these oxygen metabolites has been attributed to their strong oxidizing effect on the bacterial cell as well as destruction of nucleic acids and cell proteins (Dahl, *et. al.*, 1989; Piard, and Desmazeaud, 1992). Also, H_2O_2 could react with other cellular and milieu components to form additional inhibitory substances. H_2O_2 formation by LAB and its effect on various microorganisms has been documented for years (Klebanoff, *et. al.*, 1966; Dahiya, and Speck, 1968). LAB strains have been reported to produce H_2O_2 under aerobic conditions in a complex glucose based media.

2.3.3 Bacteriocins

The gastrointestinal tract contains many antimicrobial proteins such as colicins, defensins, cercropins and magainins. These are low-molecular weight, cationic, amphiphilic molecules; they tend to aggregate and are benign to the producing organism. LAB also produce wide range of similar antagonistic factors that include metabolic products, antibiotic like substances and bactericidal proteins, collectively termed bacteriocins. Bacteriocins vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties. Bacteriocins can be produced spontaneously or

induced. The genetic determinates of most of the bacteriocins are located on the plasmids, with a few exceptions, which are chromosomal encoded. The release of bacteriocins requires the expression and activity of released proteins and the presence of detergent resistant phospholipase A in the bacterial outer membrane of the LAB. These antimicrobial agents are species specific and exert their lethal activity through adsorption to specific receptors located on the external surface of sensitive bacteria, followed by metabolic, biological and morphological changes, resulting in the killing of such bacteria. Bacteriocins from many bacterial genera also share these characteristics.

A large number of new bacteriocins in LAB have been characterized and classified into four major classes:

- Lantibiotics
- Small heat-stable peptides
- Large heat- labile proteins
- Complex proteins whose activity requires the association of carbohydrate or lipid moieties (Nes, *et. al.*, 1996)

Most of the new bacteriocins belong to the class II bacteriocins that are small (30-100 amino acids) heat stable and commonly not post translationally modified. While most bacteriocin producers synthesize only one bacteriocin, it has been shown that several LAB produce multiple bacteriocin (2 to 3). Based on common features, some of the class II bacteriocins can be divided into separate groups such as the pediocin like (strong antilisteria) bacteriocins, the two-peptide bacteriocins and bacteriocins with a sec-dependent single sequence. The best known bacteriocin from LAB is nisin, produced by *L. lactis* (Hurst, 1981). Gibson *et. al.,* (1997) reported that *L.reuteri* produces the bacteriocin reuterin which affects gram-negative (*Salmonella & Shigella*) and gram-positive (*Clostridia & Listeria*) bacteria. Wolf et. al., (Wolf, *et. al.,* 1995) demonstrated that the oral administration of *L. reuteri*, the producer of the bacteriocin reuterin, was safe and well tolerated in adult male subjects. Speck *et. al.,* (1993) indicated that *L. reuteri* is a common lactobacillus used in the food industry and consumed without any known adverse effects.

2.4 Physiological Effects

The metabolic products of LAB range from simple short-chain fatty acids (SCFA) to essential organic acids such as folic and orotic acid. These compounds create an acidic milieu in the gut by bringing down the pH, and modulate a series of physiological events to benefit the host. Various enzymes released into the intestinal lumen by LAB exert potential synergistic effects on digestion and alleviate symptoms of intestinal malabsorption. The nutraceutical role of LAB in reduction of serum cholesterol, management of diabetes, and prevention of osteoporosis has been investigated by various laboratories.

2.4.1 Absorption of Nutrients

The human large intestine contains a complex and metabolically diverse microflora. Its primary function is to salvage energy from carbohydrate not digested in the upper gut. This is achieved through fermentation and absorption of the major products, short-chain fatty acids (SCFA), which represent 40 to 50% of the available energy of the carbohydrate. Intestinal LAB also have a role in the synthesis of the B & K vitamins and also the metabolism of bile acids, sterols, and xenobiotics.

SCFA (e.g., lactic acid, propionic acid, butyric acid) are metabolic byproducts of LAB metabolism that accumulate and exhibit significant biological activity.(Cummings, and Branch, 1986) Fermentation of protein and lipid reaching the large intestine also contributes to SCFAs in the colon, particularly the branched-chain SCFAs. The mucosa of the colon readily absorbs SCFA from the lumen, particularly acetic and propionic acids, which may then contribute to the available energy pool of the host (Rombeau, *et. al.,* 1990). In addition, some SCFAs may protect against pathological changes in the colonic mucosa because butyric acid has been shown to inhibit the expression of certain neoplastic characteristics in mammalian cell cultures (Leavitt, *et. al.,* 1978). The SCFA concentration is an important factor determining the pH of the colonic lumen. As the expression of many bacterial enzymes is influenced by the pH of the medium, changes in

amounts of SCFA produced could have important implications for foreign components and carcinogen metabolism in the gut (Mallett, *et. al.,* 1989).

2.4.2 Alleviation of Lactose Intolerance Symptoms

Lactose maldigestion is present in approximately 70% of the population world-wide. In infants, primary lactose intolerance is virtually nonexistent. *L. bulgaricus* and other lactobacilli commonly used in the fermented milk industry present sufficient active β-galactosidase to significantly decrease the lactose in the product. Kilara and Shahani (Kilara, and Shahani, 1975) suggested that yoghurt containing *L. bulgaricus* and *S. thermophilus* had a beneficial effect for lactose intolerant individuals because of the endogenous lactase. These findings were further supported by the research of Gilliland and Kim, (1984) Kolars *et. al.,* (1984) Marteau *et. al.,*(1990). This aspect has been reviewed extensively by Sanders(1994). Lin *et. al.,* (1991) demonstrated the importance of selecting strains for their β-galactosidase activity. Martini *et. al.,* (1991) indicated that lactose from yoghurt was digested better by lactose-deficient adults partly due to the microbial β-galactosidase activity. The yoghurt in that study contained *Lactobacillus delbrueckii* ssp. *bulgarcius* and *Streptcococcus salivarius* ssp. *thermophilus.* Shermak *et. al.,* (1995) examined the effect of the consumption of yoghurt and milk on lactose absorption in 14 lactose-malabsorbing children aged 4 to 16 years. They found that considerably fewer children experienced symptoms of lactose maldigestion after consuming yoghurt containing active cultures of *L. bulgaricus* and *S. thermophilus* than after consuming milk.

2.4.3 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is clinically characterised by two overlapping phenotypes, Crohn's disease (CD) and ulcerative colitis (UC), which predominantly affect the colon and/or the distal small intestine. The aetiology of the disease is not completely understood, but a genetic predisposition and the normal intestinal microflora are thought to play an important role. Modifying the composition and activity of the normal microflora may thus improve the disease. Indeed selected probiotics have been

observed to reduce the number of relapses and prolong the period of remission. Interestingly, not only LAB like *L. salivarius UCC118* and *L. rhamnosus GG*, but also *S. cerevisiae (boulardii)* and a strain of *E. coli* (Nissle) have been observed to be effective in alleviating the symptoms of IBD (Mattila-Sandholm *et. al.*, 1999; Gupta *et. al.*, 2000; Guslandi *et. al.*, 2000; Hamilton-Miller 2001).

2.4.4 Colorectal cancer

The aetiology of colorectal cancer is diverse and diet has clearly been indicated to be involved (Greenwald *et. al.*, 2001). Diets, especially high in meat and fat or low in fibre, have been observed to cause changes in the composition of the intestinal microflora, with increasing levels of Bacteroides and Clostridium and decreased levels of Bifidobacterium (Benno *et. al.*, 1991). This change in microflora composition is associated with an increase in faecal enzyme activity, β-glucuronidase, azoreductase, urease, nitroreductase and glycocholic acid reductase. These enzymes convert procarcinogens into carcinogens and may thus contribute to an increased risk for colorectal cancer. The consumption of selected lactobacilli has been observed to reduce this faecal enzyme activity. Whether this also reduces the actual risk for colorectal cancer remains to be proven. However, most, but not all, epidemiological studies suggest that regular consumption of fermented dairy products are related to lower risk for certain types of cancer (Hirayama & Rafter 2000). A certain degree of positive effect of probiotic LAB on the risk for colorectal cancer can therefore be anticipated although definite proof remains to be presented.

2.4.5 *Helicobacter pylori* Infections

Helicobacter pylori is a gram-negative bacterial pathogen responsible for type B gastritis and peptic ulcers and may be a risk factor for gastric cancer. There are some *in vitro* and animal data to indicate that lactic acid bacteria can inhibit the pathogen's growth and decrease the urease enzyme activity necessary for it to survive in the acidic environment of the stomach (Aiba, *et. al.*, 1998; Coconnier, *et. al.*, 1998; Kabir, *et. al.*,

1997; Midolo, *et. al.*, 1995). In humans, there is also evidence that probiotic strains can suppress infection and lower the risk of recurrences (Felley, *et. al.*, 2001; Michetti, *et. al.*, 1999). In thefirst study (Canducci, et. al., 2000), 120 *H. pylori* positive patients were randomly assigned to a 7-day triple therapy based on rabeprazole (20 mg twice a day), clarithromycin (250 mg three times a day) and amoxicillin (500 mg three time a day) (RCA group; 60 subjects), or to the same regimen supplemented with a lyophilized and inactivated culture of *Lactobacillus acidophilus*. Eradication of the pathogen occurred in 72% of the antibiotic-treated patients and in 88% of the patients supplemented with live lactobacilli (P = 0.03) and 87% given dead organisms (P = 0.02). The mechanisms involved are unclear, especially with the dead bacterial preparation, but there is a presumption that the lactobacilli either induced a host response to negatively affect of the survival of *helicobacter* or inhibited their spread through competitive adhesion to glycolipid receptors.

2.5 Supplementary Effects

There are numerous studies indicating that fermentation of food with LAB cultures increase the quantity, availability, and digestibility of nutrients. Yoghurt, like milk, is a good source of protein, riboflavin, folic acid, and calcium. The basis for this conclusion comes from direct measurements of vitamin synthesis and from increased feed efficiency when fermented products are fed to animals. (Gorbach, 1990) Fermentation has been reported to increase folic acid in a variety of products, including yoghurt, bifidus milk, and kefir. (Shahani, and Chandan, 1979; Deeth, and Tomine,1981; Alm,1982) There have also been studies showing an increase in niacin and riboflavin in yoghurt, B12 in cottage cheese, and pantothenic acid (B6) in Cheddar cheese (Deeth, and Tomine, 1981; Alm,1982). Thiamin and riboflavin have also been shown to increase during the preparation of LAB-fermented products

2.5.1 Production of Vitamins

Several LAB cultures synthesize certain B vitamins in fermented dairy products. In contrast, directly acidified dairy products do not exhibit such enhancement in B vitamins. Reddy et al. (1976) studied the effect of various factors on B vitamin content of cultured yoghurt and compared the B vitamin contents of cultured and direct acidified yoghurt. Incubation of yoghurt culture at 42°C for 3 h yielded maximum vitamin synthesis concurrent with optimal flavor and texture qualities. Acidified yoghurt showed a slightly higher content of certain B vitamins than the cultured yoghurt. Both cultured and acidified yoghurt showed good keeping quality and freedom from microbial contaminants during storage at 5°C for 16 days. However, folic acid and vitamin B12 content decreased 29 and 60% in cultured yoghurt and 48 and 54% in acidified yoghurt. Leim et al. (1977) found that the major source of vitamin B12 in commercial tempeh (fermented soybean food product) was a LAB that co-exists with the mold during fermentation. Reinoculation of the pure LAB in dehulled, hydrated, and sterilized soybeans resulted in the production of vitamin B12. Similarly, nutritionally significant amounts of vitamin B12 were also found in the Indonesian fermented food ontjom.

2.6 Immunomodulatory effect

Probiotics such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum* have been shown to influence the select aspects of immune function. Such altered function can involve one or several components of an immune response, e.g., humoral, cellular or non specific immunity. Although several *in vitro* and *in vivo* studies on probiotic effects on immunity have been reported, the specific mechanisms of the observed changes remain unclear. Moreover, many probiotic preparations have been tested in several separate laboratories with diverse and sometimes contradictory results. Reports of probiotic induced alteration are not limited to the localized mucosal immune system, effects on systemic immune responses have also been reported.

2.6.1 Humoral responses

There have been several reports describing the effects of probiotics on IgA in both rodents and humans (Table 2.1). Although the specific results varied, generally an enhanced IgA production was observed during probiotic treatment. For example, *L. casei*, *L. acidophilus* and yoghurt enhanced the number of IgA-producing plasma cells in a dose dependent manner (Perdigon *et. al.,* 1995). In another study, *L. casei* was shown to significantly increase the amount of IgA in response to *Salmonella typhimurium* inoculation (Perdigon *et. al.,* 1991). This increased secretion of IgA was sufficient to prevent enteric infection. Similarly, the effect of feeding heat-killed *L. casei,(Shirota)* on IgE production in mice was evaluated after intraperitoneal preinjection with ovalbumin (Matsuzaki *et. al.,* 1998). *L. casei,* (Shirota) reduced serum IgE levels and IgE production in response to ovalbumin. In addition, *in vitro* production of IgE by spleen cells from mice fed *L. casei* (Shirota) in response to restimulation with ovalbumin was inhibited in contrast to spleen cells from the control group (Matsuzaki *et. al.,* 1998). From these limited studies, it appears that *Lactobacillus* was able to enhance IgA production in experimental animal models

Table 2.1: Probiotic modulation of humoral immunit

Probiotics	Species	Assessment	Effect
Lactobacillus casei Shirota, oral (heat-killed)	Rodent	Systemic antibody response to ovalbumin	Inhibited splenocyte immunoglobulin (Ig)E in vitro and serum IgE
L. casei, oral (live)	Rodent	Infection and antibody production in malnourished animals	Increased sIgA and reduced enteric infection
L. acidophilus + *Peptostreptococcus*, oral (live)	Rodent	Translocation of Escherichia coli and serum total anti-E. coli IgG, IgE and IgM	Decreased translocation and increased anti-E. coli IgM and IgE
Bifidobacterium bifidus, oral (live)	Human	Total IgA and response to polio virus	Increased sIgA

2.6.2 Cellular response

Perhaps the most intriguing aspect of probiotic modulation of immune response is through its effects on cytokine production. Cytokines and their regulation of the immune system have been studied intensively in the last several years in cell lines and primary cells of both rodents and humans (Ha *et. al.,* 1999, Marin *et. al.,* 1998, Miettinen *et. al.,*1998, Nicaise *et. al.,* 1993, Tejada-Simon *et. al.,* 1999a and1999b). Several studies have shown that cytokine production by cells of the immune system can be altered by probiotic use (Table 2.2). For example, the effects of four commercial strains of *Streptococcus thermophilus* found in yoghurt on cytokine production were evaluated with a macrophage cell line and a T-helper cell line and compared with active strains of *L. bulgaricus, Bifidobacterium adolescentis,* and *B. bifidum* (Marin *et. al.,* 1998). All cytokines studied, tumor necrosis factor (TNFα), interleukin (IL-6), IL-2 and IL-5, were affected by heat-killed *S. thermophilus* in a strain and dose-dependent fashion. All bacteria induced significant increases in the IL-6 production in the macrophage cell line with *S. thermophilus*, showing the greatest activity. The four *S. thermophilus* strains also strongly induced TNFα production. IL-6 and TNFα to a lesser extent, production were also increased when the macrophages were costimulated with lipopolysaccharides (LPS) and cells of the three groups of LAB. After concurrent stimulation of a T cell line with phorbol 12-myristate-13-acetate, seven of the eight strains enhanced IL-2 and IL-5 production significantly (Marin *et. al.,* 1998). In another study, the effect of bacterial flora on cytokine production from mouse resident peritoneal macrophages was investigated (Nicaise *et. al.,* 1993). The production of IL-1, IL-6 and TNFα was determined in germ-free mice and mice implanted with either *Escherichia coli* or *B. bifidum*. Macrophages from the implanted mice produced significantly more IL-1 and IL-6 *in vitro* than macrophages from germ-free mice (Nicaise *et. al.,* 1993).

Table 2.2: Probiotic effects on cytokine production*

Probiotics	Species	Assessment	Effect
Lactobacillus casei, oral (dry)	Human	Serum IFNγ	Increased
Lactobacillus GG, oral (live)	Human	TNFα in patients with food allergy	Decreased fecal TNFα
Lactobacillus, *Bifidobacterium, and streptococcus* (several strains), oral (live)	Rodent	Mitogen-induced IL-6, IL-12, IFNγ, and TNFα production by intestinal lymphoid cells	Enhanced IL-6 and IL-12 (L. casei and acidophilus) Enhanced IFNγ and NO (L. acidophilus

* IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; NO, nitric oxide.

The studies have assessed the effects of probiotics on cytokine gene transcription. For example, there was no effect of repeated oral exposure to viable or nonviable *L. acidophilus, L. bulgaricus, L. casei* or *S. thermophilus* on basal cytokine mRNA expression in Peyer's patches, spleen or lymph nodes of mice, after 14 days of exposure (Tejada-Simon *et. al.,* 1999b). In another study, human peripheral blood mononuclear cells were stimulated with three nonpathogenic *Lactobacillus* strains and with one pathogenic *Streptococcus pyogenes* strain. All bacteria strongly induced IL-1b, IL-6 and TNFα mRNA expression and secretion of the cytokine protein. *Streptococcus pyogenes* was the most potent inducer of secretion of IL-12 and interferon (IFN-γ) , and two of the *Lactobacillus* strains induced IL-12 and IFN-γ production. All strains induced IL-18 protein secretion (Miettinen *et. al.,* 1998). Additional effects of probiotics have been to reverse the age-related decline in the production of cytokines (Famularo *et. al.,* 1997). For example, supplementing the diet of aging mice with several probiotic species restored IFNγ and IFNα levels compared with control mice (Muscettola *et. al.,* 1994). The mechanism of this reversal is unknown but may involve the ability of lactic acid bacteria to adhere selectively to M cells of Peyer's patches.

2.6.3 Nonspecific immunity

Several studies have demonstrated the beneficial effects of LAB in boosting a nonspecific immune response. Probiotic bacteria have been shown to influence immune responses nonspecifically by enhancing phagocytosis of pathogens as well as modifying cytokine production (Table 2.3). Most studies that have reported the effects of probiotic treatment on phagocytosis have used macrophages isolated from treated animals. However, in one study, a strain of *L. acidophilus* isolated from a human newborn was inoculated into germ-free and conventional mice, and phagocytosis of *E. coli* was assessed *in vivo* (Neumann et al. 1998). The monoassociation of germ-free mice with this LAB for 7 days improved macrophage phagocytic capacity, as demonstrated by the clearance of *E. coli* inoculated intravenously. In another study, probiotic bacteria appeared to modulate the nonspecific immune response in normal, healthy subjects compared with hypersensitive subjects (Pelto *et. al.,* 1998). Milk-hypersensitive and healthy adults were challenged with milk, with or without *Lactobacillus GG*. In contrast, milk with *Lactobacillus GG* prevented the increase of the receptors expressed. In healthy control subjects, milk challenge did not influence receptor expression, whereas milk with Lactobacillus GG significantly increased the expression of CR1, CR3, FcgRIII and FcaR in neutrophils. From this work, the authors concluded that the response was immunostimulatory in healthy subjects, but down-regulatory in milk-hypersensitive subjects. Collectively, it appears that probiotic bacteria may have a selective influence on components of nonspecific immunity, but the mechanisms by which that occurs remain to be determined.

Table 2.3: Effects of probiotics on nonspecific immunity

Probiotics	Species	Assessment	Effect
Lactobacillus casei Shirota, intravenous	Rodent	Peritoneal macrophages	Increased phagocytosis
L. acidophilus or *Bifidobacterium bifidum*, oral (live)	Rodent	Peritoneal or peripheral blood macrophages	Enhanced phagocytosis
L. acidophilus or casei, oral (live)	Rodent	Resident peritoneal macrophages	Enhanced phagocytosis
L. casei Shirota, oral (live)	Human	Peripheral blood	No effect on natural killer cell cytolysis in vitro

2.7 Clinical management of diarrhea

The use of probiotics in diarrhea is a wide and controversial field. Different types of diarrheas can benefit from probiotics use in its prevention, treatment or recovery. Travellers diarrhea, antibiotic associated diarrhea, other infectious diarrheas or AIDS related diarrhea are a few examples. Infectious diarrhea lacks a microflora balance as a consequence or cause, promoting the development of dismicrobism, which is the alteration of the optimal rate between different types of bacteria, favouring the development of harmful species. Probiotics can act in the process of dismicrobism, i.e. growth, adherence, expression of virulence factors of pathogens, which in turn interferes with peristaltic activity and non-specific immune response. The results of several trials have been reviewed [Gismondo , *et. al.,* 1999].

2.7.1 Acute diarrhea

Rotaviral diarrhea occurs mainly in infants aged 6 months to 2 years. Vomiting and subsequent rapid watery diarrhea continue for a short period. For treatment, fluid replacement for dehydration and nutritional management are performed. Effects of various probiotics on rotaviral diarrhea have been investigated by double-blind placebo-

controlled randomized studies (Isolauri, *et. al.,* 1991; Shornikova, *et. al.,* 1997; Guandalini, *et. al.,* 2000). For example, in a multicenter study performed in Europe (Guandalini, *et. al.,* 2000), 291 neonatal patients aged 1–3 months admitted for diarrhea were randomly divided into two groups, and 10^{10} CFU of *Lactobacillus rhamnosus* GG strain or placebo was administered after treatment of dehydration 4–6 h after admission. The duration of diarrhea was significantly shortened in the *L. rhamnosus* GG group, compared to the placebo group. In a double-blind placebo-controlled random ized study performed in patients aged 6–36 months (75% were infected with rotavirus), ingestion of *L. reuteri* SD 2222 strain (1010 –1011 CFU) for 5 days shortened the duration of watery diarrhea, compared to the placebo group (Shornikova, *et. al.,* 1997). Furthermore, in a study performed in 175 nursery school children aged 6–36 months in Thailand, the test group was divided into powdered milk group, *Bifidobacterium* Bb12-supplemented powdered milk group, and *Bifidobacterium* Bb12- and *Streptococcus thermophilus*-supplemented powdered milk group, and the anti-rotavirus IgA antibody titer in saliva was measured as an index of rotaviral infection (Saavedra, *et. al.,* 1994). The antibody titer increased 4 times or more during the 8-month study period in 30.4% of the subjects in the control group that ingested powdered milk alone, but no increase in the antibody titer was noted in most subjects in the group that ingested *Bifidobacterium* Bb12 and the group that ingested *Bifidobacterium* Bb12 and *S. thermophilus.*

Preventive administration of probiotics for rotaviral infectious disease has been investigated. In a double-blind placebo-controlled randomized study performed in 220 in-patients aged 1–18 months, the incidence of rotaviral infection was significantly lower in patients fed with maternal milk than in patients fed with artificial milk, but daily preventive administration of 10^{10} CFU *L. rhamnosus* GG during hospital stay did not decrease the incidence, compared to placebo administration (Mastretta, *et. al.,* 2002). Similarly, preventive administration of *Lactobacillus* GG reduced diarrheal symptoms, but no obvious prevention of rotaviral infection was noted in other studies (Szajewska, *et. al.,* 2001).

2.7.2 Antibiotic-induced diarrhea

Antibiotics cause diarrhea due to an imbalance of intestinal bacterial flora in 20% of patients treated. In double-blind placebo-controlled randomized studies, probiotics such as *Saccharomyces boulardii* (Surawicz, *et. al.,* 1989;McFarland, *et. al.,* 1995), *Lactobacillus rhamnosus* GG strain (Pant, *et. al.,* 1996), *Bifidobacterium longum* (Colombel,1987), and *Enterococcus faecium* SF 68 strain (Buydens, and Debeucklaere, 1996) significantly decreased the incidence of diarrhea in healthy subjects and patients treated with antibiotics. Diarrhea induced by antibiotics such as clindamycin, cephalosporin, and penicillin, due to proliferation of *Clostridium difficile* in the intestine is well known. The above antibiotics disturb endogenous intestinal bacterial flora, and allow abnormal proliferation of endogenous *C. difficile,* which normally exists in the intestine at a low level. Diarrhea may aggravate to pseudomembranous enteritis, and the recurrence rate after discontinuation of eradication treatment is high. For these problems, the preventive effect of probiotics on recurrence of *C. difficile* infection has been investigated. When *S. boulardii* was concommitantly administered (1 g daily for 28 d) with vancomycin for eradication, recurrence was significantly prevented, compared to the placebo group (*S. boulardii* group: 16.7%, placebo group: 50%, P=0.05) (McFarland, *et. al.,* 1995). Digestion of toxin A or B of *C. difficile,* which are important for the pathogenicity of *C. difficile,* and receptors of these toxins on intestinal mucoepithelium by proteolytic enzyme produced by *S. boulardii* is considered to be the infection preventive mechanism of *S. boulardii* (McFarland, *et. al.,* 1995). Meta-analysis of the effects of probiotics on antibiotic induced diarrhea in nine double-blind placebo-controlled studies has been performed, and the results clarified the significance of the actions of probiotics such as *S. boulardii* and *Lactobacillus* (D'Souza, *et. al.,* 2002).

2.7.3 Traveller's diarrhea

Traveller's diarrhea is defined as the passage of >3 unformed stools in a 24-h period in individuals living in industrialized countries and who travel to tropical and semitropical areas.It affects 20-50% of travellers. The prevention of traveller's diarrhea by probiotics (a mixture of *Lactobacilli, bifidobacteria and streptococci* as well as

Saccharomyces boulardii and Lactobacillus rhamnosus GG) could be a safe alternative to antibacterial drugs (De Roos and Katan, 2000). Based on these data, travellers may expect a 25-50% reduction in the risk of diarrheal illness. Other *lactobacilli* species have not shown any significant positive effects. The effects might not be uniform or consistent, and may depend on the geographic area or populations studied. The geographic dependency was studied by Oksanen *et. al.,* (1990), who investigated two groups of people travelling to different destinations in Turkey. In one group, the use of probiotics showed a significant decrease in the occurrence of diarrhea, whereas in the other group no differences were found (Marteau *et. al.,* 2001) (LR 12). Another study was performed in 282 soldiers, in which the preventive effects of *L. acidophilus* LA strain and *L. fermentum* KLD strain on diarrhea were investigated, but no significant effect was noted (Katelaris, *et. al.,* 1995). For studies of traveler's diarrhea, it is necessary to obtain more reliable results by appropriate selection of travelling regions.

2.7.4 Treatment of diarrhea in developing countries

In developing countries, diarrhea is a common disease. It is responsible for many deaths annually because of dehydration. In the few published studies performed in these countries, the early administration of *Lactobacillus rhamnosus* strain GG in addition to oral rehydration therapy resulted in faster correction of acidosis and shorter duration of diarrhea, although not in persons with blood discharge diarrhea. This can be explained by the fact that these episodes of diarrhea may be caused by rotavirus which has been shown to be effected beneficially by the use of probiotics (Marteau *et. al.,* 2001).

2.7.5 The effect of probiotic on Diarrhea: Proposed mechanisms

One of the most important hypothesis regarding the effect of probiotics on diarrhea might be the competition for binding sites on the intestinal epithelium. When lactobacilli are ingested, they will compete for binding sites, leaving less binding sites open for pathogens. Pathogens will pass through the gut and leave the body sooner if no binding site is available. Another mechanism concerns competition for nutrients. When

many harmless bacteria are present in the gut, they utilize more nutrients, leaving less nutrients for pathogenic bacteria, which may not be able to survive because of starvation. In addition, entrance of probiotics in the gut may also stimulate the production of IgA. The importance of the IgA production on the immune system has become clear from studies performed in mice, which were kept germ-free after birth (Isolauri *et. al.,* 2001). In the absence of intestinal microflora, the intestinal immune system was underdeveloped and intestinal morphology was disrupted. The mice had underdeveloped peyer's patches, decreased macrophage chemotaxis, and a lower capacity for intracellular killing of pathogens compared with macrophages from conventionalised animals. The number of lymphocytes in germ-free mice in the intestine was also greatly reduced. In most cases, they had a predominant production of IgM, little IgG, and no IgA at all. When these mice were given probiotics or were transferred to a conventional area, the immune system would develop into a normal regular immune system. They were found to produce a greater diversity of antibody isotypes, including antibodies specific for resident intestinal bacteria.

A specific effect of probiotics on rotavirus is the decrease of fecal urease. Urease is a proinflammatory mediator that predisposes gut mucosa to ammonia-induced destruction and thus to the overgrowth of urease-producing bacteria, and is stimulated by rotavirus. Oral probiotics appeared to normalise fecal urease concentration, thereby stabilising the gut microbial environment (Isolauri *et. al.,* 2000).

2.8 Applications

In recent years, the retail sales of biomilks, bio yoghurts, and other probiotic products continue to grow rapidly in the markets of Europe, North America, and many other developed countries. Such products are made using a single genus, or in combination with other LAB as mixed starter cultures. In the absence of published surveys, it is impossible to predict the extent to which expected health benefits have contributed to this sales pitch, but it would seem unlikely that consumers would have acquired this intense probiotic loyalty without a genuine belief in its therapeutic and/or prophylactic properties (Tamime, *et. al.,* 1995).

2.8.1 Fermented Foods

Fermented foods have a long history of safe usage and are found in diets throughout the world. Fermentation is a process in which foods are modified by the action of microorganisms or enzymes in order to achieve a desired biochemical change. Production and consumption of fermented foods dates back to thousands of years. Even today, fermentation continues to be used for household production and preservation of foods in many countries, where mass produced food is not widely available (Hull, *et. al.*, 1992). With modern food processing technology, large-scale production of fermented products can be accomplished with precise controls over the microorganisms and enzymes used in the fermentation process.

Fermentation of meat is a traditional method for protecting it from spoilage. LAB, either alone or in combination with micrococci, yeasts, or molds are essential for the fermentation process (Hammes, and Tichaczek, 1994). Throughout the world, fermented foods continue to constitute a significant proportion of the diet. Production of fermented foods is highest in Europe, North American, and sub-Saharan Africa, with significant production and consumption in South America, the Middle East, India, and Southeast Asia. In terms of total production and consumption worldwide, dairy foods, beverages, and cereals constitute the majority of fermented foods (Campbell-Platt,1994). Traditionally, LAB has been used in the production of fermented foods. These include species of *Lactobacillus and Bifidobacterium* and the species *S. thermophilus*. This may have begun as a process in nature, in which nutrient availability, environmental conditions selected particular microorganisms, which modified and preserved the food (Campbell-Platt,1994). Approximately 80 bifid-containing products are now on the world market.Most are of dairy origin and include products such as yoghurt, buttermilk, sour cream, powdered milk, fortified milk, cookies, and frozen desserts (Modler, *et. al.*, 1990).

2.8.2 Supplemented Foods

The species of LAB used in the preparation of probiotic products include *L. bulgaricus, L. lactis, L. salivarius, L. plantarum, S. thermophilus, Enterococcus faecium, E. fecalis,* and *Bifidobacterium* sp.

Considerable attention has been given in recent years to the use of *bifidobacteria* in probiotic foods, particularly in Japan and Europe (Ishibashi, and Shimamura,1993). Hughes and Hoover (1991) reviewed and summarized a number of probiotic applications for bifidobacteria. In the 1940s, bifidus milk was used as a treatment for infants with nutritional deficiencies. In Japan, the first bifidus product (low-fat fresh milk containing *B. longum* and *L. acidophilus*) was developed by Morinaga Milk Industry Company in 1971; full scale production began in 1977 when the company started a home delivery service (Ishibashi, and Shimamura,1993). By 1984, there were 53 Bifidus products in the market in Japan. Today, many products, including yoghurts, have been reformulated to include bifidus cultures; total yoghurt sales in Japan have nearly doubled from the 1980s to the 1990s (Hughes, and Hoover, 1991).

Today, many products containing LAB are available worldwide. Probiotic preparations are manufactured in various forms (tablets or powders) and incorporated in a number of foods (milk, chewing gums, fiber preparations, sweets, cakes, beer, and soymilk). Some of probiotic products available worldwide and the LAB used in their production are summarized in Table2.4.

2.8.3 Pharmaceutical Products

Special dietary preparations containing viable cells of LAB are available in different markets as freeze-dried tablets. The primary objective of these products is to achieve persistant colonization of the bacteria in the gut during the treatment of a variety of conditions such as gastrointestinal disorders (e.g., post-antibiotic therapy, adjustment of microbial imbalances in the gut, liver diseases, chronic constipation, chronic duodenitis, peptic ulcers in children, and after irradiation therapy) (Tamime, *et. al.*, 1995). Some of Probiotic products available commercially in the pharmaceutical market are summarized in the table 2.5

Table2.4: LAB-Supplemented Foods Currently Available in Different Markets

Product or trade name	Origin	LAB culture
A B milk products	Denmark	*L. acidophilus, B. bifidum*
Acidophilus bifidus yoghurt	Germany	*L. delbrueckii subsp. Bulgaricus, S. thermophilus L. acidophilus B. bifidum or B. longum*
B A®	France	*B. longum*
Bifidus milk	Germany	*B. bifidum or B. longum*
Bifidus milk with yoghurt flavor	UK	*B. bifidum, B. longum, or B. infantis*
Bifidus yoghurt	Many countries	*B. bifidum or B. longum*
Bifighurt®	Germany	*B. bifidum or B. longum*
Bifilakt® or Bifilact®	USSR	*Lactobacillus sp., Bifidobacterium. sp.*
Biogarde®	Germany	*L. acidophilus, B. bifidum, S. Thermophilus*
Bioghurt®	Germany	*L. acidophilus, B. bifidum, S. thermophilus*
Biokys®	Czechoslovakia	*B. bifidum, L. acidophilus, P. acidilactici*
Biomild®	Germany	*L. acidophilus, Bifidobacterium sp.*
Cultura®	Denmark	*L. acidophilus, B. bifidum*
Diphilus milk®	France	*L. acidophilus, B. bifidum*
Mil-Mil®	Japan	*B. bifidum, B. breve, L. Acidophilus*
Ofilus®	France	*S. thermophilus, L. acidophilus, B. bifidum or Lc. lactis ssp. cremoris, L. acidophilus, B. Bifidum*
Progurt®	Chile	*Lactococcus lactis biovar diacetilactis, B. Bifidum Lactococcus lactis ssp. cremoris, L. acidophilus*
Sweet acidophilus bifidus milk	Japan	*L. acidophilus, B. longum*
Sweet bifidus milk	Japan/Germany	*Bifidobacterium sp.*
Prolife®	India	L.acidophilus

Tamime et al. (1995).(392)

Table 2.5 : Commercially marketed pharmaceutical formulations of probiotics		
Product name	Probiotic bacteria present	Description and properties of the product
Kyo-Dophilus capsules (Wakunaga Probiotics)	*Lactobacillus acidophilus* *Bifidobacterium bifidum,* *Bifidobacterium longum*	Contains human strains of bacteria. Each capsule contains about 1.5 billion live cells.
Kyo-Dophilus tablets (Wakunaga Probiotics)	*Lactobacillus acidophilus*	Stable preparation not requiring refrigeration, completely vegetarian, dairy and sugar free, chewable, tasty and convenient for travel.
Acidophilas (Wakunaga Probiotics)	*Lactobacillus acidophilus*, Lipase, protease, amylase and lactase enzymes	Enzymes assist in breakdown of fats, proteins and carbohydrates, lactase assists in digestion of milk sugar lactose in lactose intolerance
Probiata tablets	An internationally accepted strain of *Lactobacillus acidophilus*	Bacteria pre-adapted for growth in human intestine. Each tablet contains one billion live cells per tablet, preparation requires no refrigeration and is heat resistant. Survives when taken with food and resistant to acid of stomach (pH 3–4)

TH1 Probiotics (Jarrow Formulas)	*Bifidobacterium longum, Saccharomyces boulardi, Lactobacillus casei, Lactobacillus plantarum*	Contains one billion *Bifidobacterium longum* per serving along with 1/2 billion *Saccharomyces boulardi*, both *Lactobacillus casei* and *Lactobacillus plantarum* are heat treated so as to be safe for use in severely immune compromised persons who have leaky gut.
Bifa 15 (Eden Foods)	*Bifidobacterium longum*	Contains microencapsulated *Bifidobacterium longum* designed to get past the stomach acids and reach the colon. Produces a very hostile environment for HIV, Candida and other infections of the large intestine.
Replenish (Innercleanse 2000)	*Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus bifidus, Lactobacillus bulgaricus, Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus brevis, FOS* (Fructo-oligosaccharides)	Causes an increase in production of beneficial short -chain fatty acids, reduction of serum cholesterol and blood pressure, improved liver function and improved elimination of toxic compounds. FOS helps promote the growth of friendly bacteria, while simultaneously reducing the colonies of detrimental bacteria.

Sporlac (powder)	*Lactobacillus sporogenes*	
Pre Pro (Fourrts)	*Streptococcus faecalis* *Clostridium butyricum* *Bacillus mesentericus* *Lactobacillus acidophilus* *Fructo-oligosaccharides*	
Bifilac (Tablets)	*Streptococcus faecalis* *Clostridium butyricum* *Bacillus mesentericus* *Lactobacillus sporogenes*	
VSL[3 (CSL)	5310 cells /g of three strains of *Bifidobacteria,* four strains *of lactobacilli* and one strain of *Streptococcus salivarius ssp. thermophilus*	Colonize the intestine and help in the remission of ulcerative colitis in the patients intolerant or allergic to 5-aminosalicylic acid.

2.9 Market potential for probiotics

The most active area within the functional foods market in Europe has been probiotic dairy products, in particular, probiotic yoghurts and milks. In 1997 these products accounted for 65% of the European functional foods market, valued at US$889 million, followed by spreads, valued at US$320 million and accounting for 23% of the market (Hilliam,1998). In a study undertaken by Leatherhead Food RA, the market for functional foods in the United Kingdom, France, Germany, Spain, Belgium, Netherlands, Denmark, Finland, and Sweden was reviewed. The results of the study showed that the probiotic yoghurt market in these 9 countries totalled >250 million kg in 1997 (Hilliam,1998), with France representing the largest market, having sales of ~90 million kg, valued at US$219 million. The German market for probiotic yoghurts is growing rapidly; for example, during 1996–1997, it increased by 150%, whereas the UK market grew by a more modest 26% during the same period. On average, probiotic yoghurts accounted for ~10% of all yoghurts sold in the 9 countries studied, with Denmark having the highest proportion (20%) of probiotic yoghurts, followed by Germany and the United Kingdom (both at 13%) and then France (11%). On the lower end of the scale were the Netherlands and Belgium (both at 6%) and then Finland and Sweden (both at 5%) (Hilliam,1998). Seen as crucial to market expansion in Europe is further clarity on the use of health claims. The market for functional foods in Europe could ultimately account for ~5% of total food expenditure in Europe, which, based on current prices, would equate to ~US$30 billion (Young, 1996).

The US functional foods market is comparatively underdeveloped by European standards, with fortified dairy products, particularly those containing active cultures, gaining popularity only recently. In contrast with the situation in Europe, there is a notable lack of development of probiotics in the United States. Vitamin- and mineral-enriched products continue to be among the more successful functional foods in the United States. Market development has been held back by criticism leveled at companies that have introduced products bearing vociferous health claims. It is predicted that the US market for functional foods will experience the fastest growth rates compared with other

countries in the future (Young, 1996). An important aspect in this context will be the development, clarification, and testing of the laws relating to health claims. As is the case in Europe, the issue of health claims will be important for the future growth and expansion of the market for probiotics and functional foods in the United States.

2.10 Mass Production

Development of consistently effective probiotics is still at a very early stage. The probiotic concept will come to acceptance if the underlying mechanisms of claimed health benefits are elucidated and appropriate selection criteria for probiotic microorganisms are defined. Probiotic strains therefore must fulfill a number of general and specific criteria (Havenaar, *et. al.*, 1992). The general selection criteria are determined by biosafety aspects, methods of production and processing, and the method of administration of the probiotic and the location in the body where the microorganism must be active. Specific selection criteria must include phenotypic traits such as specific enzyme activities or stimulation of the immune system. A rational selection and validation of promising probiotic LAB is only possible from evidence obtained in validated *in vitro* models with a high predictive value and followed by *in vivo* studies, including human trials.

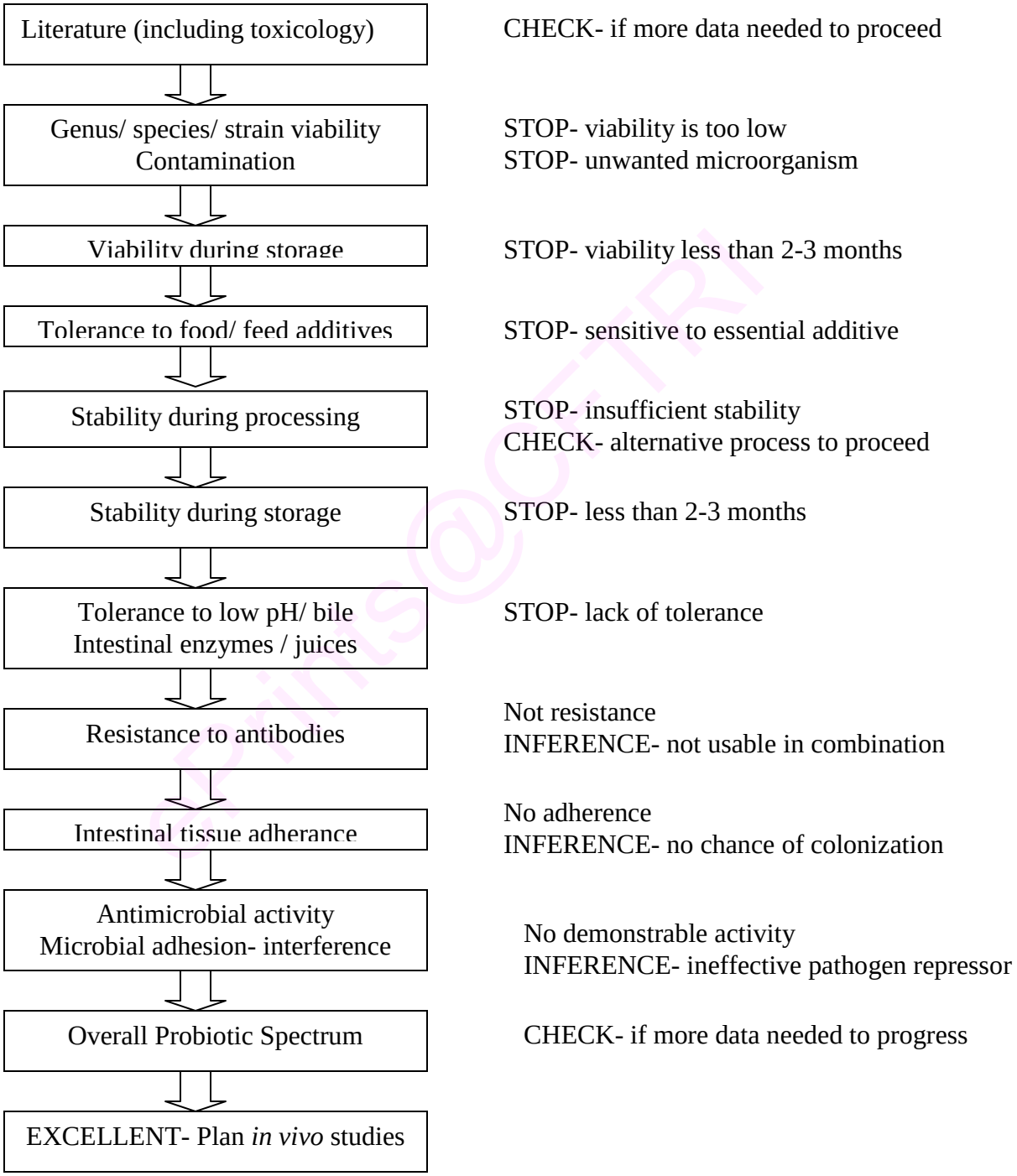
2.10.1 Strain Selection

LAB strains for probiotic use must be representative of microorganisms that are Generally Recognized As Safe (GRAS microorganisms). The use of newer strains without proven track record of biosafety should undergo extensive toxicological and tolerance testing prior to acceptance (Figure 2.1). The next important basic step in the selection procedure is the choice of origin of the strain. This choice is mainly determined by the specific purpose of the probiotic. The generally desired properties of LAB human probiotic use are

- Human origin with a proven safety and tolerance record
- Ability to survive acidic conditions and bile in the gut
- Contribute to positive regulation of intestinal functions, correction of bowel disorders

Figure 2.1:Flow diagram for *In vitro* selection of strains for probiotic use.

(Havenaar *et. al.*, 1992.)



• Produce vitamins and release beneficial enzymes that would facilitate digestion of food, absorption of essential nutrients and inactivate carcinogens and toxic compounds

• Capacity to adhere to human intestinal epithelia, colonize the mucosal surface, and proliferate in the human gut

• Produce antimicrobial substances and demonstrate a wide spectrum inhibitory activity against human pathogens

• Possess probio-active cellular components that could stimulate immune responses, augment host defense functions of reticuloendothelial cell cascade

2.10.2 Technological hurdles in the development of probiotic foods

While it is undoubted that clinical evidence supporting the health-promoting activity of probiotic cultures is of paramount importance, it is probably less well appreciated that the technological suitability of these strains is also critical for their exploitation. In this respect, it is not surprising that, human intestinal isolates, many of which are obligatively anaerobic grow very poorly outside their natural habitat, the human gut. Indeed, much of the human intestinal flora are at present uncultivable and can only be studied using culture independent approaches. Consequently, the large-scale cultivation and subsequent storage of probiotic lactobacilli and bifidobacteria in high numbers often present a major bottleneck to the realization of their commercial potential. For this reason, intensive research efforts have recently focused on protecting the viability of probiotic cultures both during product manufacture and storage and during gastric transit. These studies have demonstrated that cultures can be significantly protected via encapsulation in a variety of carriers, which include milk proteins and complex (prebiotic) carbohydrates. In many cases, the resultant products not only have better probiotic viability but can also be regarded as 'synbiotics' given the presence of probiotics and probiotics (Roberfroid 1998). The physiological state of the probiotic cultures added to a product can also be a major factor affecting overall culture viability. In this respect, the induction of stress responses in probiotic strains can have a dramatic effect on the ability of cultures to survive processing, such as freeze drying and spray drying and during gastric transit. The authors have generated probiotic cultures that over

express the heat shock proteins GroESL and have demonstrated improved performance of the culture under a variety of conditions including heat, spray drying and exposure to gastric acid (Desmond *et. al.,* 2004). The addition of various protective compounds to probiotic cultures can also improve their viability during manufacture – examples include glucose to energize cells on exposure to acid (Corcoran, *et. al.,* 2005) and cryoprotectents such as inulin to improve survivability during freeze drying (Carvalho, *et. al.,* 2004). In conclusion, a number of novel technologies are now emerging which can improve the viability of human intestinal strains for probiotic applications. It indicates that, it may be possible to exploit many 'sensitive' cultures which hitherto have been difficult to propagate and maintain at high cell numbers.

2.11 Metabolic engineering of lactic acid bacteria

It is possible that a potential probiotic LAB strain would fulfill a number of specific criteria for a product development but lack one important property. A preferable manipulation would be locating and isolating the genes that are responsible for a specific factor, such as adherence, and to transfer these genes to a probiotic LAB strain without altering other beneficial properties. Use of gene technology for the construction and improvement of microorganisms could provide great promise for future development of probiotic strains. Natural gene transfer processes are of significance both in the evolution of LAB and as a tool for the construction of genetically manipulated strains. This approach may have advantages over recombinant DNA methods that require regulatory approval and consumer acceptance (Gasson, and Fitzgerald, 1994). The advent of high voltage electroporation greatly simplified the genetic manipulation of LAB. The tools for the genetic engineering of LAB are clearly available. The catalog of cloned genes and regulatory and expression signals available will allow thorough exploration of the expression and secretion signal sequence requirements and the potential of these bacteria (Mercenier, *et. al.,* 1994) Knowledge thus gained could be put to rational application in the design and modification of strains.

LAB are promising targets for metabolic engineering, because their energy and carbon metabolism is relatively simple and energy metabolism is not generally connected to biosynthetic activity. Therefore, the sugar metabolism pathways can be manipulated without disturbing the synthesis of cell components (Kleerebezem & Hugenholtz 2003). Many metabolic engineering strategies for LAB have focused on rerouting pyruvate metabolism to produce commercially important end products (sweeteners, flavors and aroma components) (Smid et al. 2005a). Metabolic engineering of more complex biosynthetic pathways leading, for example, to exopolysaccharides, vitamin B2 and B11 has been reported (Kleerebezem & Hugenholtz 2003, Sybesma et al. 2004). Being one of the model organisms in microbial metabolism, *L. lactis* has obviously also been the main target of metabolic engineering in LAB.

Lactococcus lactis is by far the most extensively studied lactic acid bacterium, and over the last decades elegant and efficient genetic tools have been developed. These tools are of critical importance in metabolic engineering strategies that aim at inactivation of undesired genes and/or (controlled) overexpression of existing or novel ones. Especially, the nisin controlled expression (NICE) system for controlled heterologous and homologous gene expression in Gram-positive bacteria (de Ruyter et al. 1996; Kleerebezem et al. 1997; Kuipers et al. 1998) was proven to be very valuable and has been employed in most of the metabolic engineering strategies discussed there (Hols et al. 1999; Hugenholtz et al. 2000; Boels et al. 2001; Sybesma et al. 2002).

2.12 Safety of probiotic

Food fermentation is one of the oldest known uses of biotechnology. Fermented foods and beverages continue to provide an important part of human diet and constitute 20 to 40% of food supply worldwide (Campbell-Platt, 1994). By tradition, LAB are involved in the production of fermented foods. These microorganisms constitute one quarter of our diet and are characterized by a safe history, certain beneficial health effects, and an extended shelf life when compared with raw materials (Hammes, and Tichaczek,1994). Since antiquity, people have regularly consumed large numbers of

LAB, both in fermented and nonfermented foods, comprised as the material's natural flora. While the situation requires continued monitoring, it must be emphasized, however, had these organisms posed any serious health threat, it would have become apparent a long time ago.

The emergence of microbial antibiotic resistance, side effects due to the presence of synthetic stimulants in farm animals, and a rapid rise in immuno compromised hosts have instigated the consumer as well as the manufacturer to search for alternative food processes. Probiotics are being considered to fulfill this role. Consumers in the U.S. are increasingly taking a proactive stance toward their health and are purchasing products not only to eliminate what is perceived as dietary negatives but also to increase the levels of dietary positives.

In wake of the ongoing commercial upsurge, the tolerance, safety, and GRAS (generally recognized as safe) status of probiotic LAB should be clearly established. General recognition of the safety of an ingredient requires both technical evidence of its safety and suitability for the proposed use and a basis to conclude that this evidence is generally known and accepted by the scientific community. Current regulations specify that recognition of safety must be based on published studies that may be corroborated by unpublished studies and other data. A bibliography and copies of published scientific studies after which the safety determination was made, should support the rationale. According to the U.S Food and Drug Administration (FDA), the safety of an ingredient used in foods can be established in one of the following ways.

- The ingredient may be approved by the FDA as a food additive
- The ingredient may be subject to a prior sanction or approval issued by the FDA prior to September 6, 1958
- The ingredient may have been determined as GRAS by either the FDA or by a panel of qualified scientific experts

The GRAS status of probiotic LAB should be considered based on the following four lines of scientific evidence:

1. **Human trials**:

Oral administration of probiotic LAB was well tolerated and proven to be safe in 143 human clinical trials during 1961 to 1998 no adverse effects or events were reported in any of the 7526 subjects participated in these studies.

2. **Animal models**:

LAB administration in experimental animal models *in vivo*, that is, mice, rats, guinea pigs, etc.; never elicited pyrogenicity, acute toxicity, or bactereimia. On the contrary, probiotic LAB appeared to prolong the survival of the animals.

3. **In vitro studies**:

Probiotic LAB are shown to be noninvasive in eukaryotic cell interaction experiments with differentiated human intestinal epithelial cells (for microbial adhesion) or with human lymphoid carcinoma cell lines (for eliciting immunomodulatory effects).

4. **Surveillance of probiotic market**:

Consumers are looking for ways to stay fit and healthy. Several factors contribute to the expansion of probiotic and other natural product markets. By considering the following points, a marketing splash world wide is expected with well-designed probiotic-containing products (Sanders, 1998).

• People would rather prevent than cure disease

• People are more aware of the link between health and nutrition

• People want to counteract the perceived increase of environmental hazards from pollution, pathogenic microbes, and chemicals in air, water, and food

• Health care costs are on the rise and nutraceuticals and functional foods provide a low-cost approach for maintaining health

• Controlled scientific studies are providing support for efficacy of natural approaches (including probiotics) to maintain health.

2.13 Conclusions

Currently, most probiotic LAB are used in dairy with a 'Metchnikoffian philosophy' and a telemarketing trend "so what, if the expected and claimed effects fail to appear, at least the consumer is satisfied and has had a tasteful meal". Future research on probiotic LAB should break this dogma and focus on the selection of Target-Specific Probiotics for prophylactic and therapeutic health benefits. Pathogen-specific probiotic strains are needed in the clinical management of intestinal illnesses caused, for example, by rotaviruses, enteropathogenic *E. coli, and Helicobacter pylori*. Multifunctional Prebiotics with nutraceutical properties should be explored for a positive interplay in the delivery, intestinal establishment, and performance of target-specific probiotics. Probio-Active components of LAB cells and their stimulatory role in mucosal and systemic immunity warrants higher definition and characterization. Identification of specific factors for augmentation of reticuloendothelial system and cytokine pathways has unlimited potential and can transform the probiotic area. Research trends with multinational food companies indicate that the new probiotic functional foods will include infant formulae, baby foods, fermented fruit juices, fermented soy products and cereal based products, and also disease-specific clinical foods containing viable probiotic LAB, prebiotic precursors, and/or probio-active cellular components.

Chapter 3

Isolation & Screening of probiotic Lactic acid bacteria from *Kanjika*

3.1 Introduction:

Kanjika (Indian functional food, also abbreviated as Kanji), a probiotic food, is a lactic fermented product where lactic fermentation is the terminal step in food processing and is prepared from raw materials of plant origin and devoid of dairy product. It is helpful in maintaining health of ecoorgan. *Kanjika*-satwa is a type of dried *Kanjika*. Fried lentil balls (Urid wada) are also combined with *Kanjika*; known as Kanjiwada .*Kanjika* is also prepared using barley or millet in place of rice. Sometimes bamboo leaves are added along with radish in the fermentation mixture. *Kanjika* was prescribed for a number of chronic diseases by *Ayurvedic* practitioners (Acharya Sidh Nandan Misra, 1993).

Lactic acid bacteria (LAB) are known for their probiotic properties (Seher Demirer *et. al.*, 2006; Elina Ronka *et. al.*, 2003; Erika Isolauri *et. al.*, 2001). Probiotics are live microbial food supplements or health adjuvants, which can provide a wide variety of health benefits to the mankind and animals (Lan-szu chou and Bart weimer, 1999). These organisms should possess the ability to cross the barriers from mouth to intestine, such as low pH in the stomach and bile in the duodenum. They should also adhere to the intestinal micelle and exhibit antagonistic activity against pathogenic microorganisms (Lan-szu chou and Bart weimer, 1999). The health benefits attributed to probiotic bacteria in the literature can be categorized as nutritional and therapeutic benefits (Yeong-Soo Park *et. al.*, 2002).

The ability of LAB to increase the bioavailability of metal ions such as calcium, zinc, iron, manganese, copper and phosphorous for the metabolic activities along with the synthesis of vitamins, an important nutritional benefit is well documented (Yeong-Soo Park *et. al.*, 2002). Lactic acid bacteria are also reported to have the ability to control acute gastroenteritis, which is of bacterial or viral origin. Several studies have shown that *Lactobacillus rhamnosus GG*, *Lactobacillus reuteri*, *Lactobacillus casei* and *Lactococcus lactis* can alleviate the symptoms of diarrhea by approximately one day Olimpia Pepe *et. al.*, 2004; Kaila *et. al.*, 1992; Shornikova *et. al.*, 1997; and Sugita and Togawa, 1994).

Antibiotic Associated Diarrhea (AAD), which is caused by the over growth of *Clostridium difficile*, is prevented by probiotic LAB like *Lactobacillus rhamnosus GG*, *Lactobacillus acidophilus* and *Enterococcus faecium* (Gismondo *et. al.,* 1999). Probiotic LAB such as *Lactobacillus salivarius* and *Lactobacillus rhamnosus GG* are also reported to be effective in alleviating the symptoms of Inflammatory Bowel Disease (IBD) (Gupta *et. al.,* 2000; and Guslandi *et. al.,* 2000;Mattila-Sandholm *et. al.,* 1999). LAB in gastrointestinal tract play a major role in reducing IBD in animals and humans. Probiotic lactobacilli strains decrease the quantity of some of the fecal microbial enzymes such as β-glucuronidase, β-glucosidase, nitroreductase and urease, which are the indicators of metabolic activity of a few mutagens and carcinogens (Marcel, 2000). Hirayama and Rafter (2000) have reported that the consumption of fermented dairy products is related to lowering of cholesterol. Lactobacilli have been shown to be antagonistic to *Helicobacter pylori*, which are implicated, in chronic gastritis, stomach carcinoma, gastric and duodenal ulcers and in gnotobiotic murine models (Aiba *et. al.,* 1998; Kabir *et. al.,* 1997; Midolo *et. al.,* 1995).

The present chapter deals with the isolation of potent probiotic LAB from a laboratory fermented product *Kanjika,* and evaluation of probiotic properties of the isolates. The selected isolates can be used as starter cultures for dairy products and as probiotic powder in pharmaceutical industries. 17 strains from *Kanjika* were selected based on the colony characteristics and were tested for catalase after gram staining. The gram positive and catalase negative strains were further evaluated for their probiotic properties like pH tolerance, bile salt tolerance, β- galactosidase activity, cholesterol lowering capacity and antimicrobial activity.

3.2 Materials and Methods

3.2.1 Materials

MRS broth /agar, Cholesterol, Bile salts, Calcium phytate, Nessler's reagent, Antibiotic discs (Hi-Media, India), NaCl, Glacial acetic acid, Hexane (Rankem, India), Giemsa stain (Merck, Germany) , Potassium hydroxide, HCl (Qualigens, India), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) IPTG (iso-propyl-thio-β-D-galactopyranoside) (Sigma, USA). All the chemicals used were of analytical grade.

3.2.2 Methodology

3.2.2.1 Production of *Kanjika*

Kanjika was prepared according to Acharya (Acharya Sidh Nandan Misra, 1993). Broken rice was cooked in 1L of water till the rice becomes soft. It was strained through a muslin cloth and the resulting starchy liquid (*Kanji*) was collected and made up to 1L, cooled to room temperature. Finely chopped radish (*Raphanus sativus*) (50g), ground mustard (*Brassica nigra*) (25g), rock salt (25g) and mustard oil (12g) were added to the 1L *Kanji*, mixed well and the concoction was allowed to ferment in a closed vessel at 30 ± 2C for 7 days. Every alternate day of fermentation, 5 ml of sample was drawn aseptically for further analysis.

3.2.2.2 Isolation and selection of strains from *Kanjika*:

Samples taken from fermented sample were serially diluted in sterile saline and 1ml of appropriately diluted sample was taken for enumeration of LAB in MRS agar. Based on the colony characteristics like colour, type of growth, texture on MRS agar, colonies were selected and subcultured in MRS broth. A loopful of the culture from the broth was streaked on MRS agar and the individual colony forming units were

subcultured to get pure colonies. These colonies were streaked on MRS slants for further use**.** Isolated strains were stored in MRS broth with 20% glycerol at – 80^0C and subcultured every six months. Working cultures were maintained on MRS agar slants, at 4^OC and subcultured every 2 weeks. Fresh cultures grown for two to three generations in MRS broth were used for evaluating the probiotic properties. The probiotic properties of the isolates are compared with that of *Lactobacillus plantarum* B 4496, a well known probiotic.

3.2.2.3 Evaluation of Probiotic properties

3.2.2.3.1 Acid tolerance

Acid tolerance was determined as described by Yeong *et. al.,* (2002). One ml of isolates grown in MRS broth for 3 generations, having an optical density of 0.280 (50 fold dilution) at 600 nm was inoculated to 9 ml of sterile MRS broth whose pH was adjusted to 2, 2.5 and 3 with 0.1 N HCl. The initial bacterial concentration was $10^7 – 10^8$ cfu ml^{-1} and was maintained throughout the experiments. Samples were incubated at 37^OC for 4 h. 1 ml of sample was serially diluted (6 folds) with sterile saline solution to neutralize the medium acidity and incubated for 24h at 37^OC. The viable cell population was determined by spread plate method. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial bacterial concentration.

$$\% \text{ Survival} = \frac{\log \text{number of viable cells survived (cfu ml}^{-1})}{\log \text{number of initial viable cells inoculated (cfu ml}^{-1})} \times 100 \quad \text{----------(1)}$$

3.2.2.3.2 Bile salt tolerance

Bile tolerance of the isolates was carried out as described by Young *et al* (2002). One ml of inoculum prepared as described above was inoculated to 9 ml of sterile MRS

broth containing 0.3, 0.6 and 1% (W/V) bile salt. Growth was monitored by enumerating the viable cells using the plate count method on MRS agar medium after 12 h incubation. The survival rate of test sample was calculated according to equation 1 and expressed as % of survival.

3.2.2.3.3 Presence of β- galactosidase activity

The method described by Karasova *et. al.,* (2002) was used to test for the presence of β-Galactosidase activity. The isolates were streaked on MRS medium containing 0.01% X-gal (5-bromo-4-chloro-3- indolyl-β-D-galactopyranoside) and 0.1 mM IPTG (iso-propyl-thio-β-D- galactopyranoside) as an inducer. Plates were incubated at 37^{O}C for 2 days. Colonies producing β-galactosidase appear blue in colour.

3.2.2.3.4 Cholesterol assimilation by *in vitro*

Cholesterol assimilation was studied by modified Searchy and Bergquist method (1960). LAB isolates were cultivated in MRS broth supplemented with cholesterol and cholesterol with 0.3% bile salt. 10mg of cholesterol dissolved in 500µl of ethanol was added to 100ml of MRS broth with or without bile salt. The cultures were grown for 12-24 h at 37^{0}C. Cells were harvested by centrifugation at 8,000 rpm for 10 min at 4^{0}C. Spent broth was collected and used for the cholesterol assay. The uninoculated broth was considered as control (0% assimilation). The concentration of cholesterol was determined using a cholesterol calibration graph. The percentage assimilation of cholesterol was calculated using the following formula

$$\% \text{ assimilation} = \frac{\text{Conc. of cholesterol in control} - \text{Conc. of cholesterol in test sample}}{\text{Conc. of cholesterol in control}} \times 100$$

3.2.2.3.5 Phytase production

Screening for the presence of Phytase activity was carried out as per the method described by Olimpia Pepe *et. al.,* (2004) with slight modification. Instead of using Chalmer's agar medium, MRS agar supplemented with 0.2% calcium phytate was used to screen phytase producing LAB isolates. The isolates were point inoculated on the Phytate MRS agar and incubated at 37^0C for 24 h. At regular intervals, the zone of phytate hydrolysis around the isolates was measured in mm.

3.2.2.3.6 Antimicrobial activity

For the detection of antimicrobial activity, an agar spot method was used according to Jacobsen *et. al.,* (1999). One µL of inoculum (respective cultures) with an optical density of 0.280 (50 folds dilution) at 600 nm was spotted on the surface of the MRS agar. Cell population of the isolate in one µL was in the range of Log 10-12. The spotted culture plates were incubated at 37^0C for 24 h. The inhibitory effect of MRS agar was tested as negative control. 1ml of 12 h grown indicator pathogenic organism (*Escherichia coli, Bacillus cereus, Listeria monocytogenes, Yersinia enterocolitica and Staphylococcus aureus*) was mixed with 7 ml of soft agar (0.7%) and poured on the spotted agar plates. The plates were further incubated at 37^0C for 12 h. After 12 h incubation, the zone of inhibition was measured in mm.

3.2.2.3.7 Antibiotic susceptibility:

The selected acid tolerant isolates of *Kanjika* were tested for susceptibility to antibiotics. Antibiotic susceptibility was determined semi quantitatively according to Charteris *et. al.,* (2000). Antibiotic discs were placed on the agar plate spread with cultures of the known dilution (10^2) before preincubation. This provided a moderately heavy

inoculum of about 10^6 to 10^7 CFU ml^{-1}. Inhibition zone diameter was measured using slide caliper after anaerobic incubation at 37°C for 24 h. The results (mean of three determinations) are expressed in terms of resistance, moderate susceptibility and susceptibility according to interpretative standards described in Charteris *et al* (1998a).

3.2.2.3.8 HT-29 Cell culture

Enterocyte-like HT-29 were procured from NCCS Pune, India. Cells were routinely grown in Eagle's minimum essential medium (MEM) (MEM enriched with Glutamax and HEPES; Gibco Bethesda Research Laboratories [BRL]) supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), and 0.5 ml of gentamicin (50 mg/ml) (Gibco BRL) and incubated at 37°C in a water-jacketed incubator with 5% carbon dioxide. Cells were used for adherence assay at postconfluence. Concentration of HT-29 cells in the monolayer was determined by trypsinizing the cells for 10 min at 37°C and counting them using a hemocytometer. 3 ml containing 10^5 cells ml^{-1} were transferred to 35-mm-diameter dishes (Nunclon) and incubated until a complete monolayer was obtained. Medium was changed after every 48 h.

3.2.2.3.9 Adhesion assay

HT-29 cells in a monolayer were washed twice with phosphate- buffered saline, 3 ml of MEM was added to each dish, and the dishes were incubated for 30 min before inoculation of bacteria. Overnight cultures of bacteria were appropriately diluted (10^3) with MEM to give a bacterial concentration of approximately 10^8 cells ml^{-1}, and 120 ml was used to inoculate the HT-29 cells. After incubation for 1 h at 37°C, all of the dishes were washed four times with phosphate-buffered saline to remove unbound bacteria. The cells were then fixed with 3 ml of methanol and incubated for 5 to 10 min at room temperature. After removing the methanol, the cells were stained with 3 ml of Giemsa

stain solution (1:20) and incubated for 30 min. The dishes were washed until no color was observed in the washings, dried in an incubator at 37°C overnight, and examined microscopically (magnification, X100) under oil immersion. Each adhesion assay was performed in duplicate with cells from three successive passages (8 to 13 cell passages). The adherent lactobacilli in 20 random microscopic fields were counted for each test. Bacterial strains were scored as nonadhesive when fewer than 40 bacteria were present in 20 fields, adhesive with 41 to 100 bacteria in 20 fields, and strongly adhesive with more than 100 bacteria in 20 fields.

3.2.2.4 Statistical Analysis

All assays are conducted in triplicates (n=3) and repeated atleast three times. The statistical significances and standard deviation were done using Microsoft excel (Version 5.0; Microsof,Corp; Redmond,WA)

3.3 Results

3.3.1 Acid tolerance:

Percentage survival of 17 *Kanjika* isolates in low pH of 2 and 2.5 for 4 hours incubation at 37^0C was studied (Figure 3.1). Of the 17 isolates tested 6 showed little or no decrease in the viable count when compared to initial cell count at pH 2 &2.5. The percentage survival of the isolated strains K1a, K3a, K4a, K7a, K7b and K23c at pH 2 were found to be 89, 112, 95, 98, 95, 86% and at pH 2.5 were 98, 119, 83, 100, 101and 87% respectively. Other isolated strains such as K1b, K5c, K1c, K3c, K5d, K4b, K23b K1d, K4c and K3b showed greater than 80% tolerance at pH 2.5, and less than 50% tolerance at pH 2 after 4 hours incubation. The percentage survival of the *L. plantarum* was found to be 75% and 80% at pH 2 and pH 2.5 respectively.

3.3.2 Bile salt tolerance

The acid tolerant isolates were further tested for their bile salt tolerance at different bile salt concentrations (0.3, 0.6 and 1% w/v) (Figure 3.2). All the strains including *L. plantarum* except K7b were found to be tolerant towards bile salts up to 1% till 12 h incubation at 37^0C. The percentage survival of K7b isolate was found to decrease with an increase in bile salt concentration.

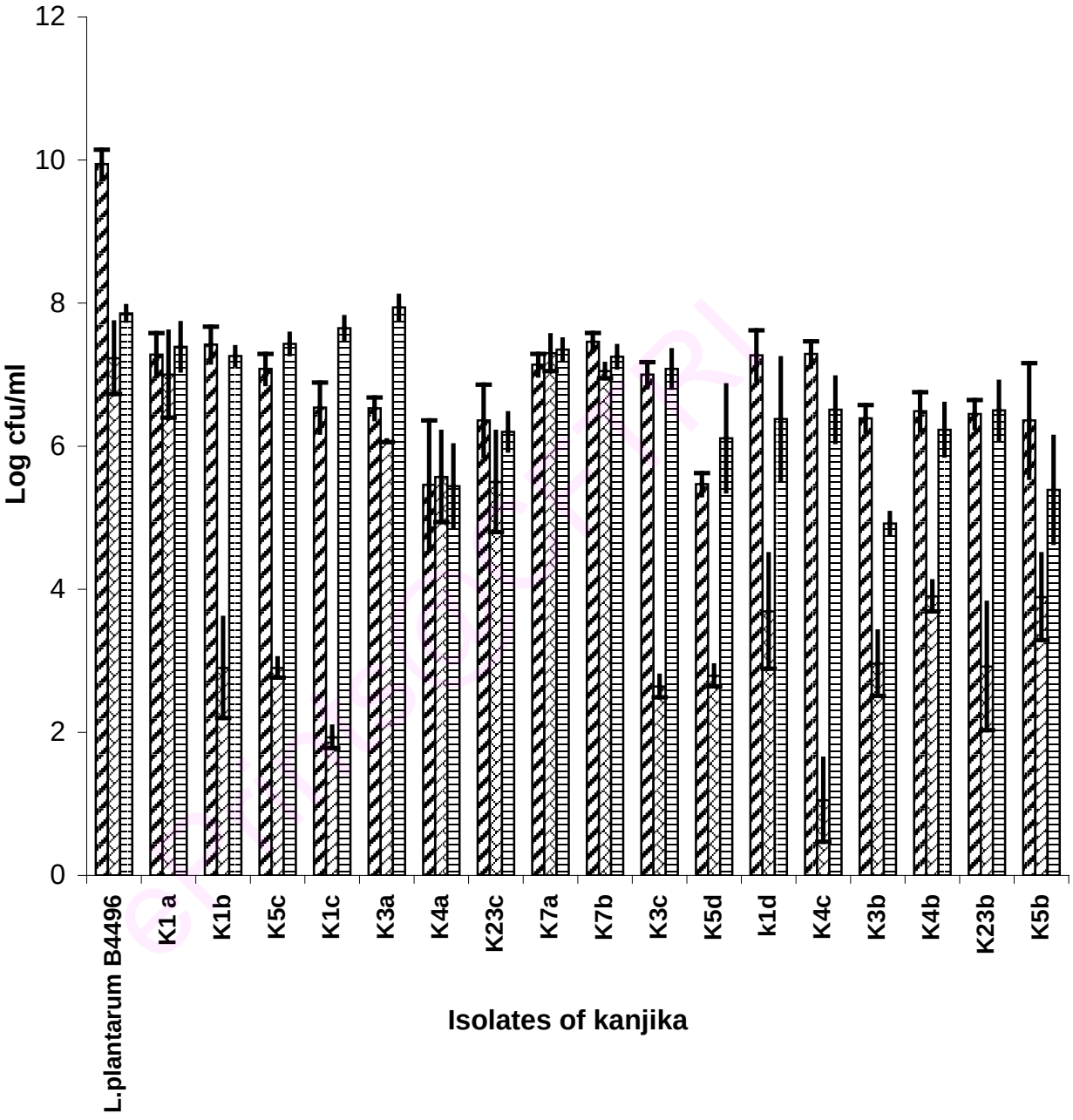


Figure 3. 1: Survival of *Kanjika* isolates in MRS broth adjusted to pH 2.0 and pH 2.5

Initial con. pH 2 pH2.5

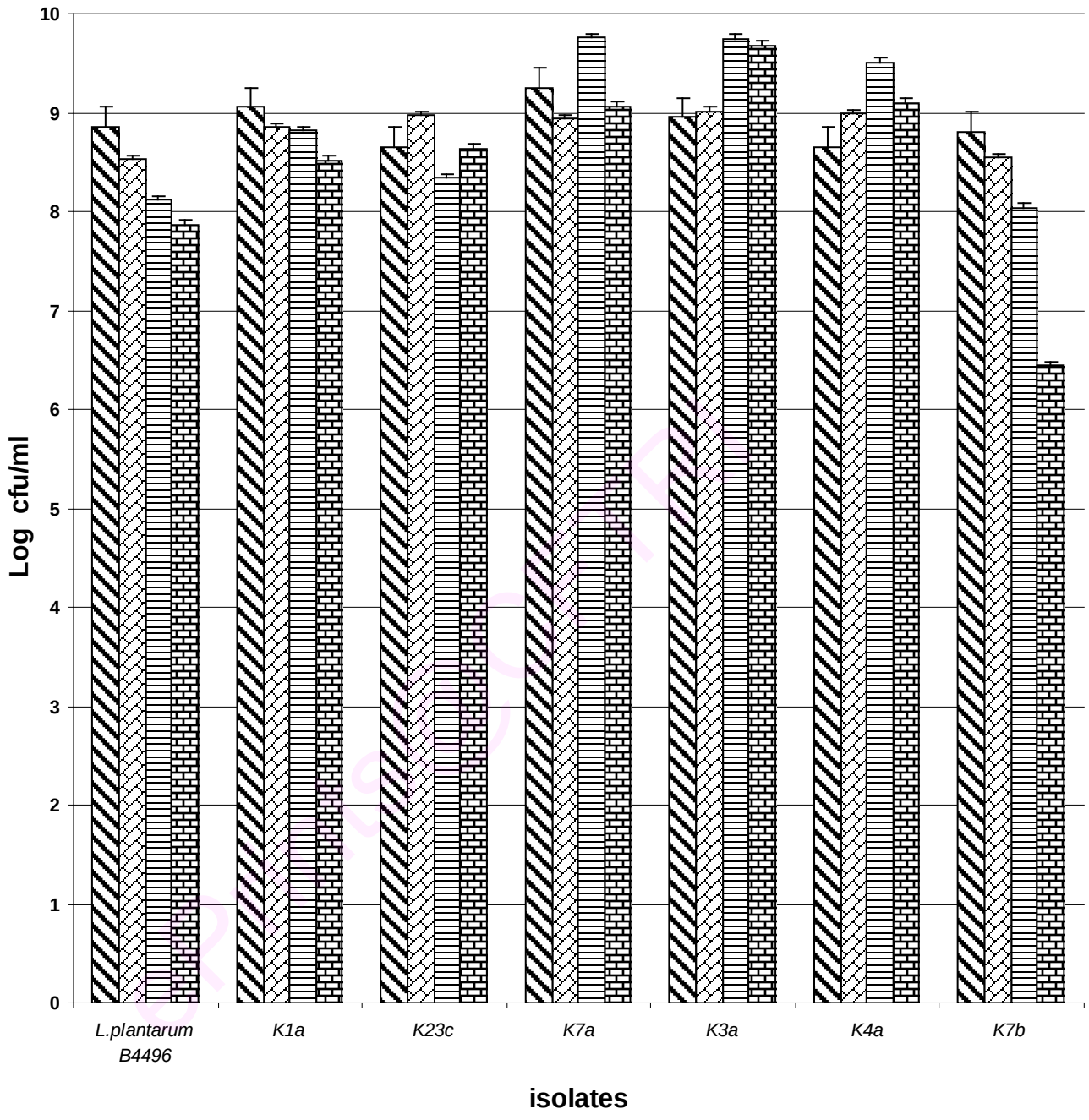


Figure 3.2: Effect of bile concentration on growth of isolates at 37^0C for 12 h

3.3.3 β-galactosidase activity

Acid tolerant *Kanjika* isolates were tested for their β-Galactosidase activity by agar screening method (Figure 3.3). After 48 h of incubation, all isolates other than K7a & K7b showed β−galactosidase activity. Appearance of the characteristic blue colour colonies on MRS agar supplemented with X-gal and IPTG indicated the presence of β□-galactosidase activity.

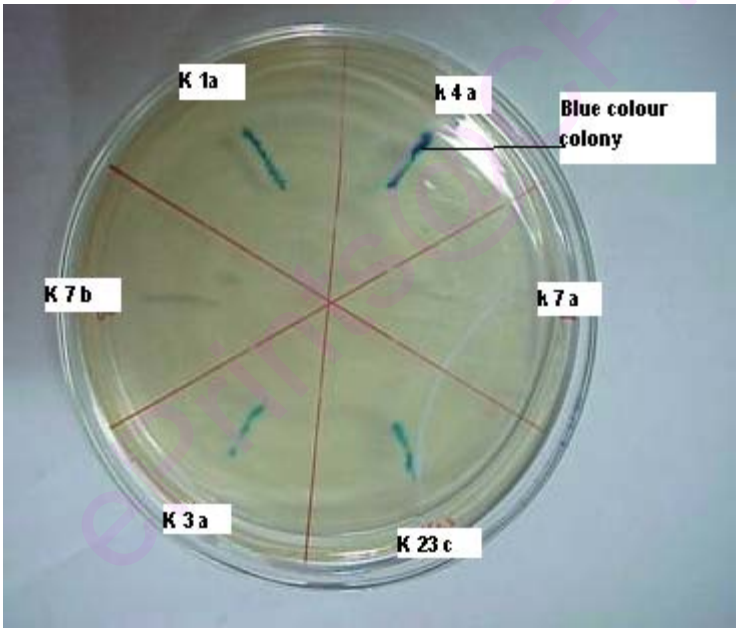


Figure 3.3: β- galactosidase activity assay by screening method showing characteristic blue colour colony formation.

3.3.4 Cholesterol assimilation assay

Assimilation of cholesterol by selected *Kanjika* isolates in the presence and absence of 0.3% bile in the spent broth is shown in Figure 4 for 24 h incubation. The assimilation of cholesterol by K3a was found to be 34% in the absence of bile salts whereas in the presence of bile salt (0.3%), it was found to be 58%. *L. plantarum* B 4496 was found to assimilate 25% and 45% of cholesterol in presence and absence of 0.3% bile respectively. The other isolates, K1a, K7a, K7b and K23c were found to assimilate cholesterol in the range of 30-40% under similar conditions. The isolate K4a show negligible assimilation in the presence of bile.

3.3.5 Antimicrobial activity:

The antimicrobial property of six selected acid tolerant *Kanjika* isolates was tested against well-known food borne pathogenic organisms like *Bacillus cereus, Listeria monocytogenes, Escherichia coli, Staphylococcus aureus and Yersinia enterocolitica*. All the selected isolates showed good antimicrobial property giving 10 to 20 mm (dia) inhibition zone against all tested pathogens (Table 3.1). Isolates like K23c, K7a and K1a showed bigger inhibition zone (20-30 mm dia) against a few selected pathogens. K23c exhibited greater inhibition against *Bacillus cereus, Listeria monocytogenes* and *Yersinia enterocolitica;* K7a against *Listeria monocytogenes, Yersinia enterocolitica* and K1a aganist *Yersinia enterocolitica.*

3.3.6 Phytase production

The selected *Kanjika* isolates were tested for the production of phytase by agar spot method (Olimpia Pepe *et al.* 2004). The presence of clear (hallow) zone indicates the capability of the selected isolates to produce phytase (Table 3.2). Isolates tested were found to degrade phytate at 12h incubation. The hydrolytic zone formed by the isolate K3a was 22mm (dia) indicating good phytase activity, whereas hydrolytic zone formed

by the isolates *K7a, K7b* and *K4a* was in the range *of 10-14mm* and that of the isolates *K1a* and *K23c* were 9 and 8 mm (dia) respectively.

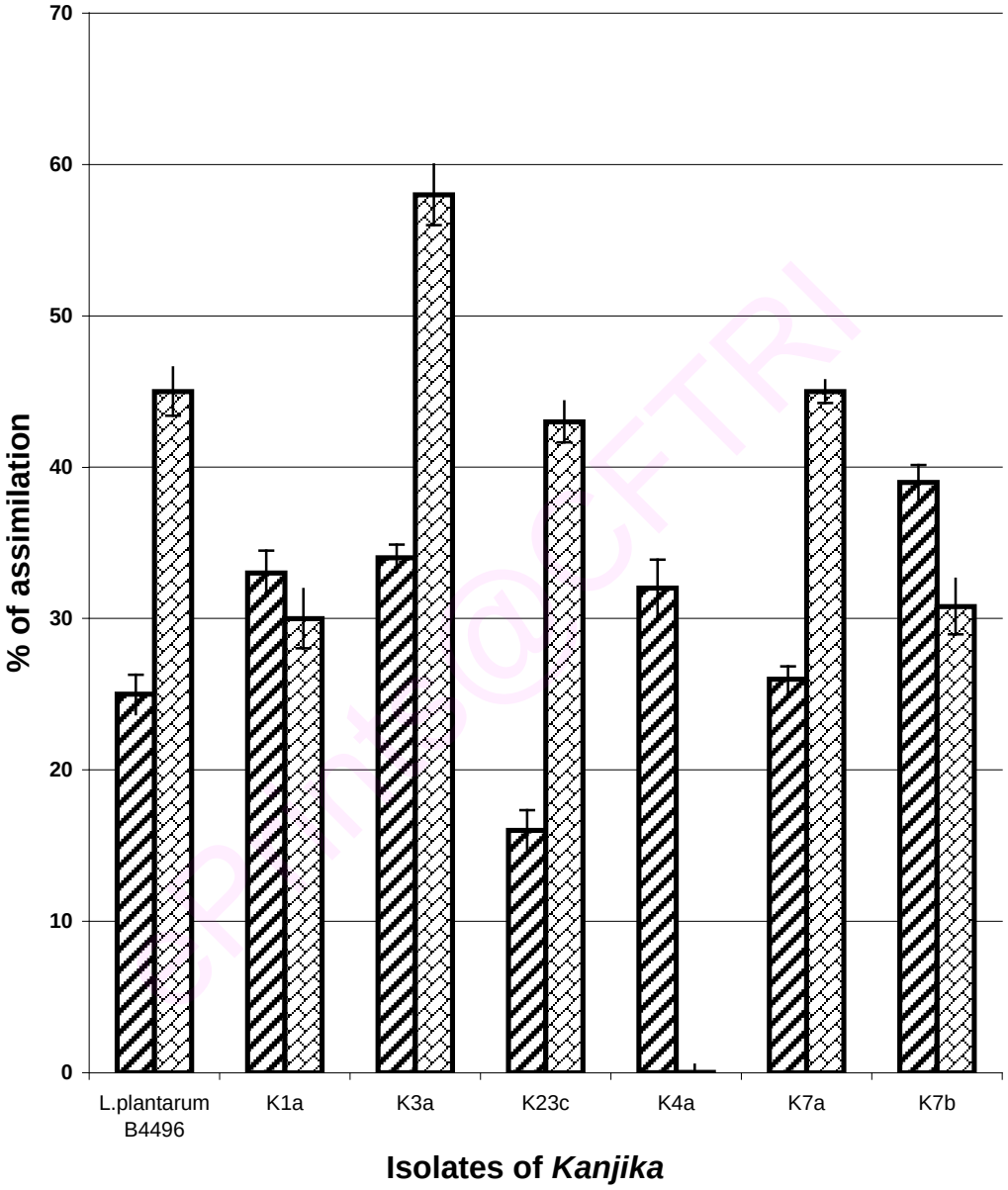


Figure 3.4: Assimilation of Cholesterol in presence and absence of bile salt (0.3%) at 24h incubation

Table 3.1: Antimicrobial activities of selected *Kanjika* strains towards pathogenic microorganisms

Kanjika strains	Pathogenic bacteria (Zone of inhibition [a])				
	E.coli **MTCC 108**	***B.cereus*** **F 4810**	***L.monocytogenes*** **Soctta**	***Y.enterocolitica*** **MTCC 859**	***SEA*** **FRI 722**
L. plantarum B-4496	++	++	++	++	+++
K23c	++	+++	+++	+++	++
K7a	++	++	+++	+++	++
K7b	++	++	++	++	++
K1a	++	++	++	+++	++
K4a	++	++	++	++	++
K3a	++	++	++	++	++

Note: [a] ++, Between 1-10mm of inhibition; +++, 11-20mm of inhibition; SEA

Table 3.2: Adherence and phytate hydrolysis activity of *Kanjika* isolates

Kanjika isolates	Zone of phytate hydrolysis in mm(dia)	Adherence index* to the HT-29 cell line
K3a	30	200±15
K23 c	27	180±20
K1a	26	150±10
K7a	25	120±18
K7b	24	100±10
K4a	20	160±15

* Each adhesion assay was conducted in duplicate with cells from three successive passages

3.2.7 Adhesion assay

The selected *Kanjika* isolates were analyzed for the adherence ability to HT-29 intestinal epithelial cell line (Table 3.2). All selected acid tolerance *Kanjika* isolates show strong adhesion to the above intestinal cell line.

3.2.7 Antibiotic susceptibility assay

The selected acid tolerant isolates were tested for antibiotic susceptibility **(**Charteris *et al* 1998b). All the strains were found to be resistant to kanamycin, metronidazole, vancomycine, co-trimoxazole and polymyxin-B and moderately susceptible to ampicillin, erythromycin and streptomycin (Table3.3). However they were susceptible to chloramphenicol. K3a, K4a were resistant to penicillin-G; K1a, K7a and K7b were moderately susceptible and K23c was susceptible.

Table 3.3: Antibiotic susceptibility testing for the *Kanjika* isolates

Antibiotic	Disc.conc. (μg)	Interpretative zone diameter (mm)					
		K3A	K1A	K23C	K4A	K7A	K7B
Penicillin-G	10	18(R)	24(MS)	28(S)	19(R)	20(MS)	25(MS)
Kanamycin	30	08(R)	11(R)	00(R)	10(R)	08(R)	10(R)
Metronidazole	5	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Vancomycin	30	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Ampicillin	10	20(MS)	25(MS)	22(MS)	19(MS)	24(MS)	23(MS)
Chloramphenicol	30	18(S)	28(S)	23(S)	22(S)	23(S)	18(S)
Streptomycin	10	08(R)	14(MS)	14(MS)	00(R)	13(MS)	12(MS)
Co-trimoxazole	25	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)
Tetracycline	30	18(MS)	19(S)	17(MS)	14(R)	18(MS)	17(MS)
Erythromycin	15	16(MS)	21(MS)	21(MS)	19(MS)	20(MS)	22(MS)
Rifampicin	5	18(MS)	20(S)	20(S)	16(R)	18(MS)	24(S)
Polymyxin-B	300	00(R)	00(R)	00(R)	00(R)	00(R)	00(R)

Susceptibility expressed as (R), resistant; (MS), moderately susceptible; (S), susceptible.

3.4 Discussion:

As a probiotic, an organism should exhibit the probiotic properties like acid and bile tolerance, antimicrobial activity and adhesion to the intestinal mucus surface. In the present study, cultures isolated from *Kanjika* (an *Ayurvedic* Lactic acid fermented product) were evaluated for their probiotic properties. Of the 17 isolates, 6 were found to be tolerant to low pH (pH 2 and 2.5) after 4 h incubation. Jin *et. al.,* (1998) have reported less than 50% survival of *L. acidophilus* isolated from chicken intestine at pH 3, where as Goldin *et. al.,* (1992) have reported survival of *Lactobacillus GG* at pH 3. At pH 2.5, Charteris *et al*. (1998) reported almost complete loss of viability for *L. casei* 2123 and F19 strains and *Lactobacillus GG* after 3 h incubation. According to Hyronimus and Rafter (2000), there was no survival of spore forming lactic acid bacteria at pH 2. Jacobsen *et al*. (1999) have reported that, out of 44 Lactobacilli strains screened in their study, none of the strains could replicate at pH 2.5. However in the present study, 6 out of 17 *Kanjika* isolates exihibited more than 90% survival, at pH 2 and pH 2.5 for 4 h incubation. Of the above 6 acid tolerant isolates, the isolate K3a showed 100% tolerance at pH 2 and 2.5 for 4 h incubation. The results suggest that the isolates are more acid tolerant in comparison with *Lactobacillus GG,* which is a known potent probiotic. Compared with the literature reports, the strains isolated in the present study show higher acid tolerance with respect to survival and replication. These acid tolerant isolates were further evaluated for other probiotic properties like bile salt tolerance, cholesterol reduction, phytase activity, β– galactosidase activity and antimicrobial activity.

Bile salt tolerance is an essential probiotic property for the evaluation of lactic acid bacteria. It plays an important role in physiological function with respect to the survival of LAB in small intestine (Yeong-Soo Park *et al*. 2002). According to Gilliland *et. al.,* (1984) 0.3% bile is considered to be a crucial concentration to evaluate a bile tolerant probiotic LAB. Among 6 acid tolerant strains, 5 strains were found to survive at the tested bile salt concentrations (0.3, 0.6 and 1%) for 12 h, whereas the survival of the isolate K7b, was found to decrease with an increase in bile salt concentration.

Yeong *et. al.,* (2002) have reported the survival of lactic acid bacteria isolated from newborn baby fecal matter up to 0.25% bile. The isolates studied under the present investigation, showed better survival upto 1% bile salt concentration, which is a very desirable characteristic.

Lactose in yoghurt with live lactic acid bacteria is better tolerated than lactose in other dairy foods, partly because of the activity of microbial β-galactosidase activity, which digests lactose *in vivo* (Martini *et. al.,* 1991). The disaccharide lactose can cause severe intestinal distress, characterised by bloating, flatulence and abdominal pain in subjects with low levels of β- galactosidase. This condition increases in severity with age and restricts the use of dairy products. Probiotics can be useful in this condition, as lactobacilli produce β- galactosidase, which hydrolyses lactose in dairy products (Barbara Mombelli and Maria Rita Gismondo, 2000). LAB probiotics are reported to reduce lactose intolerance symptoms by accelerating the digestion of lactose (Sanders, 1993). β-galactosidase plays a major role in the digestion of the milk sugar, lactose in humans. Therefore, testing for the production of this enzyme by LAB is essential to evaluate them as probiotics. In this study, out of the 6 selected isolates, 4 isolates namely K1a, K3a, K23c, K4a, were tested positive for β-galactosidase activity.

Hypercholesteroloemia results in high risk of coronary heart diseases in human (Hyronimus *et al.* 2000). A few research reports describe the use of *L. acidophilus* to decrease the serum cholesterol in human and animals (Gilliland *et al.* 1984). There are reports that lactic acid bacteria can reduce the cholesterol up to 50% in presence of 0.3% bile in 48 hours (Mishra and Prasad, 2005). This present study showed that the *Kanjika* isolate K3a, has the ability to reduce cholesterol up to 60 % in presence of 0.3% bile salt in 24 h, whereas it had the ability to reduce cholesterol up to 40% in absence of bile in 24 h. Isolates K1a and K23c were found to reduce cholesterol up to 30% in 24 h. Various species of probiotic LAB have the antagonistic activity against intestinal and food borne

pathogens (Jacobsen *et. al.,* 1999). This activity may be due to the production of any one or more of reuterin, bacteriocin like nicin, lactic acid and hydrogen peroxide (El-Ziney and Debevere, 1998). The 6 selected *Kanjika* isolates were investigated for their antimicrobial properties towards pathogenic bacteria such as *Escherichia coli, Bacillus cereus, Listeria monocytogenes, Yersinia enterocolitica* and *Staphylococcus aureus*. All the 6 selected isolates showed inhibition of all the tested pathogens. Isolates K23c, K7a and K1a showed good inhibition against *L. monocytogenes* and *Y. enterocolitica*.

Phytate is the main storage form of phosphorus in cereal grains but phytic acid-phosphorus complex is unavailable for humans, because of the lack of phosphohydrolysing enzymes in the gut that determine its total excretion through the monogastric digestive tract. Phytic acid is known to bind divalent and trivalent cations (specifically iron) and also proteins (Olimpia Pepe *et. al.,* 2004). This may lead to non-availability of essential proteins and minerals to the human body and thus acts as an anti nutritional factor. Higher quantities of phytic acid, which may come through grains and oil seeds, will bind with proteins and minerals thus making them biologically not available to human beings. (Keiko Shirai *et. al.,* 1994). Probiotic lactobacilli and other species of the endogenous digestive microflora as well, are an important source of phytase which catalyses the release of phosphate from phytate. The enzyme also hydrolyses the complexes formed by phytate and metal ions or other cations, rendering them more soluble ultimately improving and facilitating their intestinal absorption (Guiseppe Famularo, *et al.* 2005; Maria De Angelis, *et. al.,* 2003 and Janne Kerovuo, *et. al.,*1998). Phytic acid (myo-inositol hexa-phosphate) may be dephosphorylated by phosphatase enzymes or phytases (Sreeramulu, *et. al.,* 1996). Thus, phytases are considered to be of great value in enhancing the nutritional quality of plant foods and microbial phytases play a major role here (Hyronimus, *et. al.,* 2000). Only a few studies deal with the production of phytase by lactic acid bacteria. All the tested *Kanjika* isolates were found to produce phytase. This indicates that the selected isolates of *Kanjika* can increase the bioavailability of essential proteins and minerals.

Adhesion and colonisation of probiotic bacteria in the gastrointestinal tract of the host is believed to be one of the essential features required for delivering their health benefits (Bernet *et al*., 1994). In recent years, several reports have been published on the usefulness of human intestinal cell-lines, e.g. HT-29, Caco-2 and HT29-MTX, as *in vitro* model systems for assessing the colonisation potential of a bacterial strain (Tuomola and Salminen, 1998). These cell lines represent the major cell phenotypes found in the human intestinal mucosa, namely enterocytes and goblet cells. Both HT-29 and Caco-2 cell lines show the typical characteristics of enterocytic differentiation. In the present investigation, the adhesive properties of the 6 selected acid tolerant *Kanjika* isolates of probiotic strains were examined using HT-29 cell lines as *in vitro* cellular model systems. All the isolates were strongly adhesive to HT-29 intestinal epithelial cell line (Table 3.2) in the present study.

Conclusion:

A detailed study on the *in vitro* evaluation of the LAB isolated from *Kanjika* is presented in the chapter. The study indicates that the isolate K3a exhibits potential probiotic properties. Other isolates like K1a & K23c also exhibited major probiotic properties but were found to be susceptible to 3 antibiotics, which is not desirable. Isolate K4a showed promising probiotic properties except cholesterol assimilation. It has been possible to isolate Lactic acid bacteria exhibiting a profound degree of probiotic properties such as acid tolerance, bile salt tolerance, antimicrobial activity against food borne pathogens, β-galactosidase activity, phytase activity, antibiotic susceptibility and cholesterol lowering capacity from *Ayruvedic* fermented products. Table 3.4 summarized the probiotic properties of different isolates.

Table 3.4: Summary of the probiotic properties of lactic acid bacteria isolates of *Kanjika*

Isolates	pH tolerance (% log CFU/ml)		Bile salt at 1% conc. log Difference at 24h Incubation	β–Gal Activity (Blue colour colony)	Cholesterol Reduction 24h incubation	Phytase Activity Hollow zone (dia mm)	Antibiotic susceptibility Interpretative zone diameter (mm)[a]		
	2	2.5					Penicillin –G	Tetracycline	Rifampicin
K3a	112	119	↑ 0.722	Yes	58%	30 (12h)	18 (R)	18 (MS)	18(MS)
K1a	89	98	↓ 0.544	Yes	30%	26 (24h)	24(MS)	19(S)	20 (S)
K23c	86	87	↓ 0.026	Yes	42%	27(24h)	28(S)	17(S)	20(S)

[a] susceptibility expressed as R, resistance; MS, moderately susceptible; & S, susceptible, ↑ increase in log difference, ↓ decrease in log difference

Chapter 4

Phenotypic and Genotypic characterization of probiotic lactic acid bacteria

4.1 Introduction

The primary purpose of identifying lactic acid bacteria (LAB) is to provide proper strain identification as required for legal and good manufacturing practices while producing LAB supplemented products. In addition, these identification tools can be used to trace and track LAB including probiotics in the production phase and in food products as well as after consumption in the intestinal tract.

The identification and characterization of LAB from different sources is of great interest among the scientists. The identification of the LAB isolates starts with determination of morphology (Gram staining), followed by biochemistry to know the general type of metabolism, and cultural characteristics. For acquiring phenotypic information, chemotaxonomic markers may be used such as cellular fatty acid (FAME, fatty acid methyl ester analysis), mycolic acid, polar lipids, and cell wall compounds. Enzyme patterns and antibiogram patterns are also used for phylogenetic characterization.

The conventional methods are important and useful for identification, but a false result may be encountered due to reasons like impure culture, absence of appropriate tests, lack of reliable methods, wrong use of keys in the manual etc. Moreover, the observation of a similar phenotype doesn't always equate to similar or closely-related genotype. The traditional methods are often unreliable and lack the resolving power to analyze the microbial composition and activity of bacterial populations. For a more sensitive and reliable method to identify and characterize LAB, molecular biological methods are applied, particularly in the field of probiotics.

The tools that have been developed for identifying microbes and analyzing their activity can be divided into those based on nucleic acid and other macromolecules and approaches directed at analyzing the activity of complete cells. The nucleic acid based tools are more frequently used because of the high through-put potential provided by using PCR amplification or *ex-situ* or *in-situ* hybridization with DNA, RNA, or even peptide for nucleic acid probes. These probes can be designed to specifically target taxonomic groups at different levels of specificity (from species to domain) by virtue of

variable evolutionary conservation of the rDNA molecule. Nucleic acid probing is based on two major technologies: dot blot hybridization which is an *ex situ* technique and whole cell *in situ* hybridization e.g. FISH (Fluorescence *in situ* hybridization).

The popularly used 16S rDNA based methodologies are robust and superior to traditional methods based on phenotypic approaches and can be used to place the test organism into a phylogenetic frame work and can be linked to databases providing up to 100,000 sequences. A number of approaches that are based on DNA sequencing other than rDNA have been applied frequently to probiotic bacteria and have shown to be particularly useful for strain identification. These include DNA fingerprinting e.g. Pulsed Field Gel Electrophoresis (PFGE) of rare cutting restriction fragment, ribotyping, randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). PCR based approaches other than RAPD and AFLP used for molecular typing are Amplified Ribosomal DNA Restriction Analysis (ARDRA), repetitive extragenic palindromic PCR (rep PCR) and Triplicate Arbitrary Primer (RAP-PCR). Additional molecular methods being employed to determine and differenciate LAB are: Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE). These have shown to offer a discriminatory power for the identification and differentiation of LAB.

16S rDNA gene sequencing is a powerful tool that has been used to trace phylogenetic relationships between bacteria, and to identify bacteria from various sources, such as environmental and fermentation specimens. This technology is used today as routine identifications method, especially for slow-growing, unusual or fastidious bacteria, but also for bacteria that are poorly differentiated by conventional methods; Phenotypic methods present some inherent problems: there can be a substantial amount of variability among strains belonging to the same species, the corresponding database may not yet include newly described species and the test may rely on an individual and subjective interpretation. Identification based on the 16S rDNA sequence is of interest because ribosomal SSU exists universally among bacteria and includes regions with species specific variability, which makes it possible to identify bacteria to

the genus or species level by comparison with databases in the public domain (Vandamme et al., 1996). The molecular approach has been used for bacterial phylogeny and is of major importance for species definition and identification (Clarridge, 2004; Fredricks and Relman, 1996; Raoult et al., 2004; Rossello-Mora and Amann, 2001).

As described in the previous chapters a few potent probiotic LAB were isolated from *Kanjika* an ayruvedic lactic acid fermented product. Out of 17 isolates, 6 of them exhibited potent probiotic properties. As discussed earlier, 6 isolates showed significant antidiarrheal properties and antagonist to known food borne pathogens. From the results obtained, it can be said that a technologically feasible and economically viable process for the production of these isolates could be developed and hence the correct species identification of LAB is of importance from the technological, ecological and safety point of view. An effort was made to identify the LAB species by 16S rDNA method, a reliable, reproducible method for identification upto species level.

4.2 Material and Methods

4.2.1. LAB Isolates

LAB isolates from *Kanjika* (chapter 3) were maintained as glycerol stock cultures. Sub culturing of six isolates (K1a, K3a, K7a, K4a, K7b, K23c,) was done using MRS broth. 100µL of the stock culture was inoculated to 5ml of MRS broth and incubated at 37^0C for 12 h. At the end of 12h of incubation, a loopfull of the culture broth was streaked onto MRS agar slants, incubated at 37^0C for 12 h. stored at 4^0C till further use. MRS broth was prepared using dehydrated culture media from HiMedia, Mumbai, 50 mL of the broth was dispersed into test tube, sterilized at 121^0C for 15 min.

4.2.2. Identification Of Strains

For the identification of the given strains at the species level, following tests were carried out.

4.2.2.1 Morphology

4.2.2.1.1 Gram Staining

Materials: (HiMedia, Mumbai)

1. Crystal Violet
2. Lugol's Iodine (Mordant)
3. Saffranin (Counter stain)

Methodology:

1. Freshly grown cells of the isolates were transferred onto clean glass slides, smeared and heat fixed.
2. Stained with crystal violet for 1 min. Excess of stain was washed with water.
3. The stained smear was treated with Lugol's Iodine for one minute
4. Washed with water, smear was further treated with ethanol for 30 sec to remove the excess stain.

5. The smear was counter stained with saffranin, for 30 sec.
6. Washed with water, dried and examined under oil immersion. The colour taken up by the cells and the morphology was recorded.

4.2.2. 1.2 Biochemical Tests

4.2.2.1.2.1 Catalase Test

Material:

Hydrogen Peroxide solution (3%) (NICE chemical, Mumbai)

Methodology:

1. A 24 h old culture, grown on MRS agar was smeared on a clean glass slide.
2. One drop of 3% H_2O_2 was added on the smear and allowed to react for 30 sec. The presence of the effervescence was recorded as Catalase positive and absence as catalase negative.

4.2.2.1.2.2 Carbohydrate Utilization Test

A set of tests for carbohydrate utilization along with citrate, esculin, ONPG (ortho nitrophenyl β-D-galactopyranoside) was carried out using **KB009 HiCarbohydrate ™ Kit** from **HiMedia.**

Kit Contents:

The kit had three parts with media containing different carbohydrates and substrates viz ONPG, citrate, esculin.

PART A:

Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Mellibiose, Sucrose, L-Arabinose, Mannose

PART B:

Inulin, Sodium gluconate, Glycerol, Salicin, Glucosamine, Dulcitol, Inositol, Sorbitol, Mannitol, Adonitol, α-methyl D-glucoside, Ribose

PART C:

Rhamnose, Cellobiose, Melezitose, α methyl D- mannoside, Xylitol, ONPG (ortho-nitro phenol βgalactopyranoside), Esculin, D-Arabinose, Citrate, Malonate, Sorbose, Control

Preparation of inoculum

1. 2-3 well isolated colonies grown on MRS agar were picked.
2. A homogenous suspension in 2-3 ml of saline was made.
3. The density of the suspension was checked for 0.5 OD at 600 nm.

Inoculation of the Kit

1. The kit was opened under aseptic conditions in Laminar Air Flow.
2. Each well was then inoculated with 50 µl of the suspension. Lid was replaced carefully.
3. Kit was incubated at 37^{o}C for 24-48 h.

Interpretation of Results

a. **Carbohydrate utilization**:

Change in the color of wells from red to yellow indicates positive results and no change in color indicates negative result.

b. ONPG Test:

Media color change from colorless to yellow indicates positive results; no color change indicates negative results.

c. Esculin Hydrolysis:

A color change from cream to black show positive result and if remains cream, indicates negative results.

d. Citrate Utilization:

Change in medium color from yellowish green to blue shows positive results.

e. Malonate Utilization:

Color of the medium changes from light green to blue indicates positive results.

4.2.2.3. Growth conditions:

Growth at three different temperatures was checked: 15, 37 & 45^{o}C.

Material:

MRS broth was used for the growth of cultures

Methodology:

1. 12 test tubes containing 5 mL MRS broth was taken and divided into three sets for three different temperatures; each set having four test tubes for each isolate.
2. 100µL of active culture (12 h old) was inoculated to respectively labeled test tubes.
3. The inoculated sets of tubes were incubated at different temperatures viz 15^{o}C, (refrigerator); 37^{o}C (incubator); 45^{o}C (water bath), maintained at required temperature.
4. Incubated tubes were observed for growth; indicated by turbidity after 24 h.

4.2. 2.4. Thermo tolerance

Thermo tolerance was checked at temperatures 65^{o}C for 15 min and 30 min and 70^{o}C for 15 min.

Materials:

1. MRS broth

2. Peptone Water (0.1%)

Ingredients	**Amount(g/l)**
Peptone	1.0
NaCl	5.0

The ingredients were dissolved in distilled water, adjust the pH for 7 and sterilized.

Methodology:

1. Active culture (2 ml of 12 h old) was taken in three microcentrifuge tubes.
2. Centrifuged at 8000 rpm for 15 min at room temp. The supernatant was discarded.
3. Peptone water (1 mL of 0.1%) was added to the pelleted cells and mixed well to get uniform suspension.
4. The cell suspension was subjected to heat treatment: 65^{o}C for 15 min and 30 min and 70^{o}C for 15 min.
5. Treated cell suspension (100µL) was then transferred to sterile MRS broth tubes aseptically and incubated at 37^{o}C for 24 h.
6. Growth was observed visually (turbidity).
7. The process was repeated for all the isolates

4.2.2.5. Growth at different pH

Growth at four different pH (3.5, 4, 4.8, and 8.6) was checked

Material:

1. MRS broth

   MRS broths with four levels of pH (pH 3.5, pH 4, pH 4.8, adjusted with 1N HCL and pH 8.6 with 1N NaOH) was prepared for each isolate and sterilized.

Methodology:

1. Active culture (100µL, 12 h old) was inoculated to each set of

   MRS broth tubes maintained at different pH.

2. Incubated at 37^{o}C for 24 h.
3. Growth was observed visually (turbidity).

4.2.2.3.4 Growth at different Salt Concentration:

Growth at two concentrations of sodium chloride was checked.

Materials:

1. MRS broth
2. NaCl (Sodium Chloride)

Requisite quantity of MRS broth in two sets was prepared with addition of 6.5% and 10% NaCl respectively; 5 ml was dispensed in different test tubes and sterilized.

Methodology:

1. MRS broth with added salt (6.5% and 10% NaCl) were inoculated with 100µl of active culture of each isolates. Mixed well and incubated at 37^{o}C for 24 h.

4.2. 3. Molecular Biology Techniques

4.2.3.1 Isolation of Chromosomal DNA from LAB

Stock solutions:

1. Tris 1M (pH 8.0)

Tris (12.1 gm) was weighed and dissolved in 75 ml of distilled water. The pH was adjusted to 8.0 and used.

2. EDTA 0.5M (pH 8.0)

EDTA (18.6 gm) was dissolved in 75 ml distilled water. pH was adjusted to 8.0 using 1N NaOH solution.

3. Lysozyme stock solution: lysozyme 10mg/ml prepared freshly in 1M Tris (pH 8.0)
4. Lysis solution: SDS of 20% w/v in 50mM Tris: 20mM EDTA (pH 8.0)

5. Sodium Acetate solution (3M)
6. Chloroform: isoamyl alcohol (24:1) Were added separately from the stock
7. Ethanol (70%)
8. Proteinase K (Banglore Geni)
9. TE buffer:
   Tris 10mM (pH 8.0), EDTA 1mM (pH8.0)

Methodology: (Mora *et. al.*, 2000**)**

1. All the cultures grown in sterile 5mL MRS broth at 37^{o}C for 24 h was used for DNA isolation.
2. 0.5mL was transferred to sterilized eppendorfs and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded.
3. The cell pellet was washed thrice using 1mL TE buffer.
4. 400µl of TE buffer was added and then 30µL of lysozyme was added. This was kept for incubation at 37^{o}C in water bath for 1 h with mixing every 15min by inverting the eppendorfs.
5. Then 15µL of 20% SDS solution and 15µL of proteinaseK were added and kept for incubation at 55^{o}C for 15min.
6. Extraction with 250µL of Tris saturated phenol. The solution was centrifuged at 8000 rpm for 10 min.
7. The supernatant was collected in another eppendorf and extracted using 400µl of chloroform: isoamyl solution.(24:1)
8. Centrifuged at 8000 rpm for 10 min.
9. Precipitated with 15µL of sodium acetate (3M) and 400µL of ethanol (95%).
10. Centrifugation at 15,000 rpm for 30 min was carried out at 4^{o}C. The pellet was washed with 70% ethanol and centrifuged at 15,000 rpm at 4^{o}C for 10 min.
11. Dried at 40^{o}C. The pellets were dissolved in 20µL of TE buffer.
12. The quality of DNA was checked by 0.8% agarose gel electrophoresis.

4.2. 3.2 Analysis of Chromosomal DNA by Agarose Gel Electrophoresis

Reagents Required:

1. Agarose (SRL)
2. TE Buffer (50X)

   The Tris base (24.2gm), glacial acetic acid (5.71 mL) and 10ml of 0.5M EDTA (pH8.0) was added to 50ml of distilled water. The pH of the buffer was adjusted to 7.2; the volume was made upto 100ml with distilled water. The buffer was sterilized by autoclaving and stored at room temperature.
3. Loading dye (6X)

Bromophenol blue 0.25%

Xylene cyanol 0.25%

Glycerol 30%

4. *Eco. E Bst III marker* (MBI Fermenter) 3µL of marker, 7µL of sterile deionized water and 2µL of loading dye (6X) were taken in sterile microcentrifuge tubes. The tube was then incubated in a water bath at 70^{o}C for 5 min and snap cooled in ice. The entire volume was used for loading in the gel.
5. Gel running boat
6. Mini gel apparatus
7. Ethidium bromide stock solution.

   Ethidium bromide of 10mg was dissolved in 1mL of distilled water and stored in brown bottle wrapped with aluminium foil. (Note: Ethidium bromide is carcinogenic and mutagenic so it should be handled with care).

Methodology:

1. The boat was sealed with adhesive tape on either sides and wells were made with the comb fixed to a comb stand.
2. 0.8% agarose was weighed and was dissolved in 1X TE buffer and it was warmed to dissolve the agarose completely.
3. The agarose was cooled to 50^{o}C and poured into the sealed boat.

4. The gel was allowed to set for 15 min. The comb and the adhesive tape were removed and the gel was placed in the electrophoresis tank.
5. The electrophoresis tank was filled with 1X TE buffer to a depth of 1mm.
6. The prepared standard DNA marker was loaded (3µL). 5µL of DNA samples of isolates were loaded in subsequent wells.
7. Electrophoresis was carried out till the dye reached ¾ th of the gel.
8. The gel was then removed from the tank and placed in Ethidium bromide solution for 2-3 min. for staining.
9. The gel was destained in distilled water and examined under UV transilluminator (Photodyne, USA) and the gel image was documented using gel documentation unit.

4.2. 3 .3 Polymerase Chain Reaction (PCR)

PCR was carried out to amplify the targeted 1.4kb 16S rRNA gene.

Preparation of total cell lysate:

Material:

1. MRS broth
2. PBS – NaCl 13mM, NaH_2PO_4 (1mM), Na_2HPO_4 (1mM), pH7.4.
3. Triton X-100 (1%)

Methodology:(Sambrook & Russell , 2001)

1. Bacterial culture was freshly grown in MRS broth for 15 h at 37^{o}C in an incubator.
2. Cells were then harvested by centrifugation at 10,000 rpm for 10 min.
3. The supernatant was discarded and the cell pellet was washed twice with 1X PBS buffer and finally washed with sterile distilled water.
4. Cell pellets were lysed by centrifugation with 25µL of PBS buffer and 25µl of Triton-X 100.

5. The cell lysate was kept in boiling water bath for 10-15min and transferred rapidly into an ice-bath. Snap cooled and kept in ice bath for 10 min

PCR reaction components:

1. Template DNA
2. 16S rRNA gene specific primers
   Forward primer (1:10)
   Reverse primer (1:10)
3. Taq DNA polymerase
4. 10x reaction buffer (100mM Tris, pH9.0, 500mM KCl, 15mM $MgCl_2$ and 0.1% gelatin)
5. Nuclease free water
6. dNTP mixture (10mM of each dNTP)

Components used in PCR reaction mixture:

Component	**Volume (µl)**
Nuclease free water	11.2
10x reaction buffer	2.5
dNTP mixture (10mM)	5
Taq DNA polymerase	0.3
Primer	1
Template DNA/Genomic DNA	5
Total volume	25µl

Primer sequence: (Banglore genei)

Forward sequence- 5^1GAG TTT GAT CCT GGC TCA GG 3^1

Reverse Sequence- 3^1TCA TCT GTC CCA CCT TCG GC5^1

PCR Cycle parameters:

Reaction	Temperature	Time	
Denaturation	95^{o}C	3 min	
Denaturation	94^{o}C	40 sec	34 Cycles
Primer annealing	48^{o}C	20 sec	
Extension	72^{o}C	20 sec	
Final extension	72^{o}C	15 min	

Methodology:

1. The master mixture was prepared by mixing all the components except template DNA and sterile water.
2. This master mixture was taken in PCR tubes containing template DNA and sterile water.
3. The contents of the tubes were mixed by brief spin in a microcentrifuge.
4. The tubes were then placed in thermocycler and the reaction was carried out at desired program.

4.2.3.4 PCR product analysis:

1. The PCR product was analyzed by using 1.5% Agarose gel electrophoresis.
2. A 10µl of the amplified PCR product was taken, mixed with 2µl of loading dye and loaded on to the well.
3. The size was confirmed by comparing with 3 Kb ladders which was used as a molecular size standard.
4. Electrophoresis was carried out at 100 volts till the dye reaches 3/4th of the gel.
5. The gel was stained, destained and the amplicon band was observed under UV- transilluminator.

4.2.3.5 Purification of PCR product using Qiangen Gel Extraction Kit*:*

Materials:

1. Qiangen gel elution column (GENETIX BIOTECH ASIA PVT. LTD)
2. Solubilization buffer
3. PE wash buffer
4. Elution buffer
5. Sterile eppendorfs

Methodology:

1. The amplicon from the gel was excised with a sterile sharp scalpel and it was taken in sterile pre-weighed eppendorfs.
2. The eppendorfs containing the gel slice was weighed again and for one volume of gel fragment, three volumes of solubilization buffer was added (100mg/300µl).
3. The mixture was incubated at 50^{o}C for 10 min until the gel gets completely dissolved.
4. The contents in the tube were mixed often by vortexing for every 2-3 min. during the incubation to dissolve the gel. Once when the gel is completely dissolved the color of the mixture turns yellow.
5. The tube was then vortexed, spinned and the solubilization fluid was then transferred into the Qiangen's elution column.
6. The column was centrifuged at 10,000 rpm for 2 min. The flow through was discarded and 750µl of PE wash buffer was added to the column which retains the DNA fragment and it was incubated at room temperature for 10 min.
7. The column was then centrifuged at 10,000 rpm for 2 min and the flow through was discarded.
8. The PCR product was eluted by adding 20µl of deionized sterile water and stored at -20^{o}C.

Confirmation of purified PCR product:

Around 3µL of PCR product was run on 0.8% agarose gel electrophoresis to confirm its concentration and purity.

4.2. 3.6 A-tailing of PCR product:

A protocol described by supplier (MBI, Fermentas- Lithiana) was followed.

Materials:

1. Purified PCR fragment
2. Taq DNA polymerase
3. Reaction Buffer (10 X)
4. dATP (10mM stock)
5. Taq DNA polymerase
6. 25mM $MgCl_2$

Methodology:

1. 30 µl of purified PCR fragment was taken in a sterile microcentrifuge tube. To this 5 µl of reaction buffer (1X) and 2 µl of 25mM $MgCl_2$ was added.
2. dATP was added to a final concentration of 0.2mM from 10mM stock of dATP, 1:10 dilution was made.
3. To the reaction mixture 2 µl Taq DNA polymerase was added.
4. Deionized sterile water was added in the reaction mixture and the volume was made upto the desired quantity.
5. The reaction mixture was incubated at 70^oC for 30 min.
6. The PCR product was purified using Qiangen Gel extraction kit as described above.

4.2.3.7 Ligation of purified A- tailed PCR product to the T-tailed pTZ 57R/T vector.

Different components used for ligation:

Components	Experimental (Vol in µl)	Control (Vol in µl)
Plasmid vector pTZ 57R/T	2.0	1.0
Purified A tailed PCR fragment	5.0	-
PEG 4000 sol.	2.0	1.0
T_4 DNA ligase 10 unit/µl	2.0	1.0
10X ligase buffer	2.0	1.0
Deionized water	6.5	-

Methodology:

1. All the components were taken in sterile microcentrifuge tubes and incubated at 22^0C for overnight.
2. The reaction was then stopped by inactivating the enzyme. This can be done by incubating at 65^0C for 15 min followed by cooling at 45^0C for 10 min. This ligation mixture was used in transformation.

4.3.3.8. Preparation of competent cells of E.coli using $CaCl_2$.

Materials:

1. LB broth (50mL)
2. Overnight grown *E.coli* DH5α culture.
3. 0.1 M $CaCl_2$
4. Plasmid DNA

Methodology:

1. A single colony of *E.coli* DH5α was inoculated into 2 ml LB broth and grown overnight at 37^0C.

2. The 250 mL conical flask containing 50 mL of LB broth was seeded with 0.2 mL of overnight grown culture and was incubated at 37^0C at 225 rpm.
3. The culture was allowed to grow till OD_{600} reaches 0.4-0.6
4. The inoculum was then transferred to sterile propylene tubes under aseptic condition and chilled for 10-15 min by storing in ice.
5. Cells were harvested by centrifugation at 6000 rpm for 12 min at 4^0C.
6. Discard supernatant and remove last traces of medium.
7. Resuspend pellets in 10 mL of 1.0M $CaCl_2$ and incubated in ice for at least half an hour.
8. Harvest cells by centrifugation at 6000 rpm for 12 min at 4^0C and decant supernatant completely.
9. Resuspend pellet in 2.0 mL of ice-cold 0.1M $CaCl_2$.
10. The competent cells were stored at 20^0C for use.

4.2.3.9 Transformation of Competent Cells

Materials:

Selection Media: Luria Bertani Agar (LB Agar) 2%

Composition	**Amount (g/L)**
Peptone	10
Yeast extract	5
NaCl	10
Agar-agar	20

Methodology:

1. To 200µL of competent cells taken in microcentrifuge tubes, 10µL of ligated product was added and mixed gently. Incubated in ice for 30 min.
2. The following samples were also included as control:

   Competent cells.

   Competent cells mixed with pTZ vector.

3. The cells were given a heat shock treatment by placing the tubes in water bath (45^0C) for 90 sec.
4. The tubes were then cooled immediately by transferring them to ice for 1-2 min.
5. LB broth of 800 µL was added to each tube and the culture was incubated at 37^0C for 12 h at 180 rpm.
6. The tubes were centrifuged at 6000 rpm for 5 min to concentrate the cells. Portion of the media was discarded and around 200µL was taken from the remaining concentrated cells and plated in appropriate selection media.

4.2.3.10 Selection of Recombinants/ Transformants

1. 200µL of the transformation mixture was spread on LB agar plates containing X-Gal and IPTG.
2. The control mixture i.e. competent cells transformed with pTZ were also spread.
3. The plates were incubated overnight at 37^0C.
4. White colour colonies were picked.

4.2.3.11 PCR fragment analysis of 16S rRNA

The PCR fragment of size 1.4kb was cloned in pTZ57R/T and was sequenced using M13 forward and reverse sequencing primers. Sequencing was carried out by the facility provided by the DBT at Bangalore Genei on a commercial basis. The sequence was searched against NCBI databank using BLAST.

4.2.3.12 **Sequence analysis**

The 16S rDNA sequences were compared with those available in the NCBI Database. NCBI is a web tool combining a blast research, alignment with CLUSTAL W and phylogeny analysis with the neighbour joining method. The combination of these well-known tools in an automated program facilitates the bacterial identification process (Clarridge, 2004).We identified the sequence, essentially taking into account the phylogeny, and not simply the percentage similarity (Fredricks and Relman, 1996;

Clarridge, 2004), and this meant that we considered the node level 0 or 1, the smallest phylogenic distance, and finally the percentage similarity and length of the sequence. The vicinity of the query and type-strain in the phylogenic tree was also considered.

4.3 Results:

4.3.1 Preliminary identification:

A total of 6 gram-positive and catalase negative rods and cocci were isolated from *Kanjika* an ayruvedic lactic acid fermented product. Identification of the isolates was done based both on phenotypic and biochemical characterizations of the isolates. The isolate K1a, K7a, K7b, K7a, K4a & K3a were phenotypically identified as lactobacillus and K23c was cocci .

Initial identification of the isolates was carried out based on the physiological characterization (table 4.1). All the isolates showed luxurious growth at 37^0C & 45^0C and moderate growth at 15^0C. similarly all the isolates were tolerant to high temperature 65^0C treatment for 15 min as evaluated by the difference in the CFU of the treated and untreated samples. The isolates showed moderate tolerance at 70^0C (15 min) except isolate K23c which showed weak tolerance. This clearly indicates that K23c is not a bacillus and could probably be a Pediococcus. All the isolates exhibited good growth in a wide range of pHs(3.5, 4.0, 4.5 & 8.6) and high salt tolerance (6.5% NaCl). However all the isolates exhibited poor tolerance at 10% NaCl concentration.

Table 4.1: Physiological characteristics of probiotic LAB isolated from *Kanjika*

Culture ID.	Temperature			Temperature Tolerance			pH				%NaCl	
	15^0C	37^0C	45^0C	65^0C (15min)	65^0C (30min)	70^0C (15min)	3.5	4.0	4.5	8.6	6.5	10
K1a	+	++	++	++	-	+	++	++	++	++	+	w
K7a	+	++	++	++	-	+	++	++	++	++	+	w
K23c	+	++	++	++	-	w	++	++	++	++	+	vw
K4a	+	++	++	++	-	+	++	++	++	++	+	vw
K3a	+	++	++	++	-	+	++	++	++	++	+	w
K7b	+	++	++	++	-	+	++	++	++	++	+	w

+ : growth ; ++ : luxurious growth ; - : no growth; w : weak growth ;
vw : very weak growth

These cultures were further selected for 16S rDNA analysis.

4.3.2 Molecular Characterization:

4.3.2.1 Extraction of genomic DNA from Lactic acid bacteria:

Extraction of genomic DNA of probiotic lactic acid bacteria isolated from *Kanjika* was carried out according to alkaline method (Anderson and Mckey,1983). The quality of extracted genomic DNA of the 6 probiotic LAB was analyzed by (0.8%) agarose gel electrophoresis and is shown in figure 4.1.

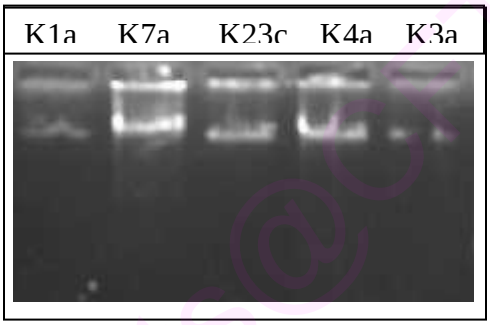


Figure 4.1: Agarose (0.8%) gel analysis of the genomic DNA of the probiotic *Kanjika* isolate, Lane 1, K1a; 2, K7a; 3,K23c; 4, K3a; 4, K4a

4.3.2.2 PCR amplification of 16S rDNA gene:

Nucleotide sequence of the rDNA of the isolated probiotic lactic acid bacteria was carried out with the forward and reverse primers viz. **BS F** : GAG TTT GAT CCT GGC TCA GG **BS R** : TCA TCT GTC CCA CCT TCG GC respectively. Average length of the primers were 20 bases since restriction sites were introduced into these primers. Total genomic DNA of the isolated cultures viz. K1a, K7a, K23c, K3a, K7b and K4a was used for the PCR amplification.. The PCR reaction parameters followed are indicated in the table 4.1. the primer annealing temperature of 48^0C was not suitable for the amplification of 16S rDNA of K23c and hence annealing was carried out at 55^0C. The PCR amplicon of 1.4kb of K23c is presented in Figure 4.2.

Table 4.2: PCR reaction parameters

PCR reaction parameter	**K1a, K3a, K7a, K4a, K7b**	**K23 C**
Initial denaturation	95^{o}C/ 3 min	95^{o}C/ 3 min
Denaturation .	94^{o}C/ 40sec	94^{o}C/ 40sec
Annealing	**48^{o}C/ 60 sec**	**55^{o}C/ 60 sec**
Extension	72^{o}C/ 20 sec	72^{o}C/ 20 sec
Final extension	72^{o}C/ 15 min	72^{o}C/ 15 min

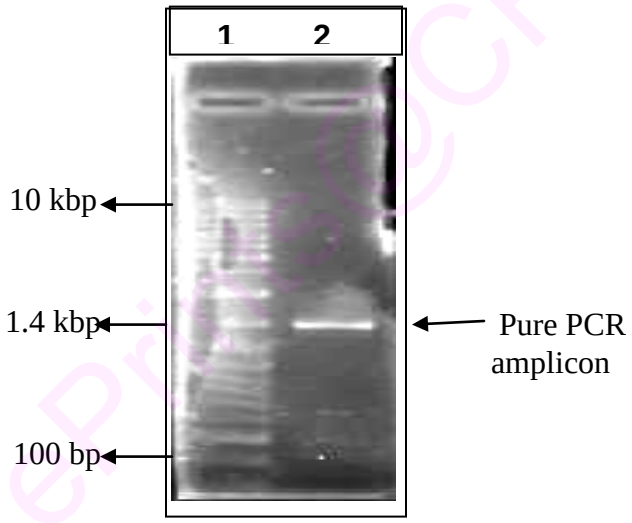


Figure 4.2: Agarose (1%) gel analysis of PCR amplicon of rRNA gene of K23c . Lane 1, marker of 1000 bp ladder; Lane 2, PCR product of K23c

4.3.2.3 Amplified rDNA restriction analysis (ARDRA)

A 1400 bp PCR product from the 16S rDNA was amplified for all of the isolates. Following digestion, different banding patterns were obtained for the *Hind III* restriction enzymes (Figure 4.3). The results of ARDRA analysis resolved by agarose gel electrophoresis were almost identical to the predicted fragments based on the nucleotide sequence data. Differentiation to the genus level was achieved using *Hind III*, as the digestion of the amplicon generated two different specific patterns, five for Lactobacillus and one for Pediococcus. The results indicates that the isolates K3a, K4a, K7a & K1a exhibits similar pattern other than K23c and K7b. By analyzing the pattern out of 6 isolates only 3 isolates showed different digestion patterns and these three isolates viz. K3a, K7b & k23c were selected for further study.

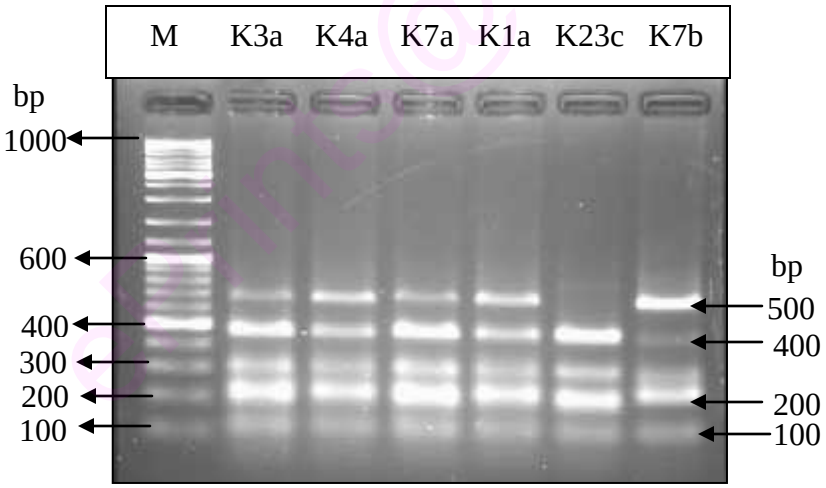


Figure 4.3. ARDRA patterns of strains isolated from *Kanjika* after digestion with HindIII. Lane M, Gene Ruler 100 bp DNA Ladder Plus (MBI Fermentas). Lanes K3a-K1a and K7b represent Lactobacillus and K23c represent Pediococcus

4.3.2.4 Molecular cloning of 16S rDNA gene:

The desired fragment was excised from the gel and purified using an QIAQuick gel extraction kit and ligated to pTZ57R/T vector using 5U of T4 DNA ligase. The recombinants were selected based on blue/white colonies and restriction analysis (Figure 4.3) 16S rDNA sequencing of the one of the clones after confirmation of the insert was carried out (Bangalore Genei, Bangalore). The sequencing data obtained using M13 forward (~ 450bp) and reverse primer (~ 450bp) was subjected to BLAST. The 16S rRNA sequence obtained from the DNA sequencer was analysis and is presented in the table 4.2

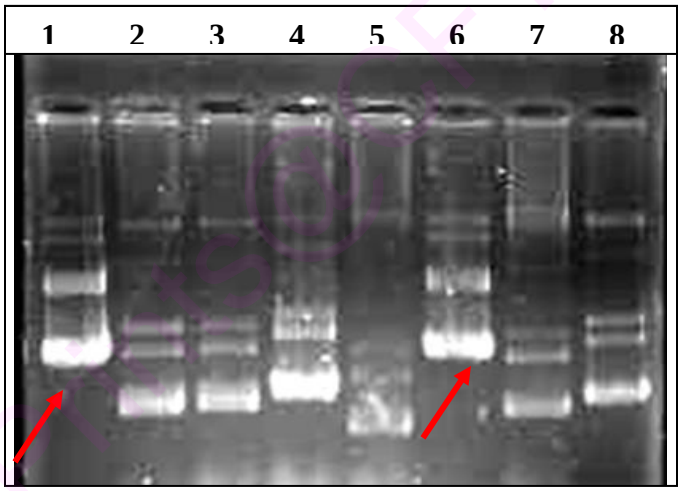


Figure 4.4: Agarose gel analysis of recombinant
Lane 1 & 6 : Recombinant pTZ vector
Lane 2 to 5 & 7 : Non recombinant pTZ vector
Lane 8: Control pTZ vector

The BLAST research data indicated that the analyzed sequence of the DNA of K23c, K3a & K7b was highly similar to *Pediococcus acidilactici*, *Lactobacillus plantarum* and *Lactobacillus fermentum* rRNA genes respectively.

Table 4.3: 16s rRNA sequence of the probiotic LAB isolates of the *Kanjika* after DNA sequence analyzer

Strain	16S rRNA sequence	Basepair
K23C	5[1]CCGATCGAGCTCGGTACCTCGCGATGCATCTAGCCTGAGTTTGATCCTGG CTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACT TCCGTTGATTGATTATGAGGTGCTTGCACTGAATGAGATTTTAACACGAAG TGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGCAGGG GATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCCT GGTTTTCTTTTAAAAGATGGCTTTGCTATCACTTCTGGATGGACCCGCGGC GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGATGATGCGTAGC CGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACT CCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGAT GGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTT GTTAAAGAAGAACGTGGGTGAGAGTAACTGTTCACCCAGTGACGGTATTT AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG GTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTC TTTTAAGTCTAATGTGAAAGCCTTCGGCTCACCGAAGAAGTGCATTGGAAA CTGGGAGACTTGAGTGCAGAAGAGGACAGTGGACTCCATGTGTAGCGGTG AAATGCGTAGATATATGGAAGACACCAGTG 3[1] 5[1]CGCTATGACCATGATTACGCCAAGCTCTAATACGACTCACTATAGGGAA AGCTTGCATGCCGGCCTCTGCAGTCGACGGGCCCGGGATCCGATTTCATCT GTCCCACCTTCGGCGGCTAGCTCCTAAAAGGTTACCCCACCGGCTTTGGGT GTTACAAACTCTCATGGTGTGACCGGCGGTGTGTAGGAGGCCCTAAACGT ATTCCCGCGGATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGTAGG CGAGTTGCAGCCTACAGTCCGAACTGAGAATGGTTTTAAGAGATTAGCTA AACCTCGCGGTTTCGCGACTCGTTGTACCATCCATTGTAGCACGTGTGTAG CCCAGGTCATAAGGGGCATGATGATTTGACGTCGTCCCCACCTTCCTCCGG TTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTGAATGCTGGCAACTAGT CATAAGGTTGCGGGCAGGTATGGCGAG 3[1]	1270
K3a	5[1]ATTCTGGTTCTAAAGGTTACCCCACCGACTTTCTCTGTTACAAACTCTCA TGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCA TGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGC CTACAATCCGAACTGAGAATGGCTTTAAGAGATTAGCTTACTCTCGCGAGT TCGCAACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAA GGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGC AGTCTCACCAGAGTGCCCAACTTAATGCTGGCAACTGATAATAAGGGTTG CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAA CCATGCACCACCTGTATCCATGTCCCCGAAGGGAACGTCTAATCTCTTAGA TTTGCATAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTA AACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTC AGCCTTGCGGCCGTACTCCCCAGGCGGAATGCTTAATGCGTTAGCTGCAGC ACTGAAGGGCGGAAACCCTCCAACACTTAGCATTCATCGTTTACGGTATGG ACTACCAGGTATCTAATCCTGTTTGCTACCCATACTTTCGAGCCTCAGCGT CAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCT ACGCATTTCACCGCTACCATGGAGTTCCCTG 3[1]	792
K7b	5[1]GCAGTCGCCGGCGGTGTGCCTATACATGCACCCCGAACGAGTTGGCCCA ATTGATTGATGTCGCTTGCACCTGATTGATTTTGGTCGCCAACGAGTGGCG GACGGGTGAGTAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAACATT TGGAAACAGATGCTAATACCGCATAACAACGTTGTTCGCATGAACAACGC TTAAAAGATGGCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAA CTTGTTGGTGGGGTAATGGCCTACCAAGGCGATGATGCATAACCGAATTG AGAAACTGATCGGCCACAATGGGACTGAAACACGGCCCATACTCCTACGG GAGGCAGCAGTAGGGAATCCTCCACAATGGGCGCAAGCCTGATGGAGCAA CACCGCGTGAGTGAAAAGGTTTCCGCTCAAACTCTGCTGCTAACCAAAAA CCCCCCGAAACCCCCCCCCC 3[1]	471

4.3.2.5 Homology sequence of rRNA gene of probiotic *Kanjika* isolates

1. K23c isolates:

The sequence of rRNA gene from K23c was homologous to an extent of 88% with that of many *Pediococcus acidilactici* strains. These include Accession Numbers AF515229.1, EF059987.1, AB018213.1, EF059986.1, AY587802.1 etc. where in the E value also was 0.0 indicating greatest similarity with sequence of rRNA from K23c (Table 4.4). Multiple sequence alignment of strains of *Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus plantarum* with the K23c was carried out by using CLUSTAL W (1.83). The multiple sequence alignment is shown the Figure 4.5.

Table 4.4: Sequences producing significant alignments with that of K23c isolate:

Accession	Description	Max score	Total score	Query coverage	E value
AF515229.1	*Pediococcus acidilactici* strain RO17 16S ribosomal RNA gene, complete sequence	1343	1965	88%	0.0
EF059987.1	*Pediococcus acidilactici* strain UL5 16S ribosomal RNA gene, partial sequence	1338	1965	88%	0.0
AB018213.1	*Pediococcus acidilactici* gene for 16S rRNA, partial sequence, strainLA 3	1338	1944	88%	0.0
EF059986.1	*Pediococcus acidilactici* strain UVA1 16S ribosomal RNA gene, partial sequence	1336	1898	85%	0.0
AY587802.1	Bacterium Te2R 16S ribosomal RNA gene, partial sequence	1328	1933	88%	0.0
DQ294960.1	*Pediococcus acidilactici* strain Uga146-3 16S rRNA gene, complete sequence	1327	1948	88%	0.0
AJ305322.1	*Pediococcus acidilactici* 16S rRNA gene, strain B1104	1321	1943	88%	0.0
AJ305320.1	*Pediococcus acidilactici* 16S rRNA gene, strain DSM 20284 (T)	1321	1943	88%	0.0

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P.pentosaceus       ------------------------------------------------------------
L.plantarum         ------------------------------------------------------------
P.acidilactici      ------------------------------------------------------------
K23 CFR-2193        ATCGAGCTCGGTACCTCGCGATGCATCTAGCCTGAGTTTGATCCTGGCTCAGGATGA 60


P.pentosaceus       ----------------------------------------------TAATTGATTATGACG 15
L.plantarum         ----------------------------------------------ATTGATTG----G 9
P.acidilactici      ----------------------------------------------TTAATTGATTATGACG 16
K23 CFR-2193        ACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTTCCGTTGATTGATTATGAGG 120
                                                                 *******     *

P.pentosaceus       TACTTGTACTGATTGAGATTTTAACACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGG 75
L.plantarum         TGCTTGCA----TCATGATTTACATTTGA-GTGAGTGGCGAACTGGTGAGTAACACGTGG 64
P.acidilactici      TGCTTGCACTGAATGAGATTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGG 76
K23 CFR-2193        TGCTTGCACTGAATGAGATTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGG 180
                    * **** *         *****  *   ** ********** ** ****************

P.pentosaceus       GTAACCTGCCCAGAAGTAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGA 135
L.plantarum         GAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAAC 124
P.acidilactici      GTAACCTGCCCAGAAGCAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGA 136
K23 CFR-2193        GTAACCTGCCCAGAAGCAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGA 240
                    * **************  ********************************** ******

P.pentosaceus       GAAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTG-CTATCACTTCTGGATGGACCCGC 194
L.plantarum         TTGGACCGCATGGTCCGAGTTTAAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGC 184
P.acidilactici      GAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTG-CTATCACTTCTGGATGGACCCGC 195
K23 CFR-2193        GAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTTTG-CTATCACTTCTGGATGGACCCGC 299
                        ***** ****     ***************  * ********* ******* *****

P.pentosaceus       GGCGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTG 254
L.plantarum         GGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTG 244
P.acidilactici      GGCGCATTAGCTAGTTGGCGAGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTG 255
K23 CFR-2193        GGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTG 359
                    **** ********* *** * ***** ********* ***  **** **************

P.pentosaceus       AGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG 314
L.plantarum         AGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG 304
P.acidilactici      AGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG 315
K23 CFR-2193        AGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG 419
                    ****************************************** ********************

P.pentosaceus       TAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG 374
L.plantarum         TAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG 364
P.acidilactici      TAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG 375
K23 CFR-2193        TAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG 479
                    ********************** ************************************

P.pentosaceus       GTTTCGACTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCC 434
L.plantarum         GTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGT 424
P.acidilactici      GTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTGAGAGTAACTGTTCACCC 435
K23 CFR-2193        GTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTGAGAGTAACTGTTCACCC 539
                    ****** ******** ******************** *   * ************ *

P.pentosaceus       AGTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG 494
L.plantarum         ATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG 484
P.acidilactici      AGTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG 495
K23 CFR-2193        AGTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG 599
                    * ************************************************************

P.pentosaceus       TAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAG 554
L.plantarum         TAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAG 544
P.acidilactici      TAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAG 555
K23 CFR-2193        TAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAG 659
                    *************** ************************************ *******

P.pentosaceus       TCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGT 614
L.plantarum         TCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGT 604
P.acidilactici      TCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGT 615
K23 CFR-2193        TCTAATGTGAAAGCCTTCGGCTCA-CCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGT 718
                    *** ******************** ************** ************ ********
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P.pentosaceus       GCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAAC 674
L.plantarum         GCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAAC 664
P.acidilactici      GCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAAC 675
K23 CFR-2193         GCAGAAGAGGACAGTGGA-CTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGA-C 776
                    ****************** ************************************* *

P.pentosaceus       ACCAGTG-ACGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGT 733
L.plantarum         ACCAGTG-GCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGT 723
P.acidilactici      ACCAGTG-GCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGT 734
K23 CFR-2193         ACCAGTGCGCTATGACCATGATTACGCCAAGCTCTAATACGACTCACTATAGGGAAAGCT 836
                    *******  * * * *     *   * *     ** *  *          * * *  * *

P.pentosaceus       AGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGATTACTAAGTGTTGG 793
L.plantarum         AGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGG 783
P.acidilactici      AGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGATTACTAAGTGTTGG 794
K23 CFR-2193         TGCATGCCGGCCTCTGCAGTCGACGGGCCCGGGATCCGATTT-CATCTGTCCCACCTTCG 895
                      **   * **  *    *  *     * **       *        *        ** *

P.pentosaceus       AGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGT------AATCCGCCTGGGGA 847
L.plantarum         AGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGC------ATTCCGCCTGGGGA 837
P.acidilactici      AGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGT------AATCCGCCTGGGGA 848
K23 CFR-2193         GCGGCTAGC-TCCTAAAAGGTTACCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGT 954
                       ** *  *  ***  *  * * * *   ** **  *        *  * * *** *

P.pentosaceus       GTACGACCGCAAGGTTGAAACTC-AAAAGAATTGACGGGGGCCCGCACAAGCGGTGG--A 904
L.plantarum         GTACGGCCGCAAGGCTGAAACTC-AAAGGAATTGACGGGGGCCCGCACAAGCGGTGG--A 894
P.acidilactici      GTACGACCGCAAGGTTGAAACTC-AAAAGAATTGACGGGGGCCCGCACAAGCGGTGG--A 905
K23 CFR-2193         GACCGGCGGTGTGTAGGAGGCCCTAAACGTATTCCCGCGGATGCTGATCCGCGATTACTA 1014
                    *  ** * *   *   **  * * *** * ***  ** **   *  *   *** *    *

P.pentosaceus       GC-ATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTT-GACATCTTCTGA 962
L.plantarum         GC-ATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTT-GACATACTATGC 952
P.acidilactici      GCCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTCGACATCTTCTGC 965
K23 CFR-2193         GCGATTCCGACTTCGTGTAGGCGAGTTGCAGCCTACAGTCCGAACTGAGAATGGTTTTAA 1074
                    ** **   *  *   *  * ** *   * **        * *  **  **    * *

P.pentosaceus       CAGTCTAAGAGATTAGAGGTTCC-CTTCGGGGAC--AGAATGACAGGTGGTGCATGGTTG 1019
L.plantarum         AAATCTAAGAGATTAGACGTTCC-CTTCGGGGAC--ATGGATACAGGTGGTGCATGGTTG 1009
P.acidilactici      CAACCTAAGAGATTAGGCGTTCCTCTTCGGGGAC--AGAATGACAGGTGGTGCATGGTTG 1023
K23 CFR-2193         GAGATTAG----CTAAACCTCGCGGTTTCGCGACTCGTTGTACCATCCATTGTA-GCACG 1129
                      *   **      **    *  *  **  * ***          **     ** * *   *

P.pentosaceus       TCGTCAGCTCGTGTC-GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTA 1078
L.plantarum         TCGTCAGCTCGTGTC-GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTA 1068
P.acidilactici      TCGTCAGCTCGTGTCTGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTA 1083
K23 CFR-2193         TGTGTAGCCCAGGTC-ATAAGGGGCATGATGATTTGACGTCGTCCCCACCTTCCTC-CGG 1187
                    *    *** *  ***  * **  *   * * *  *  **       * *   ***

P.pentosaceus       CTAGTTGCCAGCA--TTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGA 1136
L.plantarum         TCAGTTGCCAGCA--TTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGA 1126
P.acidilactici      CTAGTTGCCAGCA--TTCAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGA 1141
K23 CFR-2193         TTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTGAA--TGCTGGCAACTAGTCATAAGG 1245
                       **  ** ***   * * * * *  * *   ***   *** **  ** *  *  * *

P.pentosaceus       AGGTGGGGACG-ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA 1195
L.plantarum         AGGTGGGGATG-ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA 1185
P.acidilactici      AGGTGGGGACG-ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA 1200
K23 CFR-2193         TTGCGGGCAGGTATGGCGAG----------------------------------------- 1265
                      * *** * * * * * *
```

Figure 4.5: Multiple sequence alignment of rRNA gene from K23c with *P. pentosaceus*, *L. plantarum*, and *P.acidilactici*. ▭ regions correspond to the unsequenced 16S rRNA gene of K23c isolates. * indicates conserved region.

A phylogenic map was constructed using the rRNA gene sequences of the four strains listed below (Figure 4.6). Among the rRNA gene sequences analyzed, *L. plantarum* is separated from the rest with *P. pentosaceus* as its closest relative. The isolate K23c was near to the *P. acidilactici* indicating the nearest homology of the isolate .

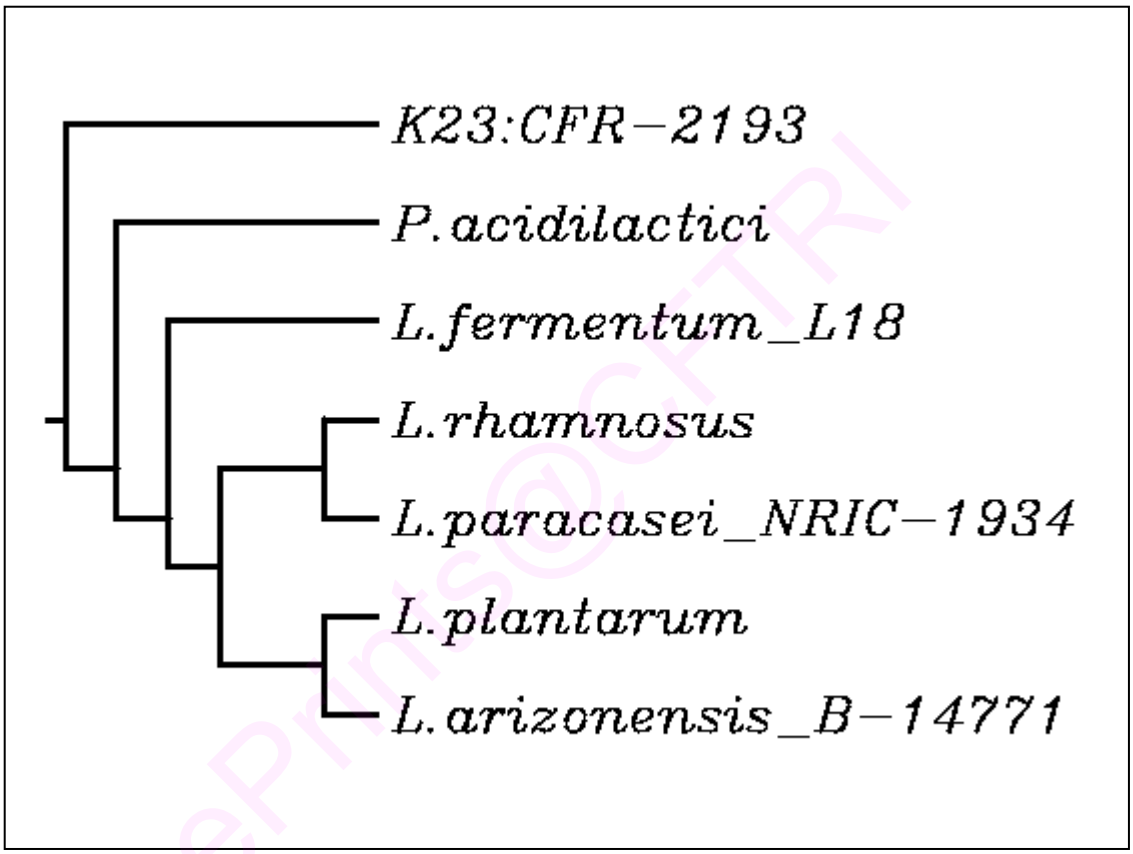

Figure 4.6: Phylogenic analysis of r DNA gene sequences.

2. **K3a and K7b Isolates**:

The sequence of rRNA gene from K3a was homologous to an extent of 99% with that of many *L. plantarum* strains (Table 4.5) and K7b to an extent of 93% with that of *L. fermentum* strains (Table 4.6). Multiple sequence alignment from strains of *L. plantarum, L. arizonensis and L. gasseri* with the K3a strain and *L. fermentum* L18& ATCC 14931 and *L.rhamnosus* GG with K7b was carried out by using CLUSTAL W (1.83). The multiple sequence alignment was represent as shown the figure 4.7 and 4.8 respectively. A phylogenic map was constructed using the rRNA gene sequences from K3a & K7b isolates (Figure 4.9).

Table 4.5: Sequences producing significant alignments with that of K1a isolate

Accession	Description	Max score	Total score	Query coverage	E value
EU147308.1	*Lactobacillus plantarum* strain BFE 8348 16S ribosomal RNA gene, partial sequence	1417	1417	99%	0.0
EU147307.1	*Lactobacillus plantarum* strain BFE 8202 16S ribosomal RNA gene, partial sequence	1417	1417	99%	0.0
EU147306.1	*Lactobacillus plantarum* strain BFE 8200 16S ribosomal RNA gene, partial sequence	1417	1417	99%	0.0
EU147305.1	*Lactobacillus plantarum* strain BFE 8239 16S ribosomal RNA gene, partial sequence	1417	1417	99%	0.0
EU074832.1	*Lactobacillus plantarum* strain TC97 16S ribosomal RNA gene, partial sequence	1417	1417	99%	0.0
EU081011.1	*Lactobacillus plantarum* strain BMG 112 16S ribosomal RNA gene, partial sequence	1417	1417	99%	0.0
AB299474.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: B50	1417	1417	99%	0.0
AB326351.1	*Lactobacillus plantarum* gene for 16S rRNA, partial sequence, strain: NBRC 15891	1417	1417	99%	0.0

Table 4.6: BLAST Sequences producing significant alignments with that of K7a isolate

Accession	Description	Max score	Total score	Query coverage	E value
DQ523484.1	*Lactobacillus fermentum* strain L18 16S ribosomal RNA gene, partial sequence	701	701	93%	0.0
EF703077.1	Uncultured Bacilli bacterium clone MS093A1_G12 16S ribosomal RNA gene, partial sequence	695	695	92%	0.0
EF535257.1	*Lactobacillus fermentum* strain KLB 261 16S ribosomal RNA gene, partial sequence	695	695	93%	0.0
EF510474.1	Uncultured bacterium clone P2D1-517 16S ribosomal RNA gene, partial sequence	695	695	93%	0.0
AM234674.1	Uncultured bacterium partial 16S rRNA gene, clone TIMGluc04	695	695	93%	0.0
AF349926.1	Uncultured bacterium clone 60CR 16S ribosomal RNA gene, complete sequence	695	695	93%	0.0
DQ399352.1	*Lactobacillus fermentum* strain SFCB2-3 16S ribosomal RNA gene, partial sequence	695	695	93%	0.0
AF302116.1	*Lactobacillus fermentum* 16S ribosomal RNA gene, complete sequence	695	695	93%	0.0
AF429506.1	*Lactobacillus fermentum* ATCC 14931 16S ribosomal RNA gene, partial sequence	695	695	93%	0.0
AB017345.1	*Lactobacillus fermentum* gene for 16S rRNA, partial sequence, strain:ATCC14931	695	695	93%	0.0
DQ779203.1	*Lactobacillus fermentum* strain 44197 16S ribosomal RNA gene, partial sequence	693	693	92%	0.0

```
L.arizonensisB-14771    GCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCA 60
L.gasseri               ------------------------------------CTAGATGAATTTGGTGCTTGCA 22
K3a-CFR 2191            ---------------------------------------------ATTCTGGTTCTAAAG 15


L.plantarum             ------------CATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCC 47
L.arizonensisB-14771    TCATG---ATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCC 117
L.gasseri               CCAAATGAAACTAGATACAAGCGAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCC 82
K3a-CFR 2191            GTTACCCCACCGACTTTC---TCTGTTACAAACTCTCATGGTGTG-ACGGGCGGTGTGTA 71
                                  *        *   *  **       *        ***     **


L.plantarum             CAGAAGCGGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCA 107
L.arizonensisB-14771    CAGAAGCGGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCA 177
L.gasseri               CAAGAGACTGGGATAACACCTGGAAACAGATGCTAATACCGGATAACAACACTAGACGCA 142
K3a-CFR 2191            CAAG-GCCCGGGAACGTATTCACCGCGGCATGCTGAT-CCGCG--ATTACTAGCGATTCC 127
                        **   *   ****    *           ***** ** ***    *  **        *


L.plantarum             TGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAG 167
L.arizonensisB-14771    TGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAG 237
L.gasseri               TGTCTAGAGTTTAAAAGATGG-TTCTGCTATCACTCTTGGATGGACCTGCGGTGCATTAG 201
K3a-CFR 2191            GACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAGAGATTAG 187
                                **     ** **    *  * ***     **       *        *****


L.plantarum             CTAGATG--GTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGA---GG 222
L.arizonensisB-14771    CTAGATG--GTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGA---GG 292
L.gasseri               CTAGTTG--GTAAGGTAACGGCTTACCAAGGCAATGATGCATAGCCGAGTTGAGA---GA 256
K3a-CFR 2191            CTTACTCTCGCGAGTTCGCAACTCGTTGTACCATCCAT-TGTAGCACGTGTGTAGCCCAG 246
                        **   *   *   * *  *  **        **   **   ****     **


L.plantarum             GTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGG 282
L.arizonensisB-14771    GTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGG 352
L.gasseri               CTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGG 316
K3a-CFR 2191            GTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGT--TTGTCACCGGC 304
                         * **  *   ***   ** *      *  *** * ** *  * **     *     **


L.plantarum             AATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTC 342
L.arizonensisB-14771    AATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTC 412
L.gasseri               AATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTC 376
K3a-CFR 2191            AGTCTCAC-CAGAGTGCCCAACTTAATGCTGGCAAC----TGATAATAAGGGTTGCGCTC 359
                        * ***  * **  *  * **  *        *****     *  * * * *  *   **


L.plantarum             GGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGA 402
L.arizonensisB-14771    GGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGA 472
L.gasseri               GGCTCGTAAAGCTCTGTTGGTAGTGAAGAAAGATAGAGGTAGTAA--------------- 421
K3a-CFR 2191            GTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGT 419
                        *   **  *                * **         *     **


L.plantarum             CGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT 462
L.arizonensisB-14771    CGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT 532
L.gasseri               ------------------------------------------------------------
K3a-CFR 2191            ATCCATGTC-CCCGAAGGGAACGTCTAATCTCTTA--GATTTGCATAGTATGTCAAGACC 476


L.plantarum             GGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAG-GCGGTTTTTTAAGTCTG 521
L.arizonensisB-14771    GGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAG-GCGGTTTTTTAAGTCTG 591
L.gasseri               ------------------------------------------------------------
K3a-CFR 2191            TGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC 536


L.plantarum             ATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAG 581
L.arizonensisB-14771    ATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAG 651
L.gasseri               ------------------------------------------------------------
K3a-CFR 2191            CCGTCAATTCCTTTG----AGTTTCAGCCTTGCGGCCGTACTCCCCAGGCG-GAATGCTT 591


L.plantarum             AAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCA 641
L.arizonensisB-14771    AAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCA 711
L.gasseri               ------------------------------------------------------------
K3a-CFR 2191            AATGCGTTAGCTGCAGCAC---TGAAGGGCGGAAACCCTCCAACACTTAGCATT-CATCG 647


L.plantarum             GTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAA 701
L.arizonensisB-14771    GTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAA 771
L.gasseri               ------------------------------------------------------------
K3a-CFR 2191            TTTACGGTATGGACTACCAGGTATCTAATCCTGTTTGCTACCCATACTTTCGAGCCTCAG 707


L.plantarum             ACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAA-TGCTAAGTGTTGGAGGG 760
L.arizonensisB-14771    ACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAA-TGCTAAGTGTTGGAGGG 830
L.gasseri               ------------------------------------------------------------
K3a-CFR 2191            CGTCAGTTACAGACCA-GACAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCA 766
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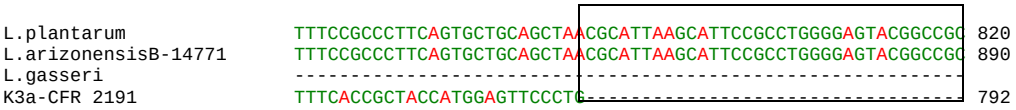


Figure 4.7: Multiple sequence alignment of rRNA gene from K3a *L. arizonensis*, *L. plantarum* and *L. gasseri*. ▭ regions corresponds to the unsequenced 16S rDNA gene of K23c isolates. * indicates conserved region.

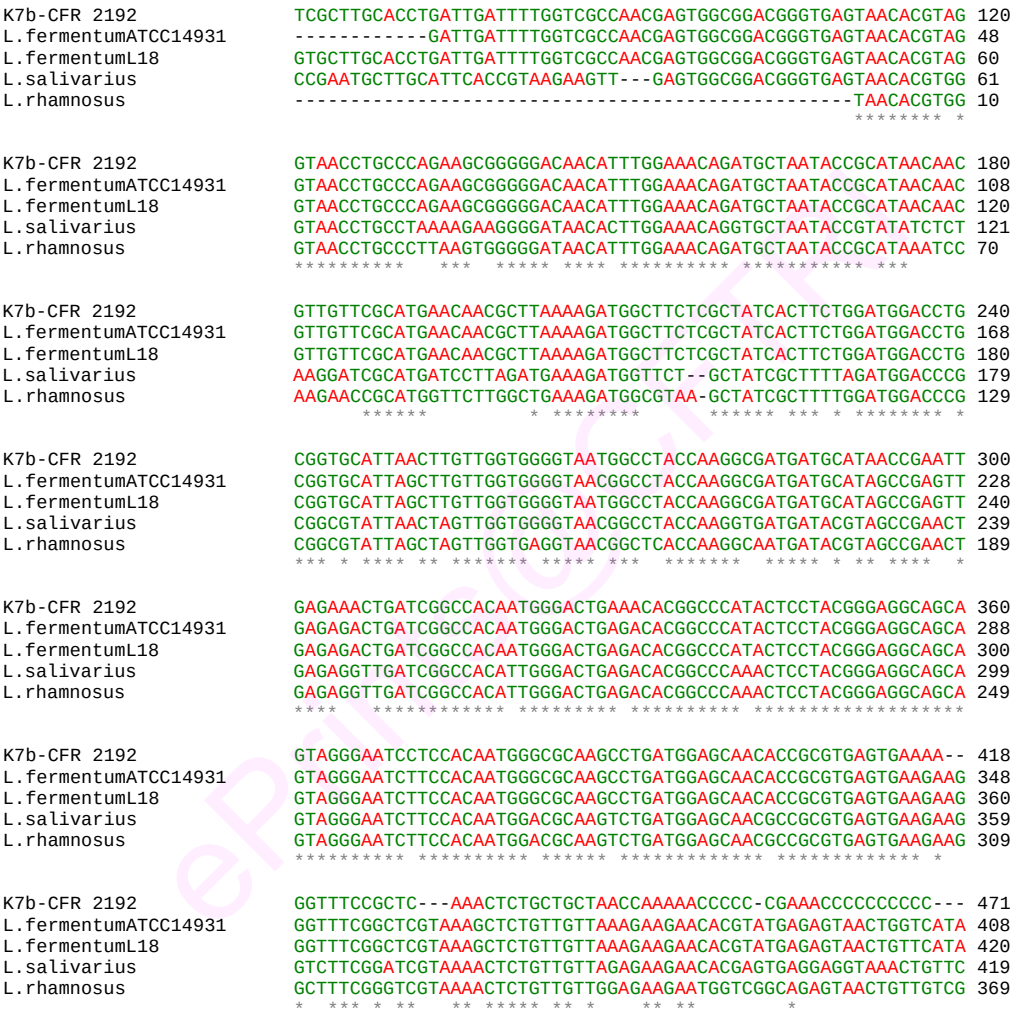


Figure 4.8: Multiple sequence alignment of rRNA gene from K7b, *L. fermentum L18 & ATCC 14931, L. salivarius* and *L. rhamnosus GG* .
*** indicates conserved region.**

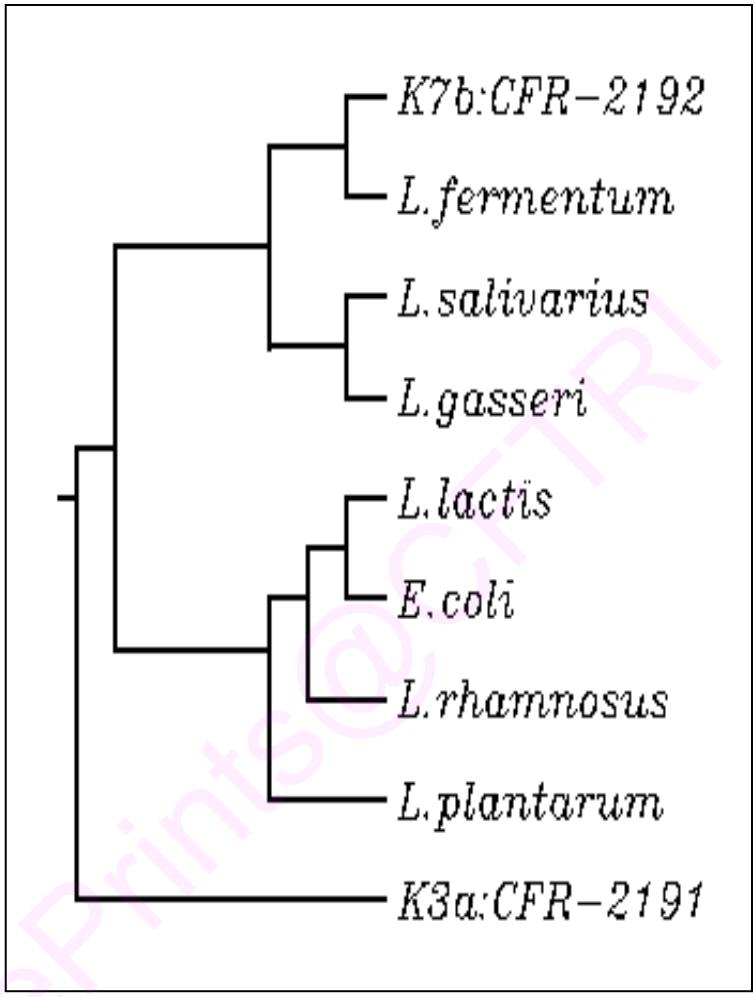


Figure 4.9: Phylogenic analysis of rRNA gene sequences of K3a & K 7b.

It is equally important to carry out the biochemical characterization of the isolated strains. Upon molecular characterization, it was found that out of the six isolates 3 of them belonged the same species; K1a, K3a, K4a – *L. plantarum*; K7a, K7b- *L. fermentum*; K23c- *P. acidilactici*; and hence detailed biochemical characterization of only the three identified species was carried out (Figure 4.10)

All the four *Kanjika* isolates were found to ferment cellobiose, fructose, galactose, mannose, raffinose, ribose, melibiose, sucrose, trehalose, xylose and esculin. While K_3a, K7b, were observed as maltose fermentors, K23c was non-maltose fermentor. Similarly K7b and K23c were found to be non-lactose fermentor. The fermentation ability of the isolates varied considerably with respect to the following substrates like mannitol, melizitose, rhamnose and sorbitol. The isolate K3a was found to ferment mannitol, rhamnose and sorbitol, however K7b and K23c were not capable of utilizing the above substrates. All the isolates except K7b utilized melizitose. None of the isolates were found to utilize starch or liquefy gelatin. Citrate and malonate were also not utilized by any of the isolates.

A comparative analysis of these properties with that of the data available for these three organisms have been done (Table 4.7)

PART – A : 1, Lactose; 2, Xylose; 3, Maltose; 4, Fructose; 5, Dextrose; 6,Galactose; 7, Raffinose; 8, Trehalose; 9, Mellibiose; 10, Sucrose; 11, L-arabinose; 12, Mannose

PART – B: 1, Inulin; 2, Sodium gluconate; 3, Glycerol; 4, Salicin; 5, Glucosamine; 6, Dulcitol; 7, Inositol; 8, Sorbitol; 9, Mannitol; 10, Adonitol; 11, α-methyl D- glucoside; 12, Ribose.

PART- C : 1, Rhamnose; 2, Cellobiose;3, Melezitose; 4, α methyl D- mannoside; 5, Xylitol; 6, ONPG; 7, Esculin; 8, D- Arabinose; 9, Citrate; 10, Malonate; 11, Sorbose; 12, Control.

Figure 4.10: Biochemical characterization of the probiotic lactic acid bacteria of the K3a *Kanjika* isolates

Table 4.7: Carbohydrate fermentation and organic substrates utilization profile: comparison between test experiment and Bergey's Manual

Substrate	(K7b) *L. fermentum*		(K3a) *L. plantarum*		(K23c) *P.acidilactici*	
	Exp.	B.M	Exp.	B.M	Exp.	B.M
Arabinose	-	d	+	+	+/-	d
Cellobiose	+	d	+	+	+	N.A
Esculin	+	-	+	+	+	N.A
Fructose	+	+	+	+	+	N.A
Galactose	+	+	+	+	+	N.A
Lactose	-	+	+	+	-	d
Maltose	+	+	+	+	-	-
Mannitol	-	-	+	+	-	-
Mannose	+	+	+	+	+	N.A
Melizitose	-	-	+	d	+	-
Melibiose	+	+	+	+	+	N.A
Raffinose	+	+	+	+	+	N.A
Rhamnose	-	-	+	-	-	N.A
Ribose	+	+	+	+	+	+
Salicin	-	-	+	+	+	+
Sorbitol	-	-	+	+	-	-
Sucrose	+	+	+	+	+	-
Trehalose	+	d	+	+	+	d
Xylose	+	d	+	d	+	+
Starch	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-
Citrate	-	N.A	-		-	-
Malonate	-	N.A	-		-	-
ONPG	-	N.A	-		-	-

+ : substrate fermented; - : substrate not fermented; d: some strains only ferment

N.A: not available in Bergey's manual

4.4 Discussion

Lactic aicd bacteria (LAB) are one of the most important groups of bacteria to the food industry because of their well documented fermentation and claimed health promoting properties. LAB have been thoroughly characterized for their metabolic properties, growth performance, resistance to industrial processes, sustainability in the end product and targeted site of action, shelf life, etc. In this context, reliable identification of LAB remains a point of crucial importance. Most industrial applications and probiotic health effects of LAB depends on the specific characteristics of a particular strain level. Although most industrial applications and probiotic health effects of LAB depend on the specific characteristics of a particular strain, it is not always necessary to identify bacteria down to the strain level. An optimal balance has to be found between the desired taxonomic resolution of a certain application and the involved workload, speed and cost.

The phenotypic methods include morphological and physiological characterization, carbohydrate fermentation patterns and protein profiling. Gonzalez, *et. al.*, (2000) identified 249 LAB isolates from freshwater fish using 44 morphological and physiological tests. A high percentage (90%) of the isolates could only be identified at the genus level. Corsetti, *et. al.*, (2001) analyzed 317 presumptive LAB isolates from sourdoughs based on morphological and physiological characteristics, but only 38% of the isolates could be identified to the species level. In the present study also only 80% similarity was found, when compared with that of the Bergey's manual for the characterization of the LAB isolates. The limitations of the phenotypic methods such as relatively poor reproducibility and a low taxonomic resolution that often, only allows differentiation at the genus level are well documented.

Genotypic techniques exhibit various levels of discriminatory power, from species level to differentiation of individual strains (typing). Many genotypic methods are based on the principle of Polymerase Chain Reaction (PCR), which enables the selective

amplification of specifically targeted DNA fragments through the use of oligonucleotide primers under controlled reaction conditions. The ARDRA technique using only one PCR reaction and one restriction enzyme has allowed us to discriminate the bacteria isolated from natural fermented products upto genus levels. This technique allows the discrimination among the similar type of the isolates of related genera with only one restriction enzyme. Therefore it can be a simple, rapid and useful method for routine identification. The identification at strain level is necessary since the beneficial effects that these bacteria exercise on the human health cannot be attributed to a genus and/or species, because these properties are strain dependent (Ouwehand *et. al.*, 2002). The identification of LAB upto species level is often not possible by phenotypic methods. Of late,16S rDNA sequencing has become the reference method for bacterial taxonomy and identification. It is nearly always possible by 16S rDNA sequencing (Janda and Abbott, 2002; Clarridge, 2004).

As discussed earlier based on the ARDA techniques, the six different cultures isolated originally were grouped to only 3 different strains. These three different strains were further characterized by 16S rDNA technique. Multiple sequence aligment of 16S rDNA gene of the isolates K3a, K7b, & K23c were compared with known cultures neareast homology. A phylogenic map based on the gene sequence has be drawn for the three selected isolates of *Kanjika.*

4.5 Conclusion:

The present chapter details the phenotypic and genotypic characterization of the potent probiotic *kanjika i*solates. Phenotypic characterization based on carbohydrate sugar fermentation pattern and conventional phenotypic properties may not always provide sufficient basis for the reliable identification of LAB, although it is a useful tool for presumptive classification and hence 16S rDNA method was chosen for the genotypic characterization of the isolates. Based on genotypic characterization, isolate K3a were identified as *L. plantarum* (CFR-2191); K7b as *L. fermentum* (CFR-2192) and K23c as *P.acidilactici* (CFR-2193). The above isolates 16S rRNA sequence were also deposited to the gene bank and accession number for K3a as *L. plantarum* (EU 263132); K7b as

L. fermentum (EU 263133) and K23c as *P.acidilactici* (EU 263134). *L. plantarum* strains are generally known to be associated with plant based food f.ermentation (Stiles and Holzapfel (1997), and Wood and Holzapfel (1995)). In agreement with this one of the isolates from *Kanjika* has been identified as *L. plantarum.* The above isolates were deposited in the departmental cultural collection.

Chapter - 5

In vitro evaluation of Lactic acid bacteria (LAB from culture collection) for potent probiotic properties

5.1. Introduction

Increase in knowledge of nutrition in the recent years has led to the development of the foods which have health benefits beyond adequate nutrition. Recent findings, different aspects of bioscience and several hypotheses supports that, beyond nutrition, diet may modulate various functions in the body (Sanders and In't Veld, 1999). The cashing in of health aspects of food products in marketing began in the 1960s and in 1970s, the trend then was to remove unhealthy components, such as salt, sugar or fat. In 1990s more healthy components, such as vitamins, antioxidants, fibers and probiotic lactic acid bacteria (LAB) were added to the functional food products. The addition of probiotic LAB to the fermented dairy products has increased tremendously since last decade. Most of the probiotics have been applied mainly in dairy products, such as yoghurt and other cultured fermented beverages. The driving force behind this is the health consciousness of today's consumer.

The concept of probiotic foods as functional foods is due to the successful cooperation of food industry and research in food science and technology as well as in clinical nutrition. Dairy products, especially fermented milk and yoghurt are prepared using viable probiotic LAB with scientifically proven health effects and safety as GRAS cleared organism. These functional dairy food products are becoming scientifically better documented and also the trend is towards the food for special health use by the consumers. Most of the colon cancer diseases caused by the *Helicobacter pylori* can be controlled by feeding probiotic LAB through fermented dairy products like yoghurt (Kuan-Yuan Wang *et. al.,* 2004). For example, *Lactobacillus GG* has been applied through yoghurt, fermented milk and dairy based products to cure many of the diseases like irritable bowl disease, traveller's diarrhea etc.

The potential therapeutic benefits associated with the consumption of fermented dairy products containing viable probiotic LAB has been detailed in a good number of research papers (Friend and Shahani, 1984; Fernandes *et. al.*, 1987; Savaianao and Lewitt, 1987; Gilliland, 1989). Shaper *et. al.*, (1963) and Mann and Spoerry (1974) observed that the tribes of Samburu and Maasai warriors in Africa showed a reduced

serum cholesterol after the consumption of large amounts of milk fermented with a wild *Lactobacillus* strain. Since then, the potential hypocholesterolemic effect of fermented milk products containing lactobacilli and/or bifidobacteria has been investigated using animal models and in human studies. Consumption of fermented dairy products increases the LAB count and decreases coliform (pathogenic *E. coli*) count in the intestine as observed in fecal analyses (Gilliland *et. al.,* 1978). Several studies also suggest that probiotic consumption through yoghurt can cure the lactose intolerance due to the hydrolysis of the milk lactose (Pochart *et. al.,* 1989; Marteau *et. al.,*1990; Martini *et. al.,*1991) and also the possible prevention of cancer initiation (Gilliland, 1989). Consumption of cultured dairy products reduces the breath hydrogen of lactase nonpersistent individuals referred to as lactose intolerants (Fernandes and Shahani, 1989). Since last two decades, several studies indicated that probiotic LAB and fermented dairy products possess anticarcinogenic activity (Kato *et. al.,* 1983). Several model experiments have been conducted to delineate the anticarcinogenic benefit of yogurt and other cultured dairy products.

The criteria for selecting potent probiotic LAB have been listed comprehensively by several authors (Lee and Salminen, 1995; Collins *et. al.,* 1998; Dunne *et. al.,* 1999). Adherence of the LAB strains to human intestinal cells is considered to be the key characteristic. In addition, probiotic strains should also have desirable properties like antibiotic resistance and sensitivity patterns, antagonistic activity towards potentially pathogenic microorganisms and metabolic activities beneficial to the well-being of the host. Immunostimulation, antimutagenicity and anticarcinogenicity are also mentioned in the context of benefits derived from probiotics. In this chapter some of the important characteristic properties of the 13 dairy LAB (Table 5.1) were studied. The strains were examined *in vitro* for tolerance to low pH, and bile salts. Acid and bile tolerance are very important in enabling the LAB to travel through the passage of the stomach and the gastro-intestinal track. Antimicrobial properties towards selected food spoilage bacteria, β-galactosidase activity for the digestion of the milk lactose, cholesterol lowering

capacity and adherence to HT-29 cell lines were evaluated for the selection of potent probiotics for dairy applications.

Table 5.1: Bacterial strains and culture conditions

LAB strains	Strain	Culture condition& media[#]
Lactobacillus plantarum	B-4496	37^0C, MRS
Lactobacillus amylovorus	B-4437	37^0C, MRS
Lactobacillus acidophilus	B-4495	37^0C, MRS
Lactobacillus casei	Lund	37^0C, MRS
Lactobacillus lactis (crimoris)	B-634	37^0C, MRS
Lactobacillus casei	B-1922	37^0C, MRS
Lactobacillus helveticus	B-4526	37^0C, MRS
Lactobacillus casei	NCIM-2586	37^0C, MRS
Lactobacillus bulgaricus	CFR-2028	37^0C, MRS
Lactobacillus casei	DSM-20011	37^0C, MRS
Lactobacillus salivarius	CFR-2158	37^0C, MRS
Bacillus coagulans	NCIM-2323	37^0C, MRS
Escherichia coli	MTCC 108	30^0C, NB
Bacillus cereus	F-4810	30^0C, NB
Listeria monocytogenes	Soctt A	30^0C, NB
Yersinia enterocolitica	MTCC- 859	30^0C, NB
Staphylococcus aureus	F- 722	30^0C, NB
Escherichia coli (ETEC)	ATCC-31705	30^0C, NB

[#] MRS= deMan, Rogosa, Sharpe; NB= Nutrient Broth; ATCC = The American Type Culture Collection; B= National Center for Agricultural Utilization Research Laboratory, NCIM= National Collection/center for Industrial Microbiology; MTCC=Microbial Type Cultural Collection; CFR= Central Food Technological Research Institute Collection Center; DSM=Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; F= International Public Health Laboratory, London

5.2 Materials and Methods

5.2.1 Materials

All the chemicals used are as detailed in section 3.2.1

5.2.2 Methods

5.2.2.1 Acid tolerance

The acid tolerance of the LAB culture was determined as described in section 3.2.2.3.1

5.2.2.2 Bile salt tolerance

The bile tolerance of the LAB culture was determined as described in section 3.2.2.3.2

5.2.2.3 Presence of β- galactosidase activity

β- galactosidase activity of the LAB culture was determined by the procedure given in section 3.2.2.3.3

5.2.2.4 Cholesterol lowering

Cholesterol lowering test was carried out according to the method described in the section 3.2.2.3.4

5.2.2.5 Antimicrobial activity

Antimicrobial activity of the LAB culture was determined as described in section 3.2.2.3.6

5.2.2.6 Cell line

Cell line for the adherence properties was maintained as described in the section 3.2.2.3.8

5.2.2.7 Adhesion assay

Adhesion assay were carried out as described in the section 3.2.2.3.9

5.3 Results

5.3.1 Acid tolerance

Survival of 13 LAB cultures at pH 2 and pH 2.5 is studied (Figure 5.1). *L. plantarum* B-4496 and *L. salivarius* CFR-2158 showed more than 70% survival at pH 2 and 82% and 90% at pH 2.5 respectively for 4h of incubation. *L. acidophilus* B-4495, *L. casei* (lund), *L. lactis* B-634 and *Bacillus coagulans* NCIM 2323 showed more than 80% survival at pH 2.5. *L. casei* (lund) and *Bacillus coagulans* NCIM-2323 showed 50-60% survival at pH 2 whereas *L. casei* B-1922, *L. bulgaricus* CFR-2028 & *L. casei* DSM-2158, only 60% of the initial cell population survived at pH 2.5. In case of other LAB like *L. amylovorus* B-4437, *L. acidophillus* B-4495, *L. casei* B-1922, *L. lactis* B-634, *L. helveticus* B-4526, *L. casei* NCIM 2586, *L. bulgaricus* CFR-2028, *L. casei* DSM- 20011 the percentage of survival at pH 2 was around 40%.

5.3.2 Bile tolerance

Tolerance of the 13 LAB cultures to bile salt (0.3%) is tested (Figure 5.2). *L. plantarum* B-4496 and *L. salivarius* CFR-2158 showed more than 85% survival at 37^{o}C for 24h incubation. *L. casei* NCIM-2528 & DSM-20011 was found to be tolerant upto 70%, however *L. amylovorus* B-4437, *L. acidophillus* B-4495, *L. casei* (Lunda), *L. helveticus* B-4526, *L. bulgaricus* CFR-2028, *B. coagulans* NCIM-2323 were found to be tolerant upto 50%-60%. *L. lactis* B-634 was found to be tolerant upto only 45% at 0.3% bile for 24h incubation at 37^{o}C.

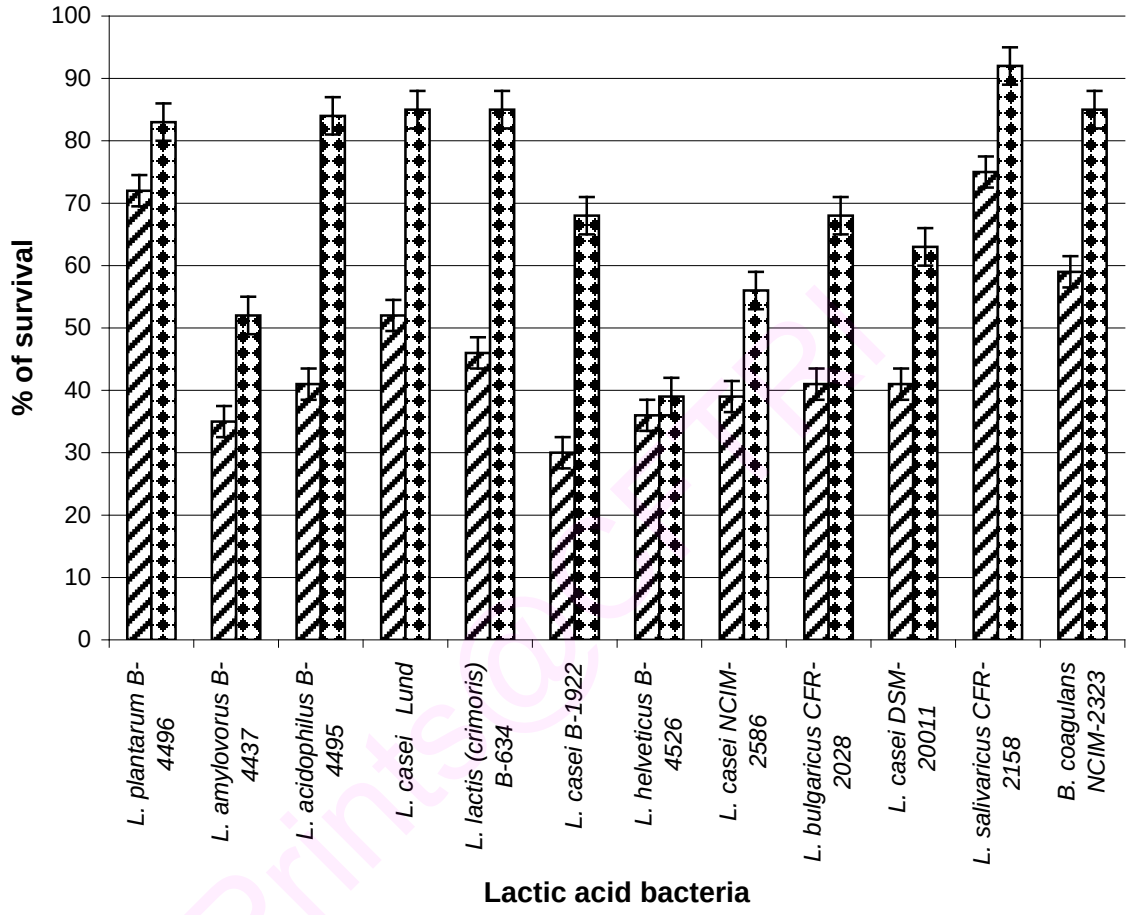


Figure 5.1: Survival of lactic acid bacteria at pH 2 and pH 2.5 after 4h incubation in MRS broth at 37^{o}C, stripped bar represents as pH 2, dotted bar represents pH 2.5. (mean values ± SD of three experiments)

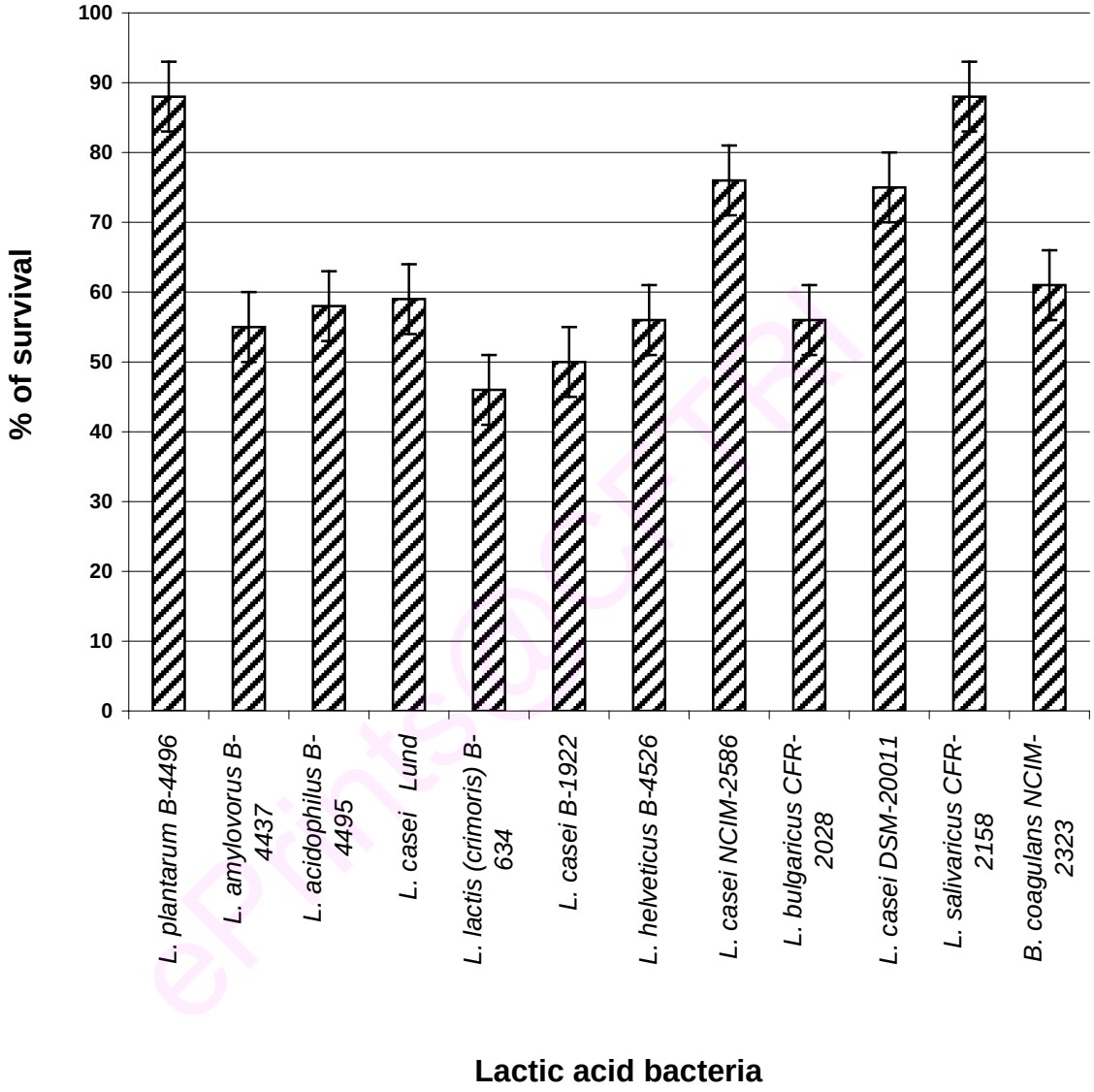


Figure 5.2: Survival of Lactic Acid Bacteria at 0.3% bile salt after 24h incubation in MRS broth at 37^{o}C. (mean values ± SD of three experiments)

5.3.3 Cholesterol assimilation test

Reduction of cholesterol level in the spent broth was observed with all the 13 lactic acid bacteria both in the presence and absence of 0.3% bile as shown in Table 5.2 for 24 h incubation. Among 13 LAB cultures, *L. acidophilus* B-4495, *L. helveticus* B-4526, *L. casei* NCIM-2586 and *L. salivarius* CFR-2158 showed reduction in cholesterol from 103.49 μg/ml to 15.93 μg/ml (84.6%), 14.57 μg/ml (85.59%), 9.83 μg/ml (90.50%) and 19.32 μg/ml (81.33%) in presence of 0.3% of bile salt in MRS broth, respectively. The reduction in cholesterol in MRS broth without bile salt is from 103.49 μg/ml to 50.84 μg/ml (50.87%), 38.64 μg/ml (62.66%), 42.71 μg/ml (58.73%) and 40.67 μg/ml (60.7%), respectively.

Table 5.2: Change in cholesterol level after *in vitro* incubation with selected LAB*

LAB Strain No.	Bile salt concentration (μg/ml) [reduction rate of cholesterol (%)]	
	0%	0.3%
Control	103.49 (0)	103.49 (0)
L .plantarum B-4496	79.32 (23.35)	45.08 (56.44)
L. amylovorus B-4437	62.71 (39.40)	31.86 (69.21)
L. acidophilus B-4495	50.84 (50.87)	15.93 (84.6)
L. casei Lund	65.08 (37.11)	38.98 (62.33)
L. lactis (crimoris) B-634	37.28 (63.97)	30.84 (70.20)
L. casei B-1922	40.67 (60.70)	22.71 (78.05)
L. helveticus B-4526	38.64 (62.66)	14.57 (85.59)
L. casei NCIM-2586	42.71 (58.73)	9.83 (90.50)
L. bulgaricus CFR-2028	59.32 (42.68)	53.22 (48.57)
L. casei DSM-20011	41.35 (60.04)	33.22 (67.9)
L. salivarius CFR-2158	40.67 (60.7)	19.32 (81.33)
B. coagulans NCIM-2323	37.96 (63.32)	30.50 (70.52)

* Amounts of cholesterol was measured after 24 hrs incubation at 37^{o}C, MRS broth containing cholesterol with (0.3%) or without bile salt.

5.3.4 β- galactosidase activity

The 13 Lab cultures were tested for the β-galactosidase activity by plate screening method (Karasová et al., 2002). All the LAB cultures except *L. acidophilus* B-4495 and *L. casei* NCIM-2586 were tested positive for the β- galactosidase activity indicated by the characteristic blue colour colony on the MRS agar plate containing x-gal as a substract and IPTG as a inducer(Table 5.3).The results indicate that *L. acidophilus* B-4495 and *L. casei* NCIM-2586 lack β-galactosidase.

5.3.5 Adhesion assay

The LAB cultures were analysed for the adherence ability to HT-29 intestinal epithelial cell line (Table 5.3). *L. plantarum* B-4496, *L. amylovorus* B-4437, *L. casei* DSM-20011, *L. salivarius* CFR-2158 and *L. helveticus* B-4526 were strongly adhesive. Whereas *L. lactis* (crimoris) B-634, *B. coagulans* NCIM-2323, *L. bulgaricus* CFR-2028, *L. casei* NCIM-2586 and *L. lactis* (crimoris) B-634 showed moderate adhesion to the HT-29 cell line. However, *L. acidophilus* B-4495, *L. casei* (Lund) and *L. casei* B-1922 showed weak adhesion to the above intestinal cell line.

5.3.6 Antibacterial activity

The antibacterial property of LAB cultures was tested against well-known food borne pathogenic organisms like *Bacillus cereus*, *Listeria monocytogenes, Escherichia coli, Staphylococcus aureus,* Enterotoxigenic *E. coli and Yersinia enterocolitica*. All the LAB cultures showed antibacterial activity against all the above tested pathogens (Table 5.4) which is indicated by zone of inhibition (1-10 mm). Expect *L. casei* DSM-20011, other LAB cultures showed good antibacterial activity against Enterotoxigenic *E. coli* which is the main culprit in causing traveller's diarrhea. *B. coagulans* NCIM-2323, *L. bulgaricus* CFR-2028, *L. acidophilus* B-4495, *L. salivarius* CFR-2158 and *L. amylovorus* B-4437 showed inhibition zone from 10 to 20 mm (dia). This indicates that all the LAB cultures have antimicrobial property which is the main criteria for the evaluation of probiotics.

Table 5.3: Adherence and β–galactosidase activity of LAB cultures

LAB strains	β–galactosidase activity (blue color colony)	Adherence index* to the HT-29 cell line
L. plantarum B-4496	+	175±20
L. amylovorus B-4437	+	167±15
L. acidophilus B-4495	-	25±17
L. casei Lund	+	20±10
L. lactis (crimoris) B-634	+	60±23
L. casei B-1922	+	30±14
L. helveticus B-4526	+	125±30
L. casei NCIM-2586	-	65±20
L. bulgaricus CFR-2028	+	75±24
L. casei DSM-20011	+	190±26
L. salivarius CFR-2158	+	220±35
B. coagulans NCIM-2323	+	80±12

*Each adhesion assay was conducted in duplicate with cells from three successive passages.

Table 5.4 :Antibacterial activity of LAB cultures on selective food born pathogens by agar spot method

Pathogens*	E.coli	SEA	YER	LM	BCE	ETEC
L. plantarum B-4496	++	++	+	+	++	+++
L. amylovorus B-4437	++	++	++	+++	++	++
L. acidophilus B-4495	++	+	++	+++	++	+++
L. casei Lund	++	++	++	++	++	++
L. lactis (crimoris) B-634	+	+	++	++	++	++
L. casei B-1922	+	++	++	++	+	++
L. helveticus B-4526	+	++	+	++	++	+++
L. casei NCIM-2586	++	+	++	+	+	+++
L. bulgaricus CFR-2028	+++	++	+++	++	++	+++
L. casei DSM-20011	++	+	++	++	++	+
L. salivarius CFR-2158	+++	++	+++	++	++	+++
B. coagulans NCIM-2323	+++	+++	++	+++	+	+++

Zone of Inhibition in mm: +, between 1-10mm; ++, between 11-20mm; +++, > 20mm. the load of the point inoculation was 10^9 CFU/ml

* E.coli : *Escherichia coli* MTCC 108; *BCE=Bacillus cereus* F- 4810*;* LM= *Listeria monocytogenes* Soctt A; YER=*Yersinia enterocolitica* MTCC- 859;
SEA= *Staphylococcus aureus* F-722;ETEC= *Escherichia coli* ATCC-31075

5.4. Discussion

Most of the lactic acid bacteria encountered in dairy products are neutralophiles, but their intracellular pH (pHi) is not as tightly regulated as that of *E. coli* (Kashket, 1987). When exposed to environments like mild acidification, lactic acid bacteria tend to protect themselves through the induction of wide range of protective measures that alter cell membrane composition, extrude protons, protect macromolecules etc. One of major mechanisms involves a heavy reliance on proton pumps, especially the F_1F_0-ATPase. In the present study, *L. plantarum* B-4496 and *L. salivarius* CFR-2158 showed more than 70% survival at pH 2 and 82% and 90% at pH 2.5 respectively for 4h of incubation. The organisms may be exhibiting any of the above mentioned mechanisms which also helps to keep the intracellular pH stable for a sufficient length of time to permit the induction of acid tolerance response (ATR). Acid tolerance response (ATR) is related to the increased ability of cells to resist changes on being exposed to unfavorable condition (Eili´S O' Sullivan and Se´amus condon, 1997). Thus, it is noticed that *L. plantarum* B-4496 and *L. salivarius* CFR-2158 which has more than 70% tolerance to low pH environment (pH 2), exhibits ATR to survive in the harsh environment.

Lactobacilli are detected in large numbers in all regions of the murine gastrointestinal tract of human and other animals (Tannock, 1992) where they are exposed to bile salts which are released periodically into the intestine (Stevens and Hume, 1995). These salts are known to be toxic to bacteria (Gilliland and Spech, 1977; Tannock *et. al.*, 1989; De Smet *et. al.,* 1995). Certain species of *Lactobacillus* have the capacity to express bile salt hydrolyses activity (BSHs) (Lee and Salminen, 1995; Jacobsen *et. al.,* 1999). These BSHs hydrolyse the conjugated bile salt and protect the LAB from toxicity. This further helps in their survival in presence of intestinal bile (0.3%). The results in this study indicates that *L. plantarum* B-4496 and *L. salivarius* CFR-2158 have more BSHs activity to hydrolysis the bile salt and are tolerant to 0.3% bile salt concentration to survive upto 85%. Two major hypotheses have been proposed to explain the capacity of the gastrointestinal bacteria to express BSHs which contributes to their functional ability in the gastrointestinal tract (Huijghebaert *et. al.,*1982; Tannock

et. al.,1989; Savage, 1992; De Smet *et. al.,* 1995). One hypothesis states that some species of lactic acid bacteria are able to deconjugate bile salts to enable them to utilize the aminoacid like taurine as an electron acceptor. This hypothesis may be supported by certain *Clostridium* species (Huijghebaert *et. al.*,1982; Van Eldere, 1988). Second hypothesis states that BSHs decreases the toxicity of conjugated bile acids for bacteria (Savage, 1992). It has also been suggested that BSHs are detergent shock proteins that protect the bacteria from the toxicity of bile acids in the gastrointestinal tract (Adamowicz, 1991; Tannock, 1992**).**

For all the LAB strains tested, results clearly indicate that the reduction of cholesterol was more in the presence of bile than without bile in MRS broth. This is in agreement with the work of Tahri *et. al.*, (1997), who reported higher cholesterol assimilation with increased oxgall concentrations in the growth medium. This could partly be explained by the co-precipitation of cholesterol with deconjugated bile acids, which is observed at pH values below 5.5 (Klaver and Van Der Meer, 1993). Since the study was performed without pH control, it is probable that part of the cholesterol present in the growth medium precipitated when the pH had dropped below 5.5, due to bacterial fermentation and short chain fatty acid formation. However this phenomenon would be relevant for only bile tolerant strains, that are able to deconjugate bile acid.

Several *in vitro* studies proposed a number of mechanisms for the purported cholesterol lowering action of probiotic bacteria (Gilliland *et. al.,* 1985; Tahri *et. al.*, 1996; Tahri *et. al.*, 1997; Noh *et. al.,* 1997; Usman, 1999). This includes physiological action of short chain fatty acids especially propionate, cholesterol assimilation by the bacteria, cholesterol binding to the bacterial cell wall, and enzymatic deconjugation of bile acids. It is noticed that the uptake of cholesterol occurred only when the culture was growing anaerobically in the presence of bile.

Assimilation of cholesterol in the small intestine may be important in reducing the absorption of dietary cholesterol from the digestive system into the blood (Gilliland *et.*

al., 1985; Walker and Gilliland, 1993). Some of the natural microorganisms inhabitating the human intestine are beneficial in lowering serum cholesterol (Mann and Spoerry, 1974; Fernandes *et. al.*, 1987; Fukushima *et. al.*, 1999). The LAB like *Lactobacillus* and *Bifidobacteria* sp. in particular, have the ability to metabolize cholesterol (De Smet *et. al.,* 1995). Assimilation of cholesterol by lactic acid bacteria is important since hypercholesterolemia is associated with cardiovascular diseases, one of the most important causes of death in the western countries (Lee *et. al.,* 1992). Decreasing serum cholesterol is, therefore very important to prevent cardiovascular disease. Gilliland *et. al.,* (1985) reported that *L. acidophilus* reduces blood cholesterol by direct breakdown of cholesterol and deconjugation of bile salt. In particular, cholesterol metabolism is closely linked to the formation of bile salts, which may be transformed by enzyme activities of some intestinal bacteria like LAB during the enterohepatic circulation. BSHs deconjugative bile acid which results the formation of free bile salt. It was hypothesized that deconjugation of bile salts may contribute to lower cholesterol levels as free bile salts may be excreted more likely from the Gastrointestinal track (GIT) than conjugated bile salts (Fukushima *et. al.,* 1999). However, the hypothesis is disputable and incompatible based on current findings with regard to the passive absorption kinetics of free bile salts in the GIT. Fecal loss of bile salts may indeed result in an increased requirement for cholesterol for maintaining serum cholesterol levels. Klaver and van der Meer, (1993) suggested that *in vitro* cholesterol reduction by some *Lactobacillus* spp. results from its coprecipitation with deconjugated bile salts. De Smet *et. al.,* (1994) also suggested that highly BSHs-active *Lactobacillus* spp. may reduce serum cholesterol levels considerably.

β-galactosidase is widely used, especially in dairy technologies. This enzyme provides two benefits that make its use attractive for dairy industry: preparation of lactose-free milk and biosynthesis of galactooligosaccharides that are interesting from the technological as well as health point of view. The valid technological reasons for partial removal of lactose from milk include higher solubility, suppression of lactose crystallisation in sweet condensed milk and ice creams, increase of sweetness, decrease in

the hygroscopicity of dried dairy products etc. Lactose in yoghurt with live lactic acid bacteria is better tolerated than lactose in other dairy foods, partly because of the of microbial β-galactosidase activity, which digests lactose *in vivo* (Martini *et. al.,* 1991). All the LAB strains in the study except *L. acidophilus* B-4495and *L. casei* NCIM-2586 show β-galactosidase activity which facilitates their use in the dairy technology.

LAB probiotics are reported to reduce lactose intolerance symptoms by accelerating the digestion of lactose (Sanders and In't Veld, 1999). β-galactosidase enzyme plays a major role in the digestion of the milk sugar, lactose which can cause severe intestinal distress, characterized by bloating, flatulence and abdominal pain in subjects with low levels of β-galactosidase. This condition increases in severity with age and restricts the use of dairy products in humans. β-galactosidase shows both hydrolysation and transglycosylation activity, i.e. they are able to synthesize oligosaccharides during hydrolysis of lactose. This in turn is beneficial since in addition to lactose being digested an additional nutrient in the form of galactooligosaccharides is also produced. Therefore, testing for the production of this enzyme by LAB is essential to evaluate them as probiotics.

LAB can inhibit the growth of the pathogens by the action of the acid, hydrogen peroxide and antimicrobial peptides like bacterocin. Based on structural, physicochemical and molecular properties, bacteriocin from LAB can be subdivided into three major classes (Klaenhammer, 1993; Nes *et. al.,*1996) - class I, II &III bacteriocins. Bacteriocins form pores in the cell membrane, thereby depleting the transmembrane potential and/ or the pH gradient, resulting in the leakage of indispensable intracellular molecules (Cleveland *et. al.,* 2001). All the LAB cultures showed antibacterial activity against food borne pathogenic organisms like *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus,* Enterotoxigenic *E. coli and Yersinia enterocolitica* (Table 5.4). The LAB cultures except *L. casei* DSM-20011, showed good antibacterial activity against Enterotoxigenic *E. coli* which is the main culprit in causing traveller's diarrhea.

As per the set of criteria put forth by Collins *et. al.,* (1998) for the selection of potential probiotic strains, one of the important criteria for a potentially probiotic strain is its ability to adhere to mucosal surfaces of the human gastro-intestinal tract. It is however, difficult to devise an *in vivo* assay to screen a large number of potential probiotic strains for their adherence characteristics. *In vitro* cellular models involving three human intestinal epithelial cell lines, namely HT-29, HT29-MTX and Caco-2, have been extensively used to assess the adhesive properties of probiotic strains (Blum and Reniero, 2000.) The advantage of these cellular models is that they express morphological and functional differentiation *in vitro* and show characteristics of mature enterocytes, including polarisation, a functional brush border and apical intestinal hydrolases. *L. plantarum* B-4496, *L. amylovorus* B-4437, *L. casei* DSM-20011, *L. salivarius* CFR-2158 and *L. helveticus* B-4526 were strongly adhesive to HT-29 intestinal epithelial cell line (Table 5.3) in the present study.

Bacterial adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesions (usually proteins) and complementary receptors (Beachey,1981).The mucus covering the epithelial cells that, the ingested micro-organisms confront in the human gut is considered an important site for bacterial adhesion and colonisation (Mikelsaar *et. al.*,1998). Mucus is continually subjected to degradation, conversely new mucin glycoproteins (the major components of mucus) are constantly secreted. Thus, bacteria that adhere to mucus but are unable to reach the epithelial cells might be dislodged from the mucosal surface with the degraded mucin and are washed away with the luminal contents. This may partly explain the transient pattern of colonisation characteristic of most probiotic bacteria. On the basis of these criteria, an *in vitro* evaluation of the bacterial adhesion to human intestinal mucus provides a good additional model for studying the ability of probiotics to adhere to intestinal surfaces.

5.5. Conclusions

In conclusion, criteria for *in vitro* selection of probiotic bacteria that may reflect certain *in vivo* effects such as modulation of gastrointestinal tract microflora, inhibition of pathogenic bacteria, and enhancement of the immune system on the host has been detailed. Considering all the probiotic properties, among the 13 LAB investigated in this study, *L. plantarum* B-4496 and *L. salivarius* CFR-2158 were selected as promising candidates, to be considered as a probiotic supplement. It has been clearly showed in the present study that the adoption of logical criteria for the *in vitro* selection of probiotic bacteria can result in the isolation of strains capable of performing effectively in the gastrointestinal tract. However, given that the human gastrointestinal tract is a complex and hostile environment, it is unlikely that a single probiotic bacterial strain will be capable of influencing the microbial ecology of the host. Hence, it is more a viable approach if these probiotic strains are not ingested as individual entities but rather as the active ingredients of the food products that are ultimately intended for human consumption.

Chapter –6

Spray drying of probiotic Lactic acid bacteria

6.1. Introduction:

Probiotic lactic acid bacteria (LAB) are of strategic importance to the dairy and nutraceutical industries because of their role in human and animal health and welfare. It also plays a key role in the production of lactic fermented foods, which represent about 20% of the total economic value of fermented foods produced throughout the world (McKay and Baldwin, 1990). There has been a considerable interest in incorporating these probiotics to functional and pharmaceutical products. It has been recommended that food containing probiotic LAB (probiotic functional foods) should contain at least 10^7 live microorganism per gram or per ml (Ishibashi and Shimamura, 1993) at the time of consumption, in order to benefit the consumer. Therefore, in addition to the requirements for good safety and functional characteristics, probiotic cultures should also be able to withstand food processing and storage conditions encountered during the manufacture of functional foods under industrial sectors (Knorr, 1998). There are reports that various health products and pharmaceutical preparations containing dried cells of LAB are used in the treatment of gastrointestinal disturbances (Gomes and Malcata 1999). From a commercial point of view, an inexpensive method for the large scale production of shelf stable product containing high levels of viable cells becomes a necessity.

Spray drying, Freeze drying, and freezing are the most common down stream process used for the preparation of dried stable probiotic cultures. The importance of starters in dairy industry is apparent from the industrial point of view. But, the preparation of starters is time consuming (Tamine, 1981). A disadvantage in using stock cultures is the need for production of large volumes of cultures. The above downstream processes acquire importance in this context. The preparation of concentrated starter cultures via spray drying for inoculating the product directly has eliminated much of the time, drudgery, and danger customarily involved in preparation and maintenance of starter culture in the dairy plant (Gilliland, 1976). The spray-drying of microorganisms began in 1914 with the drying of milk cultures of LAB (Rogers, 1914). Spray drying of large quantities of bacterial cultures as a method to replace the usual liquid bulk starter in the production of fermented foods.

products has been extensively investigated (Fu and Etzel, 1995; Kim and Bhowmilk, 1990; Metwally *et. al.*, 1989; Gilliland, 1976). Despite its low cost in comparison to other technical solutions (e.g. freeze drying), spray drying has not been developed commercially, mainly because of low survival rates during drying, and difficulties in rehydrating the product (Porubean and Sellars, 1975). However, spray drying is still one of the predominant processing tools used in the dairy industry and is used to produce large amounts of dairy ingredients relatively inexpensively, it has been estimated that the cost of spray drying is six times lower per kilogram of water removed than the cost of freeze drying (Knorr, 1998). Spray dried powders can be transported at a low cost and can be stored in a stable form for prolonged periods. However, there are obvious challenges in spray drying of viable cultures, including the requirement that the microorganisms should survive the relatively high temperatures (Daemen and Van der stege, 1982). In addition to maintaining the viability of probiotic cultures, retention of probiotic properties is highly relevant. A number of studies concerning the survival of various lactic cultures affected by spray drying have been reported by various investigators.

The present chapter deals with the study of spray drying as a method of producing concentrated probiotic lactic acid bacteria as an ingredient for the nutraceutical and pharmaceutical industry. Food grades Maltodextrin (MDX) and Nonfat skimmed milk (NFSM) were used as carriers for spray drying of probiotic LAB. The effect of spray drying on cell survival, with an emphasis on retention of probiotic properties like pH & bile tolerance, cholesterol assimilation has been detailed. Survival of probiotic LAB during storage has also been investigated.

6.2. Materials and Methods:

6.2.1. LAB Strains:

Two strains, out of 17 lactic acid bacteria isolated during the preparation of *Kanjika,* an ayruvedic lactic acid fermented product, exhibiting potent probiotic properties were identified by partial sequencing of 16S rDNA gene (Ribotyping) as *Lactobacillus plantarum CFR 2191 & Pediococcus acidilactici CFR 2193 and one Lactobacillus salivarius CFR-2158* was selected from departmental culture collection were chosen to study the effect of spray drying. All LAB strains were preserved under at -80 °C with 20% glycerol in 2 mL cryovials.

6.2.2 Preparation of feed solutions for spray drying application:

Two types of feed solution were prepared. In the first type, overnight cultures of each probiotic LAB were inoculated into MRS broth (1% v/v) and incubated at 37^0C until the stationary growth phase was reached. After centrifugation at 10,000 rpm for 15min at 4^0C, the cells were resuspended at different concentrations of 1, 3 and 5% (w/v) on wet weight basis in nonsterile 10% Non fat skimmed milk solution. This type of feed solution was termed “Non fat skimmed milk (NFSM)”. In the second type, overnight cultures of each probiotic lactic acid bacteria strain were added at different concentrations of 1, 3 and 5% (w/v) on wet basis biomass in 10% Maltodextrin. This type of feed solution was termed as “Maltodextrin” (MDX). These feed solutions with lactic acid bacteria were directly spray dried.

6.2.3 Spray drying:

A bench top scale dryer (JISL, Bombay, India) was used. The inlet air, heated to 140 ± 2°C by an electrical heater, flowed concurrently with the spray into a 12.5L drying chamber with an outlet temperature of 40± 2°C. Feed solution was delivered by a peristaltic pump into a two fluid stainless steel atomizer. The spray dried powder was collected at the bottom of a cyclone. A constant inlet and outlet air temperature of 140°C and 40°C respectively was used throughout the study. The moisture content of the resultant spray dried powder was between 5-7%. The residual viability of spray-dried cultures was determined and dehydrated samples were stored at 30 ± 2°C and 4 ± 2°C for 60 days.

6.2.4 Heat shock and growth characteristics of probiotic lactic acid bacteria:

Heat shock treatment was carried out as detailed by Prasad *et. al.,* (2003). The growth of probiotic Lactic acid bacteria was monitored by absorbance at 600 nm. Growth was initiated at 37°C, and when the OD_{600} reached 0.4 to 0.5 corresponding to the early log phase, the cultures were transferred to water bath maintained at 40, 45, 50, and 55^0C and incubated for 30 min at respective temperatures. The time taken to attain the required shock temperature was less than 3 min. At the end of 10 min, the cultures were restored back to a water bath maintained at 37°C. Growth was further monitored until the cells reached the stationary phase.

6.2.5 Cell survival:

Residual viability of spray dried samples was determined by the standard plate count method. The spray dried powder (1g) was rehydrated with 10ml of sterile distilled water to about the same solids content as the feed solution. The rehydrated samples were kept on a shaker for 30 min to allow complete dissolution. Suitable dilutions of feed solution and rehydrated samples were prepared by serial dilution, 100 µl was plated in quadruplicate using the spread plate method. CFU were determined after incubation for 48 h at 37^0C. The survival rate of the spray dried sample was calculated as

% survival= 100 X Nr/Nf, ---------- (1)

where Nr was Log CFU ml^{-1} of rehydrated sample and

Nf was the Log CFU ml^{-1} of feed solution

6.2.6 Acid tolerance

The acid tolerance of the LAB culture was determined as described in section 3.2.2.3.1

6.2.7 Bile salt tolerance

The bile tolerance of the LAB culture was determined as described in section 3.2.2.3.2

6.2.8 Cholesterol lowering

Cholesterol lowering test was carried out according to the method described in the section 3.2.2.3.4

6.2.9 Scanning Electron Microscopy of spray dried powder:

Spray dried powder were attached to brass stubs and coated with gold by using a model scanning electron microscopy coating system (Polaron). Samples were then examined with a Leo electron microscopy (model Leo-435 VP, England) using an accelerating voltage of 20 kV. Micrographs were taken at different magnifications.

6.3. Results:

A number of factors are known to affect the retention of viability of the bacteria during spray drying and consequently the subsequent recovery of cells damaged during processing. In the present study, effect of the following factors like cell concentration, carriers for the spray drying, and pre-adaptation treatment of the probiotic LAB, on the viability and also the probiotic functional properties during spray drying have been studied.

6.3.1 Effect of Cell suspension on viability of the spray dried probiotic lactic acid bacteria.

Spray drying of *Pediococcus acidilactici CFR 2193, Lactobacillus plantarum CFR 2191& Lactobacillus salivarius CFR 2158* using either MDX or NFSM as the carriers has been carried out. The effect of cell concentration (1%, 3% and 5%) on the stability of the LAB during spray drying has also been investigated. The results are depicted in Fig 6.1. As can be seen from the figure, all the three probiotic LAB exhibited more than 97% survival, when 1% cell suspension was spray dried either with MDX or NFSM (10%). This is probably due to the fact that the ratio of the carriers to the concentration of cells being higher, there could be a protective effect on the cells (Fig 6.2 a). These results in less damage to the cells during spray drying (Fig 6.2 b). However when higher cell concentration with 10% MDX was used it was found, that the percentage of viability moderately decreased with the increase in cell concentration, in case of *Pediococcus acidilactici CFR 2193.* These are gram positive tetrococcus and do not have the lipid bilayer and hence are more susceptible to heat damage. Scanning electron micrographs (fig 6.2 a) clearly indicates the oozing out of the cytoplasm from the cells damaged during spray drying. In case of *Lactobacillus plantarum & Lactobacillus salivarius CFR-2158* there was no significant decrease in viability at all the three concentrations of cells used. This indicates that bacilli are less susceptible to heat damage during drying. When NFSM was used as the carriers, there was no significant difference in the percentage survival of all the three probiotic LAB studied

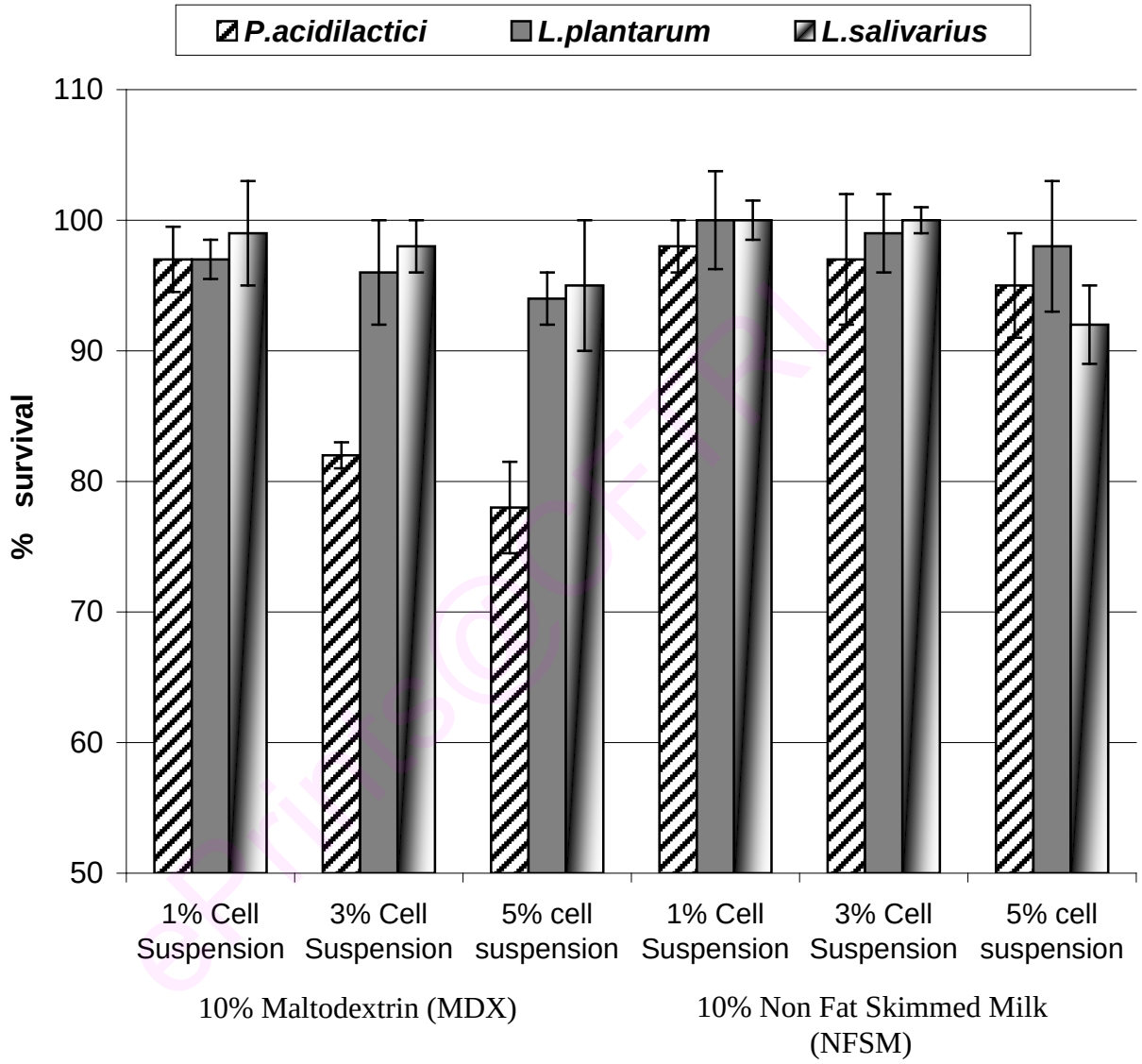
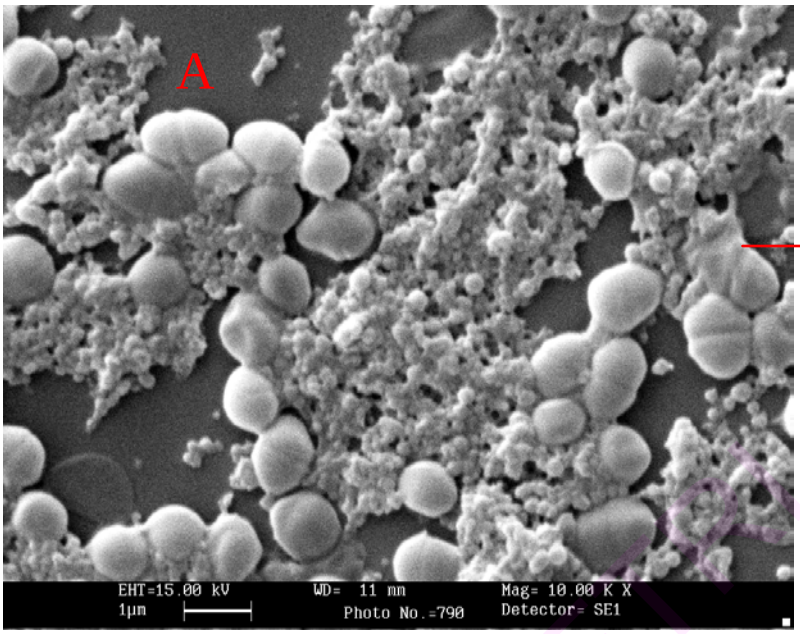
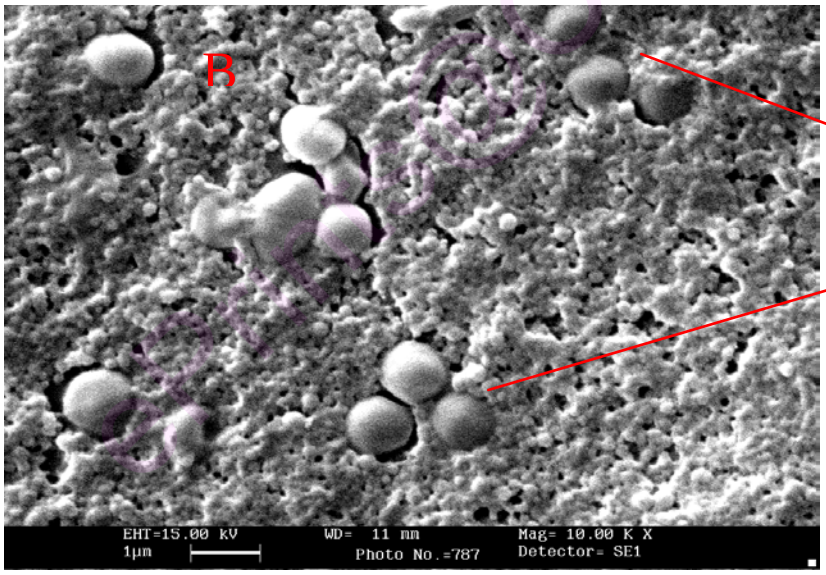


Figure 6. 1: Effect of cell suspension on survival of Lactobacillus spray dried with Maltodextrin and Non fat skimmed milk as carriers



Damaged cell with cytoplasm oozing out



Pediococcus cells covered with the additive

Figure 6.2 : Scanning electron microscope images of the spray dried *Pediococcus acidilactici CFR 2193*

a: Damaged cell with oozed out cytoplasm due to improper coating of the cells with carriers.

b: Cell protected with a coated layer of carrier.

Similarly, varying concentration of cells also did not have any pronounced effect on the percentage survival.

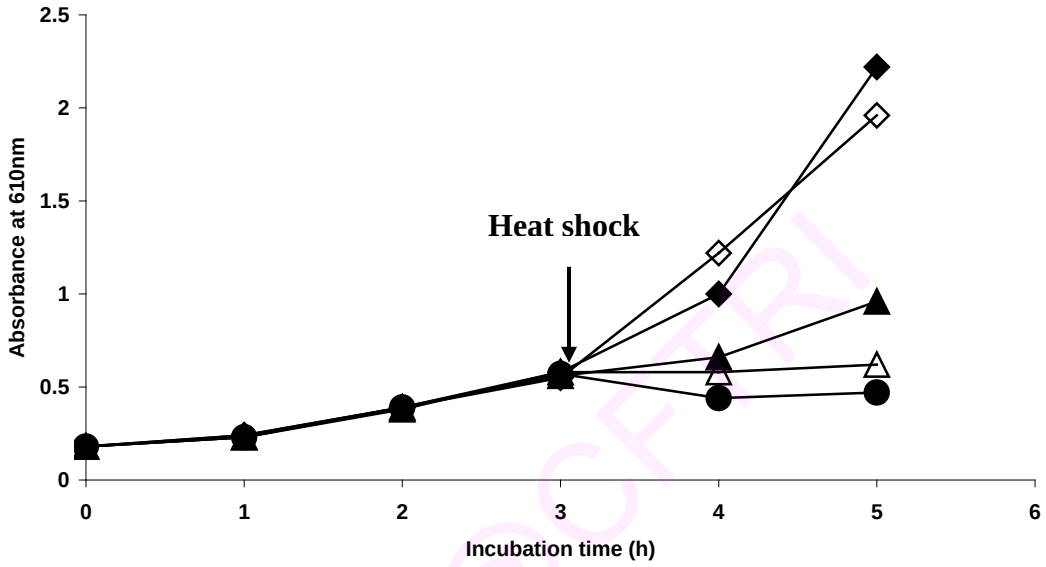
6.3.2 Effect of heat shock treatment on the growth of probiotic Lactic acid bacteria

The probiotic Lactic acid bacteria were grown in MRS broth upto 3 h and subjected to heat shock treatment at 37^{O}C, 40^{O}C, 45^{O}C, 50^{O}C &55^{O}C for 30 min during the early log phase (corresponding an OD of 0.4-0.5). The results are indicated in Figure 6.3 a, b& c for *L plantarum*, *L. salivarius* & *P. acidilactici,* respectively. As can be seen from the results there was no retardation in the growth rate where the cells were subjected to heat shock treatment at 37^{O}C & 40^{O}C. However 50% retardation at 45^{O}C, 80% at 50 ^{O}C and 100% retardation at 55 ^{O}C was observed in case of *P. acidilactici*. Similarly in case of *L. salivarius* and *L. plantarum* the retardation in growth due the heat shock treatment was of the same extent.

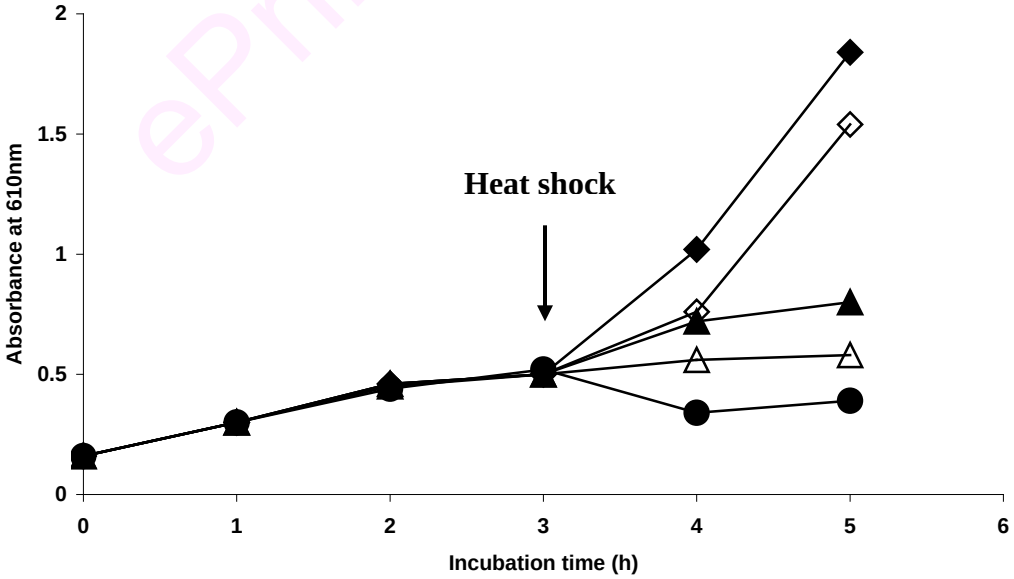
There are a few reports on induction of certain heat shock proteins in probiotic LAB by preadaptation of the cells to a set temperature for known period of time (30-60 min). There are also reports discussing the role of heat shock proteins in protecting the cells during spray drying and hence an attempt was made to induce the heat shock proteins by treating 1% cell suspension of all the three LAB probiotics with 10% MDX. The cell suspension with MDX were subjected to three different treatments like incubation at 37^{o}C/30min; 37^{o}C/60min; heat shock at 50^{o}C/30min & heat shock at 50^{o}C/30min+ incubation at 37^{o}C/30min prior to spray drying. However as can be seen from Fig 6.4, heat shock treatment did not have a significant effect on the percentage survival of the three probiotic LAB studied.

Figure 6.3: Effect of heat shock on the growth of Lactic acid bacteria. The cultures were grown in MRS broth at 37°C to an OD of 0.4 to 0.5 at 610 nm and subjected to heat shock for 30 min at 37°C (open diamond), 40°C (closed diamond) 45°C (closed triangle), 50°C (open triangle), or 55°C (closed circle).

a*: L. plantarum*



b*: L. salivarius*



c: ***Pediococcus acidilactici***

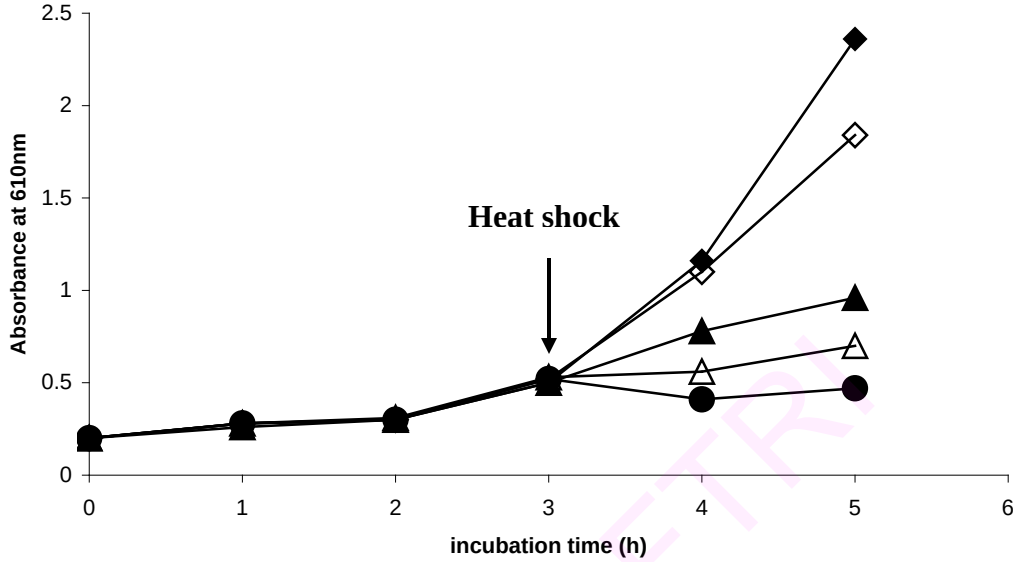
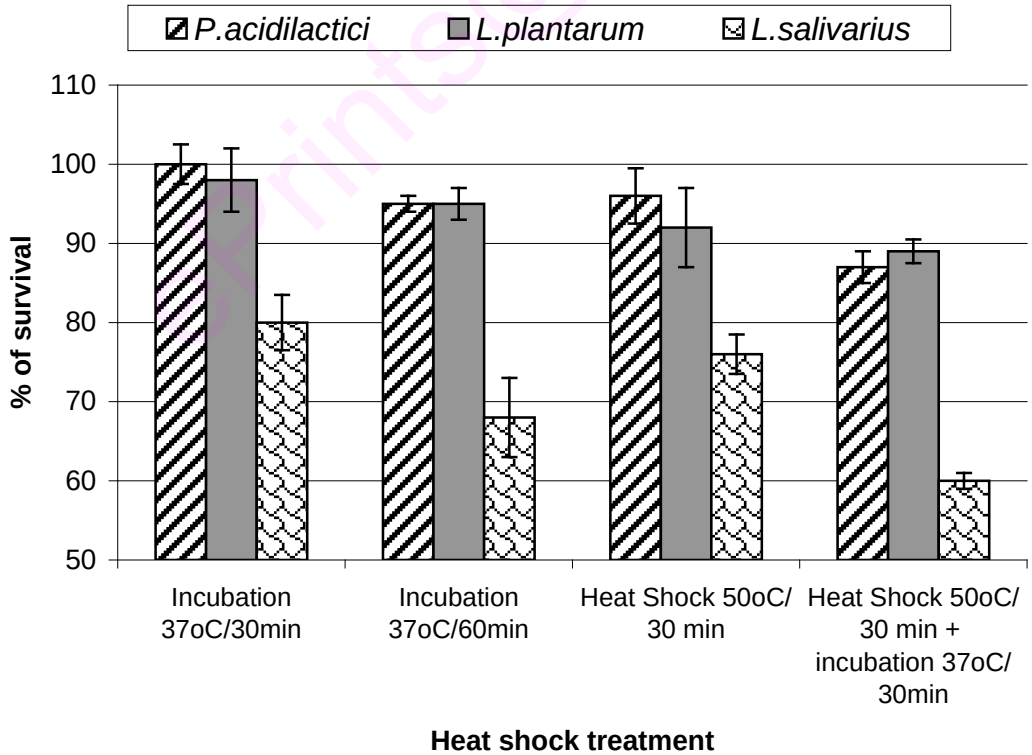


Figure 6.4 : Effect of heat shock treatment on the survival of the *Lactobacillus* species



6.3.3 Acid tolerance of spray dried probiotic Lactic acid bacteria:

Acid tolerance is one of the important probiotic properties and hence studies have been carried out to evaluate the retention of the acid tolerance after spray drying of lactic acid bacteria. *L. salivarius CFR-2158* was found to retain only 60% survival at pH 2.0 and hence spray drying of *L. salivarius* CFR 2158 was not carried out. Effect of different cell suspensions (1, 3 & 5% on wet weight basis) and of two carriers like MDX and NFSM have also been evaluated. Percentage survival of the lactobacillus species at pH 2.0 has been presented in Fig 6.5. As can be seen from the results, the active cells of *P. acidilactici* CFR 2193 exhibited a good tolerance of 85% at pH 2.0. There was a distinct decrease in the % survival of *P. acidilactici* CFR 2193 after spray drying with MDX. The decrease was more pronounced with the increase in percentage cell suspension. The % survival of spray dried *P. acidilactici* (1% cell suspension) was reduced to 70% corresponding to 17.6% loss in comparison with that of active cells (85%). The % survival decreased from 70% to 58% with an increase in percentage cell suspension from 1% to 5%.

Similarly there was a decrease in % survival of spray dried *L. salivarius CFR-2158* with an increase in percentage cell suspension. The percentage loss was more pronounced and corresponded to 21% with 1% cell suspension and was 12-15% with an increase in percentage cell concentration from 3-5%. *L. plantarum* CFR 2191 was found to retain acid tolerance to a significant level even after spray drying either with MDX or NFSM. The varying concentration of cell suspension did not affect the acid tolerant property. Based on these results it can be said that a stable spray dried preparation of *L. plantarum* CFR 2191 can be prepared using 1% cell suspension with MDX as the carrier.

Acid tolerance of the spray dried LAB species was also studied at pH 2.5 and results are presented in Fig 6.6. *P. acidilactici* CFR 2193 & *L. plantarum* CFR 2191 were found to retain the acid tolerant property (upto 95%) at all cell suspension (1-5%) tried. A considerable loss in viability was observed in case of *P. acidilactici* CFR 2193 , spray dried with NFSM at all the concentrations studied. Loss in viability (22%) was

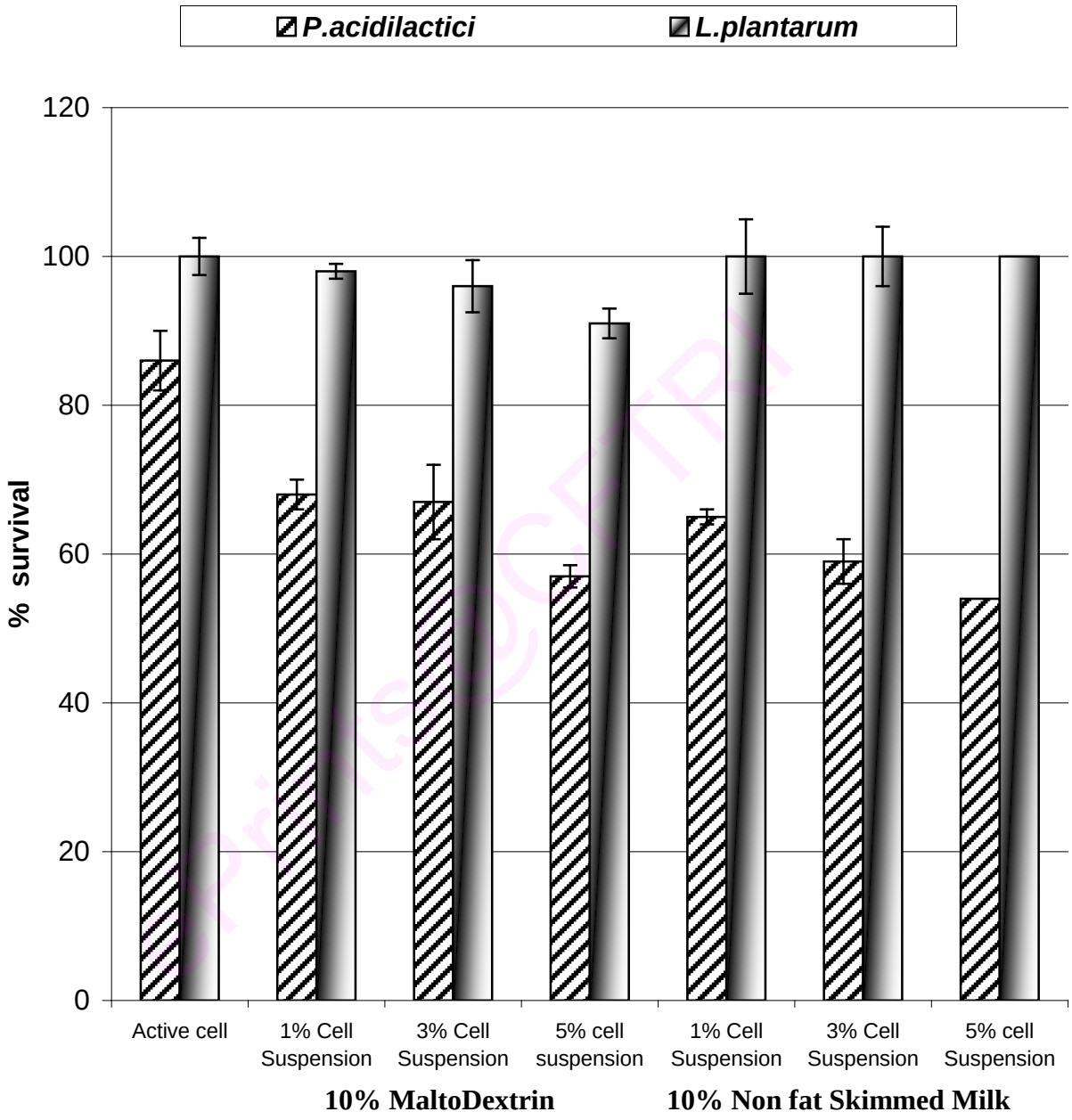


Figure 6.5 : Effect of cell suspension and carriers on acid tolerance of spray dried Lactobacillus species at pH 2: % survival in comparison with that of initial cell concentration of the feed solution

Figure 6.6: Effect of cell suspension on acid tolerance of Lactobacillus species at pH 2.5

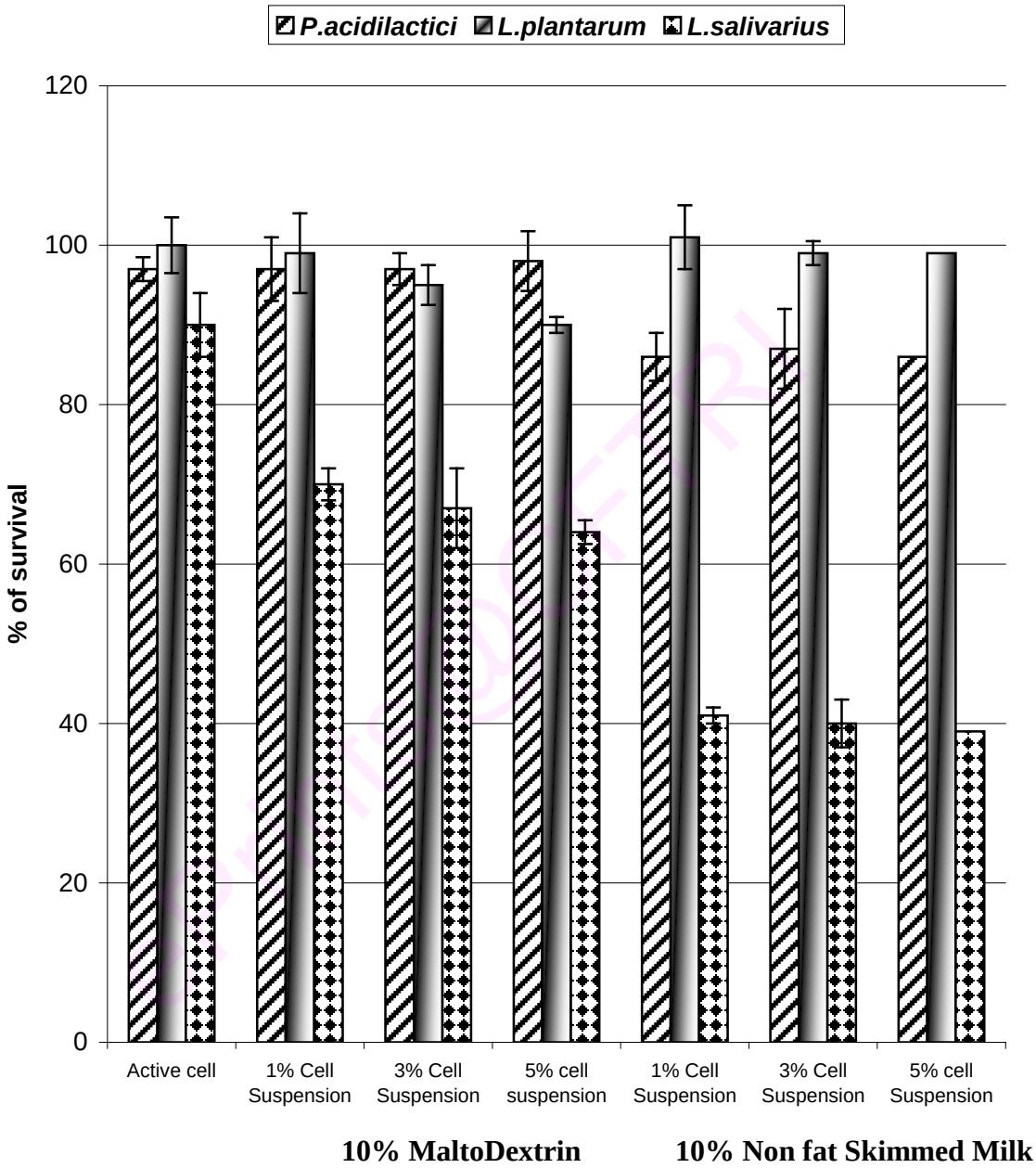
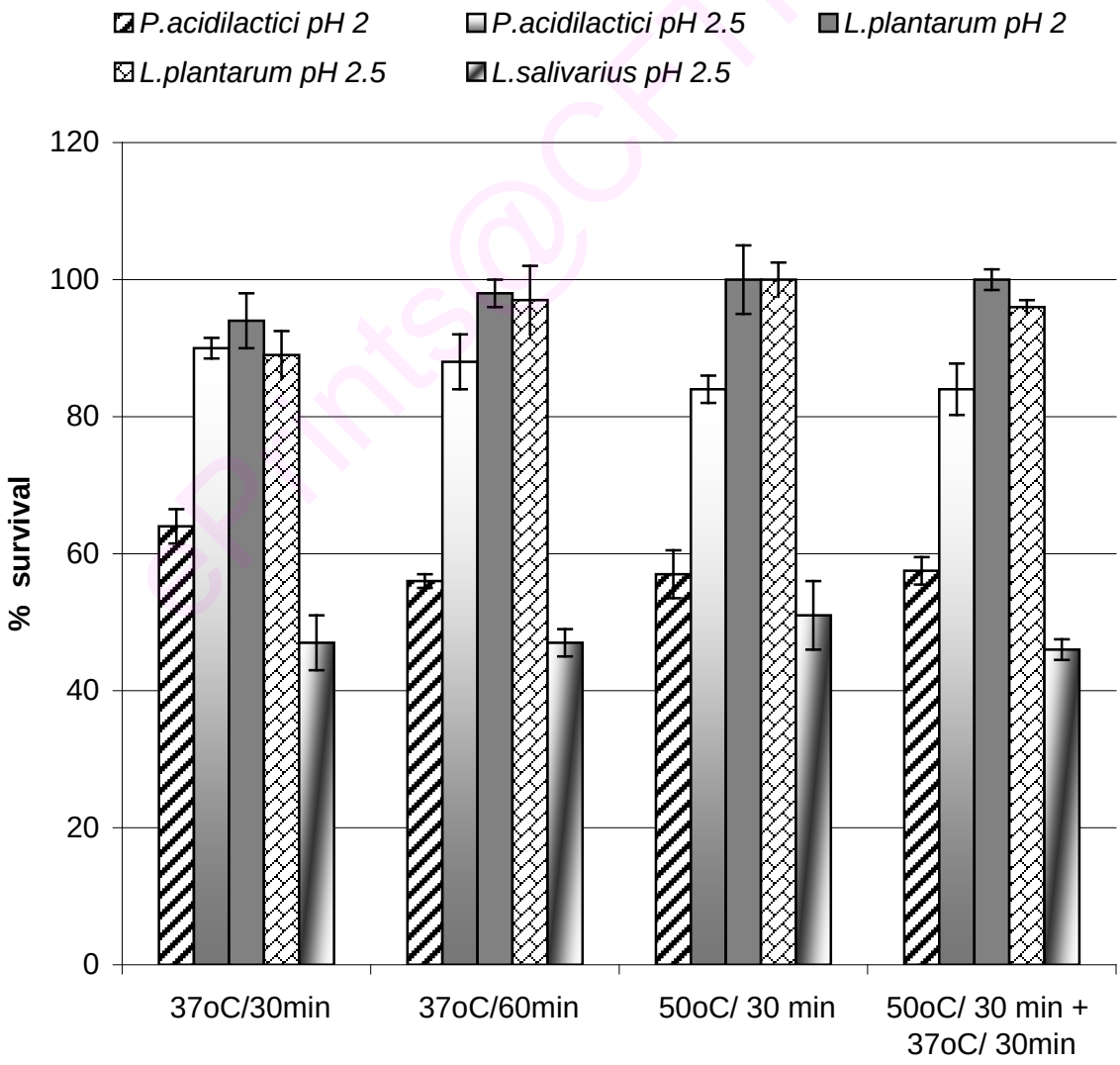


Figure 6.6: Effect of cell suspension and carriers on acid tolerance of spray dried Lactobacillus species at pH 2.5: % survival in comparison with that of initial cell concentration of the feed solution

observed in case of *L. salivarius CFR-2158* spray dried with MDX. The decrease was little more significant when NFSM was used as the carrier.. The loss in percentage survival was around 55% in comparison with that of active cells (90%). Efforts were made to subject the LAB species to heat shock treatment and preadaptation. However as can be seen from the results (Fig.6.7), the different combinations of incubation time and temperature did not result in any improvement in the retention of acid tolerance after spray drying.

Figure 6. 7: Effect of heat shock treatment on the retention of acid tolerance property.



6.3.4 Bile salt tolerance and cholesterol assimilation of spray dried probiotic lactic acid bacteria:

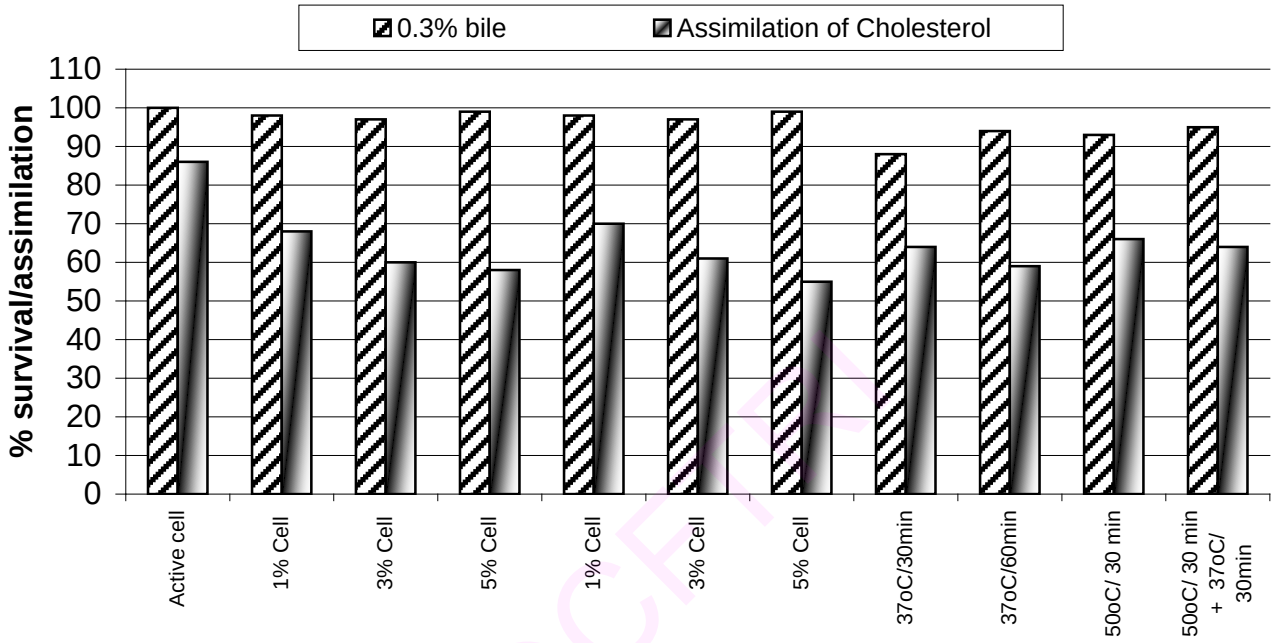
The effect of cell concentration, carrier and heat shock treatment on the retention of bile salt tolerance (0.3%) and cholesterol assimilation in the presence of bile, post spray drying has been studied. The results are depicted in Figure 6.8 a, b & c for L*. plantarum CFR 2191*, *L. salivarius CFR 2158* & *P. acidilactici CFR 2193,* respectively. As can be seen from the figure 6.8 there was no significant difference in bile tolerance property in all the combinations tested. In comparison with the active cells, there was an increase in cholesterol assimilation with an increase in cell concentration, when both MDX and NFSM was used as the carrier. With increase in cell concentration and considering the cholesterol assimilation of active cells as 100%, the loss in cholesterol assimilation varied from 21-32%, 0-8% & 6-7% for *L. plantarum CFR 2191*, *L. salivarius CFR 2158* & *P. acidilactici* CFR 2193 when MDX was used as the additive. Whereas, with the NFSM the loss in cholesterol assimilation varied from 18-35%, 31-71% & 12-22% for L*. plantarum CFR 2191*, *L. salivarius CFR 2158* & *P. acidilactici CFR 2193* respectively with increase in cell concentration from 1-5%. In case of cells subjected to heat shock treatment and then spray dried, there was a loss in cholesterol assimilation property from 25-30% for *L. plantarum CFR 2191*; 0-47% for *L. salivarius CFR 2158*; 31-64% for *P. acidilactici* CFR 2193 in comparison to active cells.

6.3.5. Survival of spray dried Probiotic cultures during storage

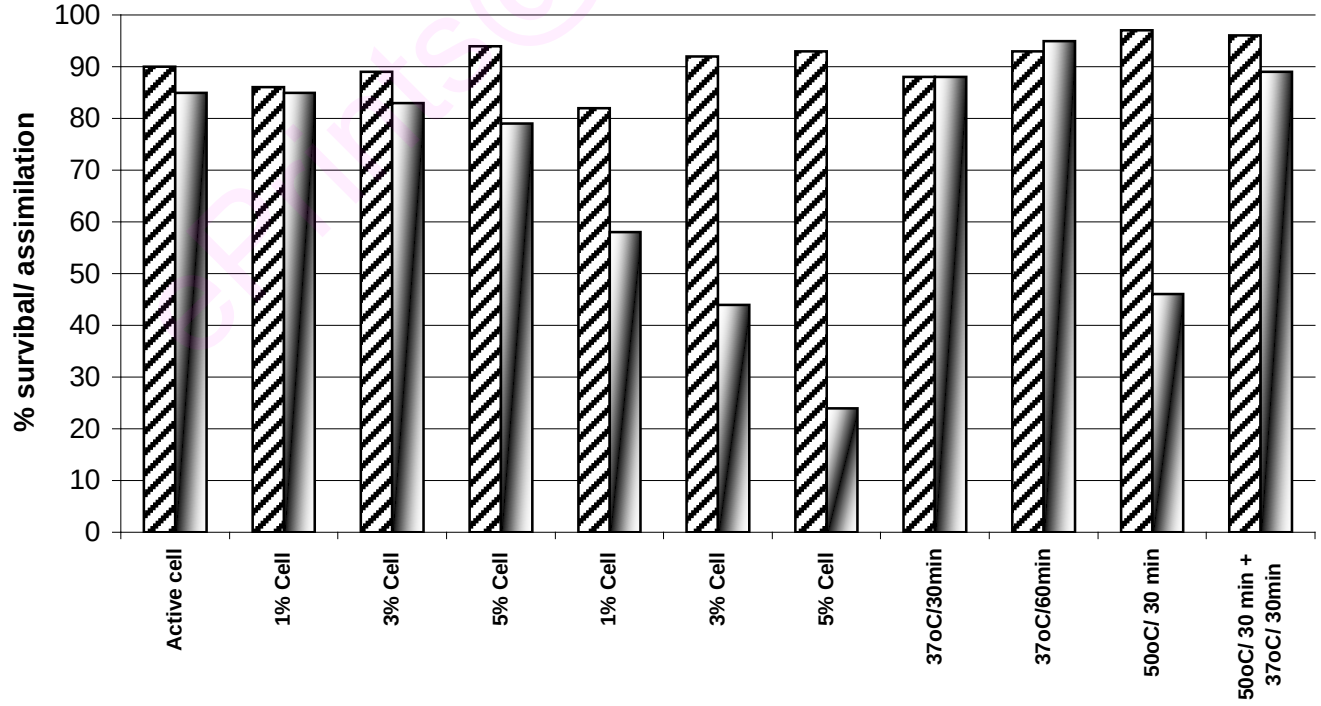
Probiotic attributes of the spray dried preparation of 3 probiotic LAB was assessed over a period of 60 days at 4 & 30^0C. Percentage survival of spray dried *L. salivarius CFR 2158; P. acidilactici CFR 2193& L. plantarum CFR 2191* over a period of 60 days (4^0C & 30^0C) is presented in Fig 6.9 a, b &c, respectively and the percentage survival was 80, 70 & 80% in case of *L. plantarum CFR 2191* , *P. acidilactici CFR 2193 & L. salivarius CFR 2158* spray dried with both the carriers(MDX & NFSM) at 4^0C for 60 days. However during storage at 30^0C for 60 days, the survival was decreased to 45% for *L. salivarius CFR 2158*, 50% for both *P. acidilactici CFR 2193 & L. plantarum* CFR

Figure 6. 8: Effect of spray drying on Bile tolerance and Cholesterol assimilation by lactic acid bacteria

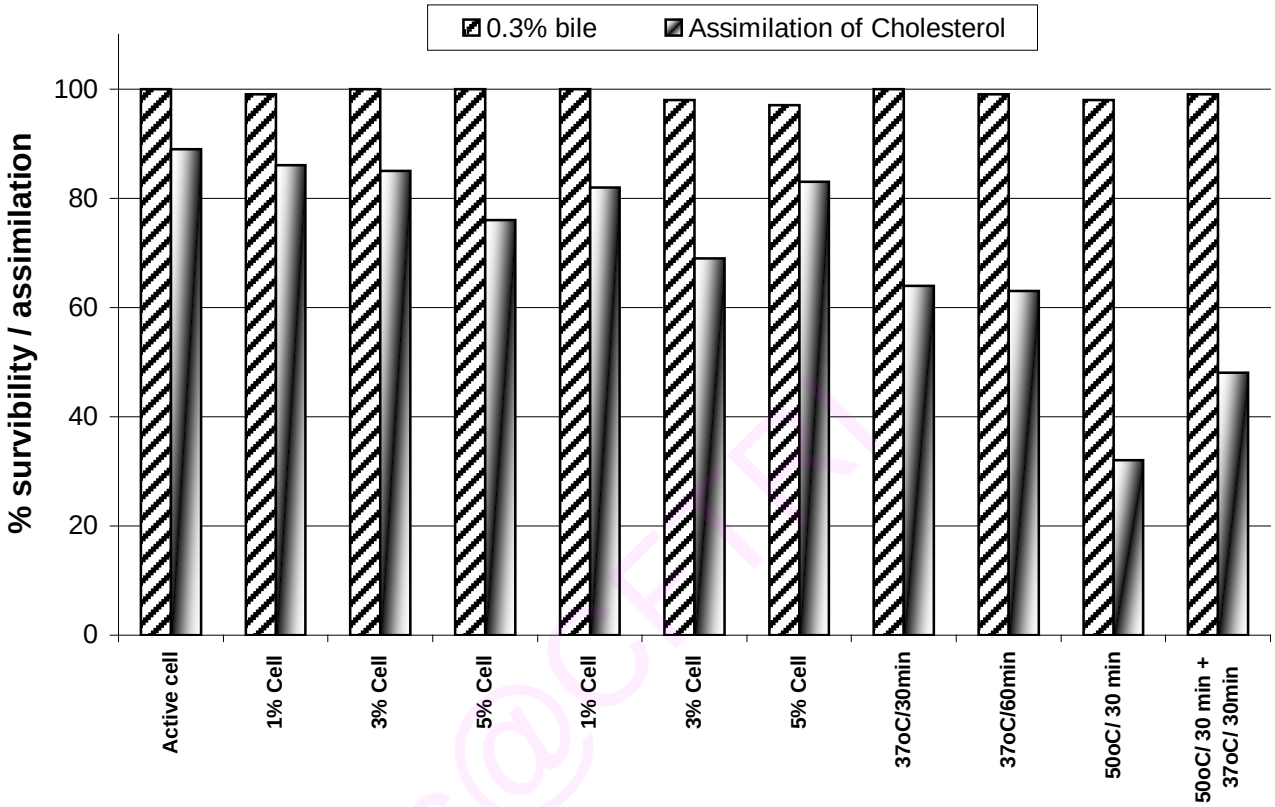
a*: L. plantarum*



b*: L.salivarius*



c: P. acidilactici



2191 when MDX used as a carrier, but survival was found to decrease to 38% for *L. salivarius* CFR 2158, 53% for *P. acidilactici* CFR 2193 & 67% for *L. plantarum CFR 2191* when NFSM used as carrier for spray drying. The study indicates that MDX can be effectively used for the spray drying of three chosen probiotic lactic acid bacteria.

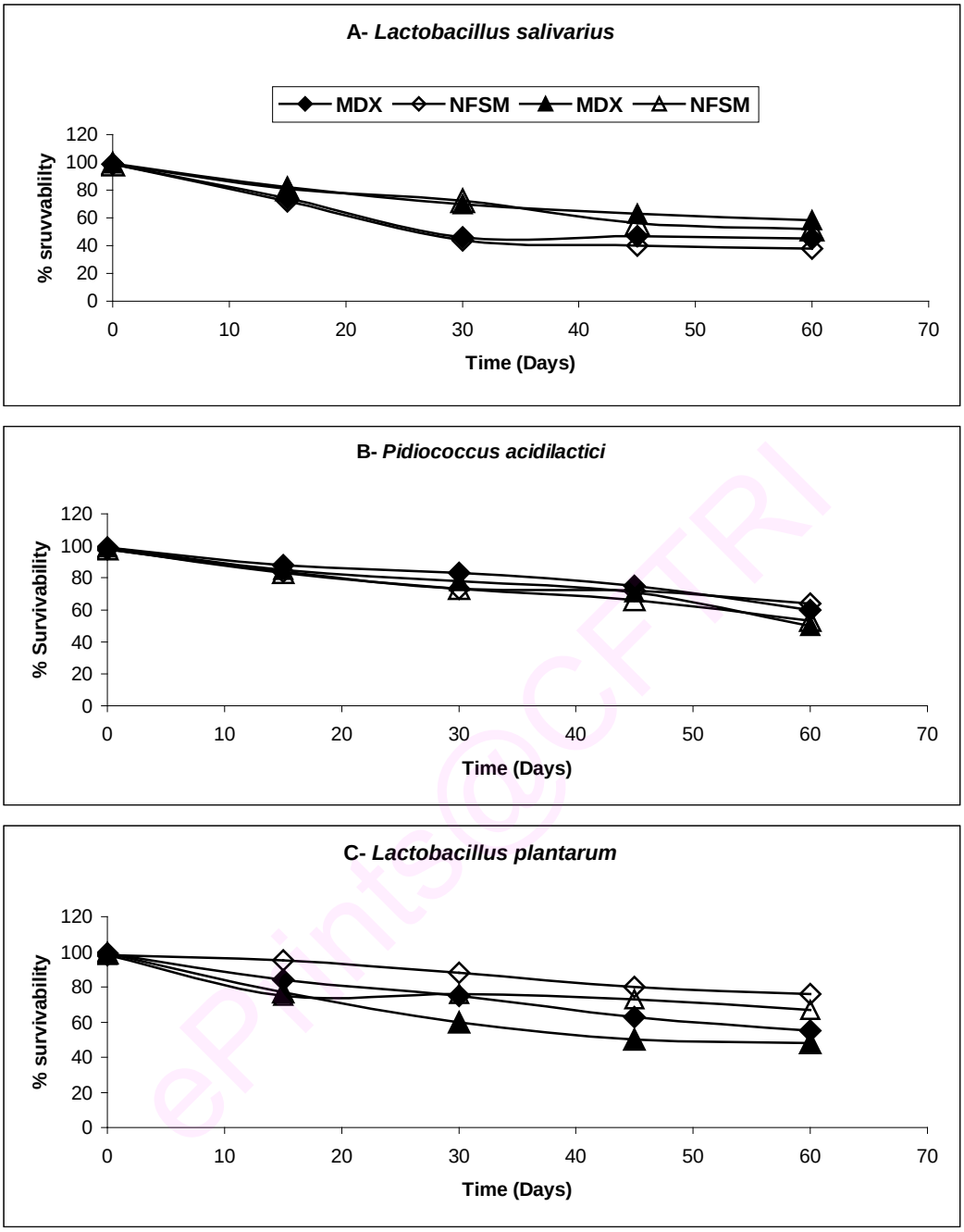


Figure 6. 9 : Percentage survival of spray dried *L.salivarius* (a), *P. acidilactici* (b) and *L.plantarum* (c) with Malto dextrin (MDX) and Non fat skimmed milk (NFSM) as a carrier during storage at 4^0C (open triangle& diamond) and 30^0C (closed triangle & diamond). The results are mean of two replicates.

6.4. Discussion:

The present chapter details the effect of different carriers, cell suspension on the spray drying of the probiotic LAB. The advantages of spray drying is that it allows large quantities of cultures to be dried at low cost (Mauriello, *et. al.,* 1999). But the disadvantage is extreme cell damage during the process and hence loss of survival of the organism during spray drying and storage (Brennan, *et. al.*,1986). Inlet and outlet temperature could also be one of the major reasons for cell damage/ death during spray drying. (Kim and Bhowmik, 1990; Mauriello, *et. al.,* 1999). The optimum temperature for the growth of most LAB is around 40^0C. Considering this factor, the experiments were carried out at a constant outlet temperature of 40^0C and corresponding inlet temperatures of 140^0C. As can been seen from our experiments (Figure 6.3), it is clear that growth retardation is significant at temperatures higher than 45^0C, due to the cell experiencing both thermal and dehydration inactivation of cells.

Experiments carried out with different cell concentrations in combinations with two carriers clearly indicated that percentage survival was more in case of spray dried *P. acidilactici* CFR 2193 (3-5% cell suspension) with NFSM in comparison with that of spray dried *P. acidilactici* CFR 2193 with MDX (Fig 6.1). Cell damage during spray drying is mainly due to the denaturing of cell membrane proteins. NFSM could probably be having a protective effect against cell damage. There are a few reports on the physiological stress responses in LAB (Angelis, 2004) but only a few reports are available on the stress induced mechanism for improving the survival of LAB during food processing. The effect of heat shock induction of proteins and stress response have been studied in some detail. (Gousebert, *et. al.,*2002; 2001; Gardiner, *et. al.*, 2000). However efforts to induce heat shock proteins to improve the survival of three LAB chosen for the study were not fruitful. In addition, heat shock treatment did not result in any protection of the probiotic properties.

It is very important to have a large number of viable cells in the finished spray dried powder. However it is much more important that the functional probiotic properties

of the spray dried probiotic LAB are retained. A number of literature reports are available only on the viability of probiotic LAB, however there are no reports on retention of the probiotic properties after spray drying and hence the present investigation emphasizes on the retention of major probiotic properties of spray dried LAB. There was no significant loss in the acid tolerance property in case of L. *plantarum* CFR 2191 at both the pH studied (2 & 2.5). *P. acidilactici* CFR 2193 exhibited tolerance only at pH 2.5. This could be due to the fact that *P. acidilactici* CFR 2193 being coccoid do not have the protective lipid bilayer. *L. salivarius CFR 2158* has been earlier reported to be sensitive to heat treatment (Gardiner, *et. al.,* 2000). In agreement, considerable loss in acid tolerance property at pH 2.5 after spray drying was observed. There was no significant change in the bile tolerance property of all the three after spray drying probiotic LAB.

There is an alarming increase in the cardio vascular diseases (CVD) in the urban population, probiotic supplementation is being viewed as an alternate therapeutic approach against CVD. Thus the cholesterol assimilation property assumes greater importance. All the three cultures studied were found to retain more than 80% of the cholesterol assimilation property when 1% cell suspension was spray dried with MDX. Of the two carriers tested, it was found that viability was retained to greater percentage with NFSM whereas better retention of the probiotic properties with reasonably good viability was observed with MDX. It is more of a general practice to use NFSM for the preparation of spray dried LAB. But the present investigation has clearly demonstrated the effective use of MDX, a cost effective, widely available carrier for the preparation of spray dried LAB.

In many events, evidence of the growing importance of probiotic microorganism to the human diet offers sufficient justification to determine survival after spray drying and short term storage. In this connection, the results obtained are very promising. *In lieu* with other studies (Teixeira, *et. al.,* 1995; Abd El gawad, *et. al.,* 1989) here also we found that spray dried LAB stored at refrigerated temperature (4^0C) exhibited better survival.

6.5. Conclusion:

In the present chapter, it was found that the concentration of cell suspension had a significant effect on the viability of spray dried probiotic LAB. The findings highlight the need to consider the technological properties of probiotic strain and emphasize more on retention of functional probiotic properties in addition to the viability of cells after spray drying . The results indicated that MDX was the better carrier for spray drying in comparison to NFSM. Even though NFSM supported better survival rate after spray drying, it failed to protect the functional properties of the probiotic lactic acid bacteria. Based on the findings, it can be said that among the three cultures studied, *L. plantarum CFR 2191* was more resistant to changes during spray drying and hence a stable spray dried preparation of *L. plantarum* CFR 2191 could be prepared. Although the laboratory scale experiments conducted in this study provide some indication on the performance of the probiotic Lactobacillus cultures during spray drying process, however studies are needed to evaluate its performance at pilot and industrial scale.

Chapter –7

Freeze drying of probiotic Lactic acid bacteria

7.1 Introduction:

Freeze drying, freezing and spray drying are some of the most useful techniques for preserving foods, agricultural products and pharmaceuticals (Rhodes *et. al.,* 1993). Freeze drying under vacuum is the most satisfactory method for the long term preservation of cultures. It is often used to preserve lactic acid bacterial (LAB) starter cultures involved in dairy and food fermentation (Kearney *et. al.,* 1990). Freeze drying is a stabilizing process in which a solution of a substance is first frozen and then the quantity of the solvent (generally water) is reduced, first by sublimation (referred to as the primary drying process) and then by desorption (secondary drying process) to a value that will no longer support biological activity or chemical reactions. This helps in preserving the viability of LAB for a long time while maintaining their technological properties. This approach is of considerable interest for research and industries, LAB are widely used in food processing industries and may be employed for the elaboration of pharmaceutical products (Corthier *et. al.,* 1998).

Freeze drying, one of the most commonly used methods of preserving bacteria is important both from the academic point of view as well as industrial application. It is suitable for the production of concentrated bacterial cultures, with an added advantage of storage at ambient temperatures (Phadtare & Inouye, 2001). However, tolerance to freezing temperature depends on freeze drying medium (protective additives), parameters, rate, cell concentration and physiological state of the cells (Broadbent & Lin, 1999; Costa *et. al.*, 2000; Peter & Reichart, 2001). Different species of bacteria display different degrees of freeze drying survival, gram-negative bacteria often show lower survival than gram-positive bacteria (Peter & Reichart, 2001). The percentage survival after freeze drying has been reported to increase with increasing initial cell concentration up to a concentration of 10^{11} CFU mL^{-1} (Costa *et. al.,* 2000). A minimal concentration 10^{7} CFU mL^{-1} is generally recommended (Pocard *et. al.*, 1994). Bacterial cells in the stationary phase are more resistant, indicating that age of the culture has a positive effect (Morice *et. al.*, 1992; Brashears & Gilliland, 1995). The composition of the fermentation medium affects the survival of LAB after freezing. Many researchers (Gomez Zavaglia

et. al., 2000; Beal *et. al.*, 2001) have shown that the addition of Tween 80 increases the proportion of unsaturated fatty acids in the membrane. This is related to modification of the membrane permeability that allows better survival. In addition, *L. delbrueckii subsp. bulgaricus* grown in the presence of calcium has been reported to exhibit better resistance to freezing. Harvesting conditions are also known to affect bacterial survival during freezing. The resistance of *Lactococcus lactis* to freezing is enhanced when the bacterial suspension is cooled before freezing (Panoff *et. al.*, 1995; De Urraza & De Antoni, 1997; Kim,& Dunn, 1997; Broadbent,& Lin, 1999). This was ascribed to cryoadaptation phenomenon by Panoff *et. al.*, (1995)

Biological materials however can be irreversibly damaged during these treatments. Freeze drying can damage the cells, which results in loss of viability during processing and also during subsequent storage (Potts, 1994; Castro *et. al.*, 1995; Miyamoto-Shinohara *et. al.*, 2000). Microscopic studies also indicate that death after freezing and thawing is correlated with membrane damage (i.e., leakage, fusion, and rupture). Therefore, it is essential to design protective agents to preserve protein activity and cell viability. The freeze drying medium should also prevent freeze drying damage, improve storage stability, and facilitate rehydration. Commonly used freeze drying media contain protective solutes such as non reducing disaccharides, sugar alcohols, polysaccharides, amino acids, proteins, and skimmed milk (Costa, *et. al.*, 2000). The protective properties of the non-reducing dissaccharides like sucrose and trehalose have been ascribed to interaction with protein and membranes (Croweet Al., 1993; Panoff *et. al.*, 1994).

As mentioned earlier, microbial cell survival during the freeze drying process is dependent on many factors, including the initial concentration of microorganism, the protective medium. Among these, protective additives have an important role in the conservation of viability. A good protectant should provide cryoprotection to the cells during freezing process, be easily dried, and provide a good matrix to allow stability and ease of rehydration. Various groups of substances have been tested for their protective

action, including polyols, polysaccharides, disaccharides, amino acids, glycine betaine, protein hydrolysates, proteins, minerals, salts of organic acids and vitamins- complex etc., (Berny, & Hennebert, 1991; Champagne *et. al.,* 1991; Cleland *et. al.,* 2004). Conrad *et. al.,* (2000) have studied in detail the stabilization matrixes on viability of *L. acidophilus* during freeze drying. They have reported that 20% trehalose exhibits better protective effects. Based on this report, in the present study the different saccharides have been tested at 20% for their protective effect. Rehydration is a critical step in the recovery of freeze dried microorganism. Cells that are subjected to sub-lethal injury may not be able to repair the damage that has occurred if they are rehydrated under inappropriate conditions (Champagne *et. al.,* 1991). The media itself, its molarity and the rehydration conditions can significantly affect the rate of recovery.

Many studies showed that bacterial resistance to freezing and frozen storage might be improved by applying moderate stress conditions before freezing. These stresses may prepare the cells to react better to unfavorable conditions. LAB are reported to react to unfavorable conditions by changing their physiological and biochemical behavior, e.g., by producing stress protein (Whitaker, & Batt, 1991; Panoff *et. al.,* 1998; Broadbent, & Lin 1999; Roberfroid, 2000) or by modifying the membrane composition (Broadbent, & Lin, 1999; Beal, 2001). The effects of different stresses like cold shock (De Urraza, & De Antoni, 1997; Kim, & Dunn, 1997; Broadbent, & Lin, 1999), heat shock (Teixeira *et. al.,* 1997; Broadbent, & Lin, 1999) starvation (Poirier, *et. al.,* 1998), and acid shock (Beal *et. al.,* 2001) on the cryotolerance of LAB have been studied. These stress conditions are known to improve the bacterial resistance to freezing and/or frozen storage.

These chapter details the effect of freeze drying on viability and the functional properties of the three chosen potent probiotic LAB. The role of cryoprotectants, age of the culture, cell concentration, cold shock treatment on the viability and the probiotic properties after freeze drying are discussed. The stability of freeze dried lactic acid bacteria stored at 4^0C over a period of 60 days is also discussed.

7.2. Material and Methods:

7.2.1. LAB Strains:

LAB strains chosen for the study and maintenance of the strains is as mentioned in section 6.2.1

A single vial of frozen culture after thawing was used for each experiment. The pre-inoculum was developed by inoculation of 100 μL of the thawed bacterial suspension to 10 mL of the MRS broth and incubated at 37^0C for 12 h. This was inoculated (1% (v/v)), the MRS broth in an Erlenmeyer flask. The cells were harvested in the early stationary phase by centrifugation (15 min, 1000 rpm, 4^0C). The pelleted cells were resuspended in saline (0.9% NaCl) after washing with distilled water 2-3 times. The cells were stored in ice bath till further use.

7.2.2 Cryoprotectants:

Based on the literature reports three dissaccharides, an oligosaccharide, a dairy carbohydrate like skimmed milk, dextrin like maltodextrin (MDX), which are very commonly used protectants were used. The different cryoprotectants used either individually or in combination are given in table 7.1.

7.2.3 Preparation of feed solutions for freeze drying :

The fresh cells harvested after each fermentation, were dispersed in distilled water (1% w/v on wet weight basis) and vortexed to get a homogenous cell suspension. Cryoprotectants (Table 7.1) at 20% (w/v) was added to the suspension cell. The volume of cell suspension was made up with distilled water to get a feed suspension of 20% (w/v) solids. The feed suspension was stored in an ice bath for about 30 min prior to use.

7.2.4 Freeze drying:

The feed suspension was frozen and then freeze dried. Each of the lyophilization flasks were filled with 100ml of feed suspension. The feed suspension was frozen quickly by rotating the flasks in dry ice and connected to a freeze drier (Heto drier, Switzerland)

operated at 0.080 Pa and -45^{0}C for 18 h. The residual viability of freeze dried cultures and moisture content of the freeze dried powders were determined and stored in air tight containers at 4^{0}C ± 1^{0}C for 60 days.

Table 7.1: List of carbohydrates used as cryoprotectants for freeze drying of probiotic lactic acid bacteria

Sl No.	Carbohydrates
1.	20% Trehalose
2.	20% Sucrose
3.	20% Lactose
4.	20% Skimmed Milk
5.	20% Maltodextrin
6.	8% Sucrose+ 1.5% gelatin+ 10% Skimmed milk
7.	5% lactose + 1.5% gelatin + 1% glycerol
8.	10% Skimmed milk+ 5% glycerol+ 0.1% CaCO3
9.	10% Skimmed milk + 10 ^{0} Brix FOS
10.	10% Maltodextrin + 10 ^{0} Brix FOS
11.	20 ^{0} Brix FOS

7.2.5 Cold shock treatment of probiotic lactic acid bacteria:

Cold shock treatment was carried out as detailed by Derzelle, *et. al.*, (2000). The growth of probiotic Lactic acid bacteria was monitored by absorbance at 600 nm. Growth was initiated at 37°C, and when the OD_{600} reached 0.4 to 0.5, corresponding to the early log phase, the cultures were incubated for 2 h at 8^0C. At the end of 10 min, the cultures were restored back to a water bath maintained at 37°C. Growth was further monitored until the cells reached the stationary phase.

7.2.6 Protein analysis:

The protein composition of cell extracts was determined by Tricine- SDS-PAGE gel electrophoresis as detailed by Derzelle, *et. al.*, (2000). Total cellular proteins were extracted from cultures grown (early stationary phase) at 37^0C. The protein was extracted both from the control cells and cold shock treated cells by homogenizing the cells with glass beads with intermittent cooling. Total proteins of the cell extract (13μg) was loaded to each well and protein spots were visualized using coomassie brilliant blue.

7.2.7 Cell survival:

Residual viability of freeze dried samples was determined as described in section 6.2.5

7.2.8 Acid tolerance

The acid tolerance of the LAB culture was determined as described in section 3.2.2.3.1

7.2.9 Bile salt tolerance

The bile tolerance of the LAB culture was determined as described in section 3.2.2.3.2

7.2.11 Presence of β- Galactosidase activity

β- galactosidase activity of the LAB culture was determined by the procedure given in section 3.2.2.3.3

7.2.10 Cholesterol assimilation

Cholesterol lowering test was carried out according to the method described in the section 3.2.2.3.4

7.3. Results:

7.3.1. Effect of cryoprotectants on survival of probiotic Lactic acid bacteria:

The results are depicted in Figure 7.1. As can be seen from the graph, there was a drastic reduction in the survival upto 50% in case of all the three probiotic lactobacillus strains freeze dried without any cryoprotectants. Significant protective effects (>98%) were observed when trehalose was used as the cryoprotectant during freeze drying. More than 90% survival was observed in case of all the three organisms freeze dried with all the different combinations of cryoprotectants.

7.3.2. Effect of cryoprotectants on protection of functional properties of probiotic lactic acid bacteria:

Commercial preparation of freeze dried concentrates are available for use in dairy industry. However most of the studies deal more on the stability and viability of freeze dried probiotic cultures. But it is equally important to ensure the sustenance of key probiotic properties of these freeze dried probiotic cultures. Keeping this in view, an effort has been made to study in detail the probiotic properties of freeze dried lactic acid bacteria in addition to their viability studies.

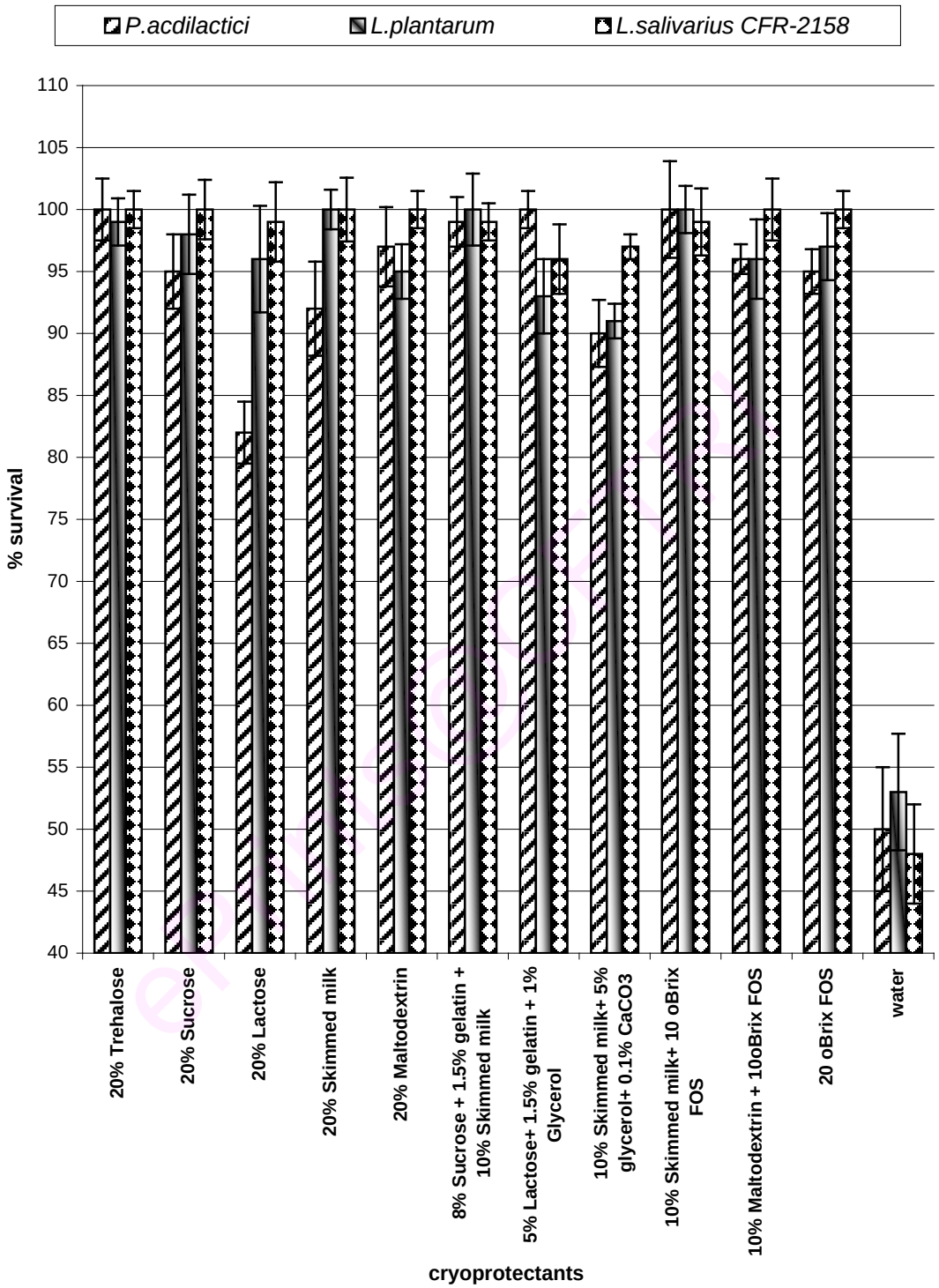


Figure 7.1 : Effect of cryoprotectants on survival of probiotic Lactic acid bacteria during freeze drying

7.3.2.1. Acid tolerance :

Having confirmed the high survival of three freeze dried lactic cultures, an experiment was carried out to evaluate the acid tolerance (pH 2.0) of these freeze dried cultures. The acid tolerance property is evaluated in terms of % survival at pH 2.5 &2.0. Results are depicted in Figures 7. 2 & 7.3.

The results (Figure 7.2) indicated that more than 95% survival in case of *L. plantarum* CFR 2191 with all the cryoprotectants studied at pH 2.5. More than 95% survival was observed in case of *P. acidilactici* CFR 2193 freeze dried with trehalose, sucrose, lactose, skimmed milk, MDX, fructooligosaccharides (FOS) and also in case of combination of skimmed milk with FOS. However only 80-85% survival was observed in case of freeze dried *P. acidilactici* CFR 2193 with a combination of the three of the cryoprotectants used. In case of *L. salivarius* CFR 2158, more than 85% survival was observed with the following cryoprotectants like trehalose, sucrose with skimmed milk, skimmed milk with gelatin, maltodextrin with FOS. The survival was further less (75%) when *L. salivarius* CFR 2158 was freeze dried with FOS alone as the cryoprotectant.

It is well known that most of lactic acid bacteria easily withstand low pH of 3.0 and exhibit high survivability above pH 2.5. However it is very important to study the pH tolerance upto 2.0 in order to ensure their probiotic property. The pH tolerance of *L. salivarius* CFR 2158 at 2.0 was not studied due to the fact that the % survival of the active cell suspension at pH 2.0 was only 60% (data not shown). Effect of cryoprotectants on the percentage survival of LAB at pH 2.0 is depicted in figure 7.3. As can be seen from the results, of the three probiotic LAB studied, *L. plantarum* CFR 2191 was resistant to the cell shrinkage and other damages during freeze drying, as indicated by the high survival of freeze dried *L. plantarum* CFR 2191 at pH 2.0.

In case of *P. acidilactici* CFR 2193, more than 80% survival was found when trehalose, sucrose and lactose were used as the cryoprotectants. But only 78% and 72%

survival was found when skimmed milk with FOS and MDX were used as the cryoprotectants respectively. The survival was further reduced to 55-65% when skimmed milk with sucrose and gelatin, skimmed milk with glycerol and calcium chloride, MDX with FOS and FOS alone was used as the cryoprotectants. About 40-50% survival was observed when skimmed milk alone was the cryoprotectant. A combination of lactose, gelatin and glycerol resulted only in 40% survival.

7.3.2.2. Effect of cryoprotectants on the bile salt tolerance of freeze dried Lactic acid bacteria:

Figure 7.4 represents the percentage survival of freeze dried LAB in the presence of 0.3% bile salt. As can be seen from the graph, freeze dried *L. plantarum* CFR 2191 with all the cryoprotectants studied, exhibited more than 95% survival. In case of *P. acidilactici* CFR 2193, only 80% survival was observed when sucrose was used as the cryoprotectant, similarly more than 75% survival was exhibited by freeze dried *P. acidilactici* CFR 2193, with lactose, gelatin with glycerol. 78% survival was noticed in case of samples freeze dried with skimmed milk, glycerol and $CaCO_3$. In case of *L. salivarius* CFR 2158 there was a decrease in the percentage survival from 90% (active cells) to 75% when sucrose alone and MDX with FOS was used as the cryoprotectants and to 70% with lactose and skimmed milk. In case of other cryoprotectants more than 80% survival was observed.

7.3.2.3. Effect of cryoprotectants on cholesterol assimilation:

The ability of the three potent LAB to assimilate cholesterol is depicted in Table 7. 2. The ability of the *P. acidilactici* CFR 2193 and *L. plantarum* CFR 2191 to assimilate cholesterol was not affected after freeze drying with any of the cryoprotectants used in isolation or in combinations, in comparison with that of active cells. However a significant reduction was found in case of *L. salivarius* CFR 2158 in comparison with the active cells (67% assimilation). *L. salivarius* CFR 2158 when freeze dried with MDX, FOS and a combination of FOS, glycerol with skimmed milk, 65-68% cholesterol assimilation was found. The cholesterol assimilation was 45-55% in case of the cryoprotectants trehalose, lactose and MDX with FOS. This was further reduced to 22-

28% in case of sucrose, lactose with gelatin, skimmed milk with FOS, sucrose + gelatin + skimmed milk.

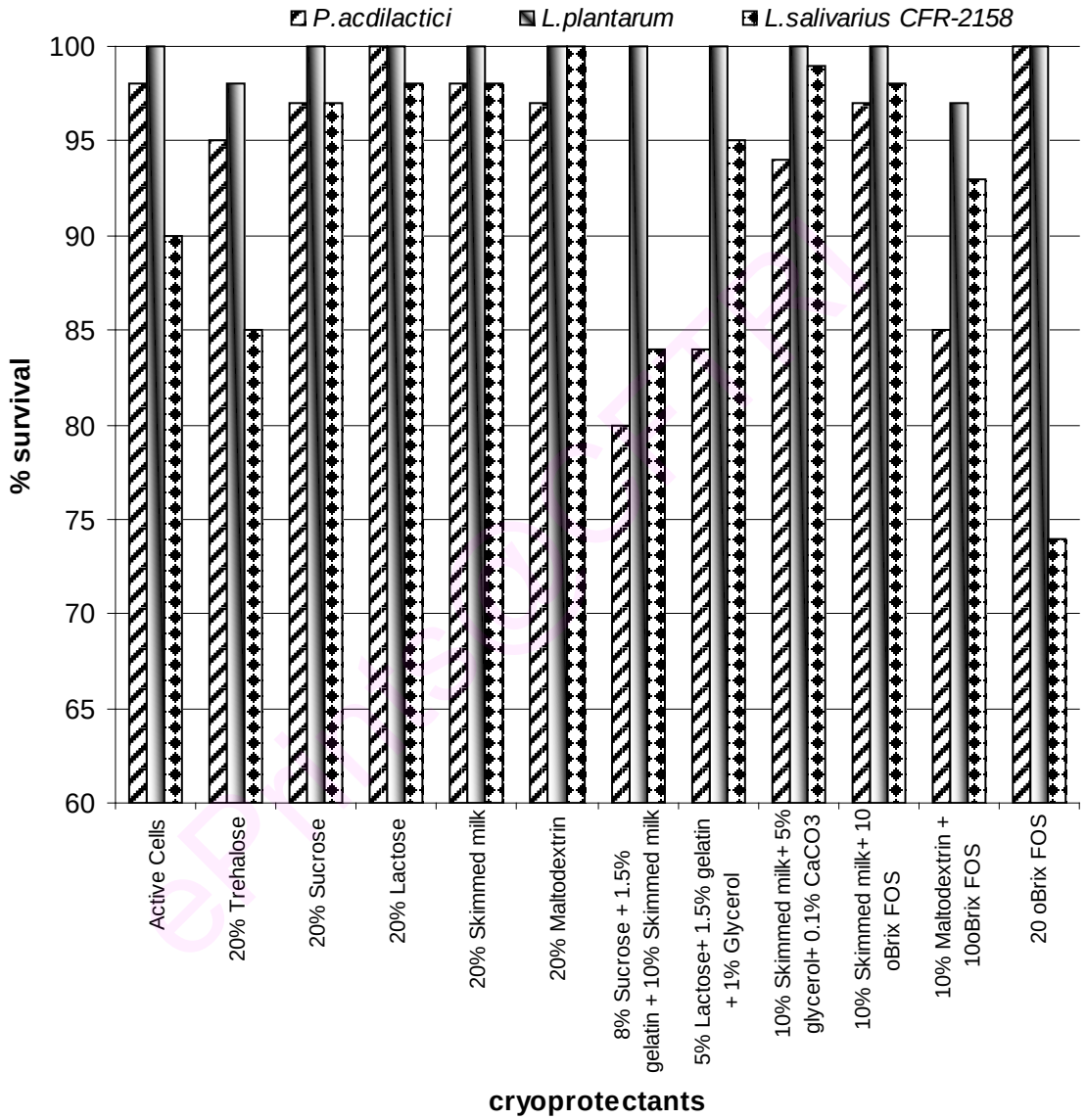


Figure 7.2: Effect of cryoprotectants on protection of pH tolerance (pH 2.5) of probiotic Lactic acid bacteria after freeze drying. Results are represented as % of survival in comparison with that of initial viable cells of feed solution for freeze drying.

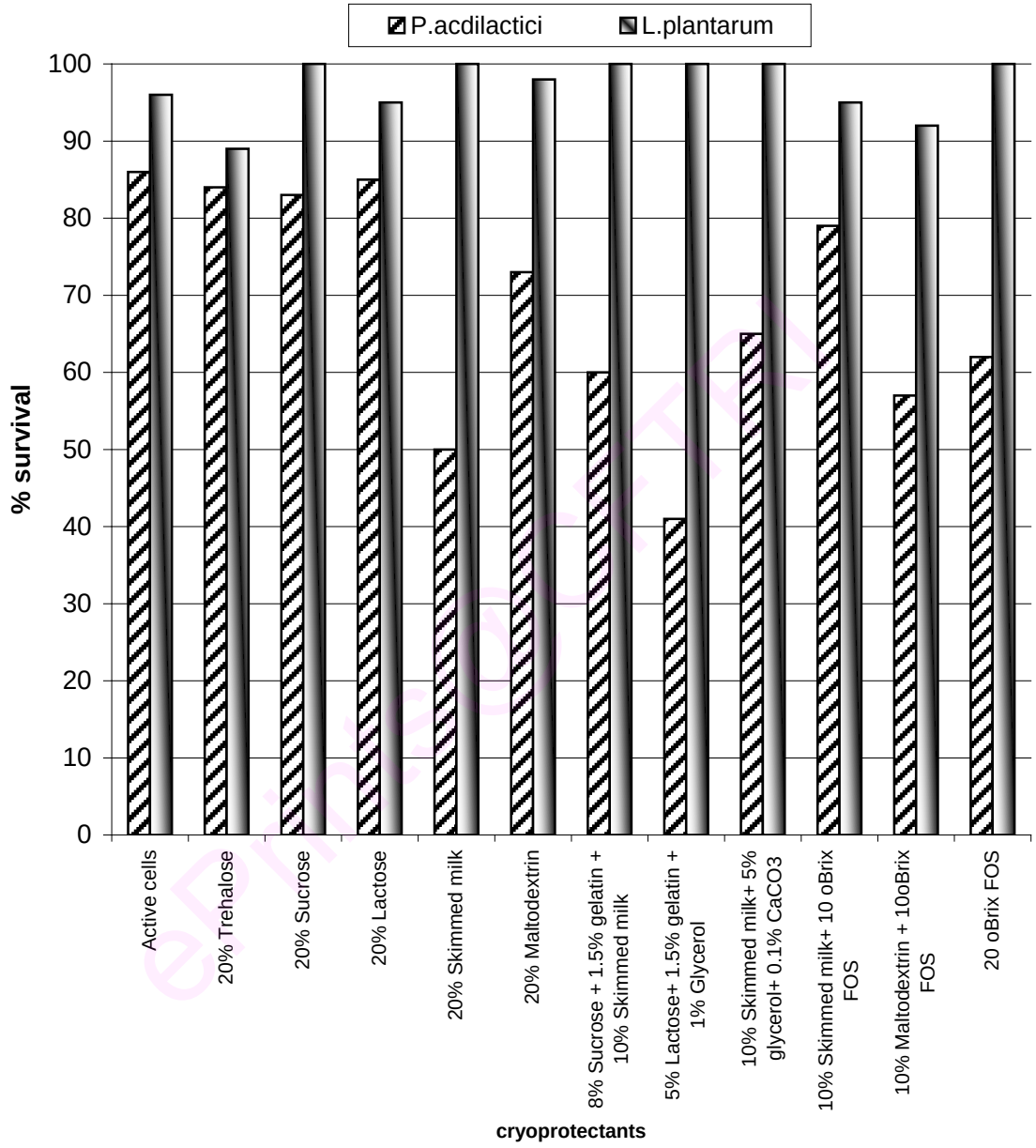


Figure 7.3: Effect of cryoprotectants on protection of pH tolerance (pH 2.0) of probiotic Lactic acid bacteria after freeze drying. Results are represented as % of survival in comparison with that of initial viable cells of feed solution for freeze drying.

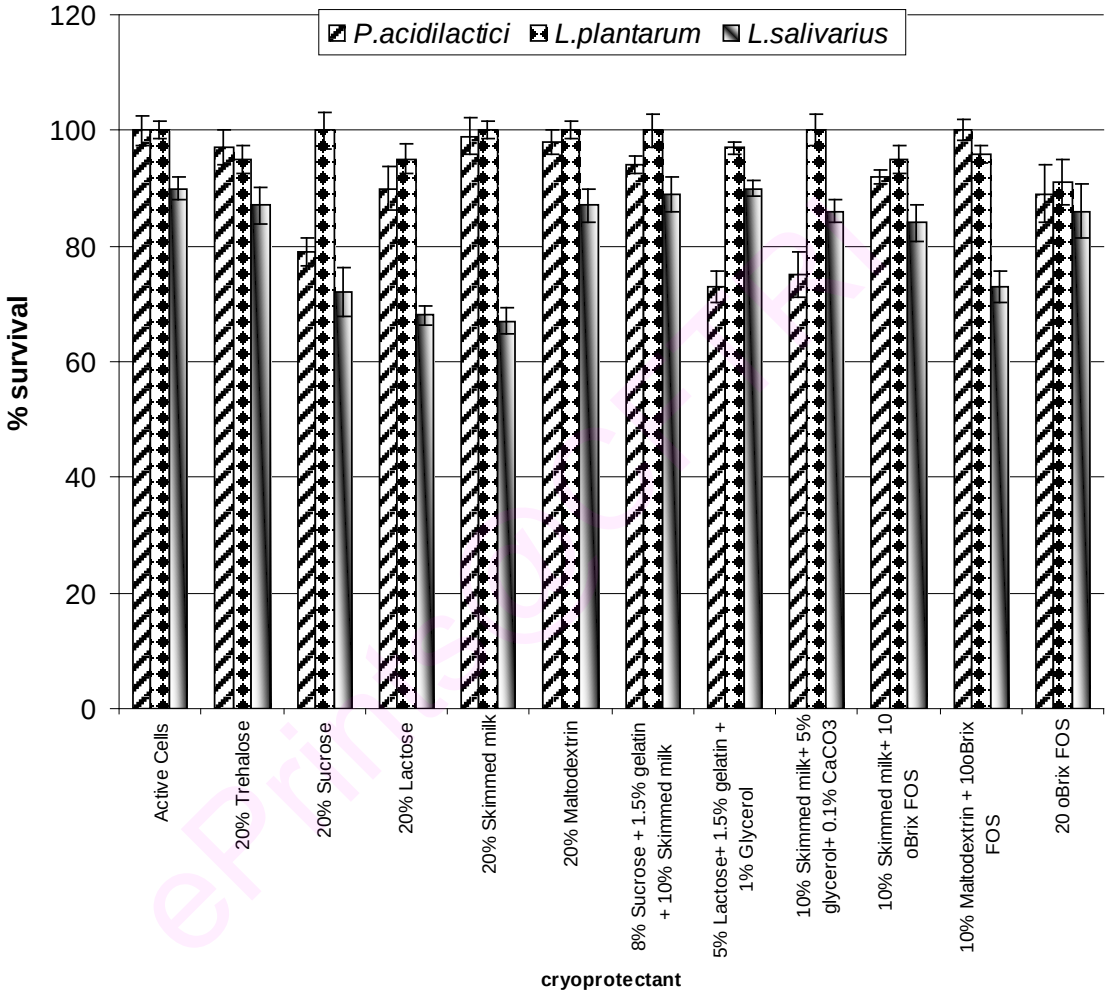


Figure 7.4 : Effect of bile salt (0.3%) concentration on % survival of freeze dried probiotic lactic acid bacteria with different combination of cryoprotectants

Table 7.2: Changes in cholesterol level after i*n vitro* incubation with freeze dried probiotic lactic acid bacteria.*

Cryoprotectants	Residual cholesterol μg/ml (reduction of cholesterol [%])#		
	P . acidilactici	***L . salivarius***	***L . plantarum***
Control	**103 (0)**	**103 (0)**	**103 (0)**
Active cells	**59.74 (42)**	**33.99 (67)**	**22.66 (78)**
20% Trehalose	**52.53 (49)**	**54.59 (47)**	**28.84 (72)**
20% Sucrose	**60.77 (41)**	**74.16 (28)**	**33.99 (67)**
20% Lactose	**50.77 (51)**	**52.53 (49)**	**25.75 (75)**
20% Skimmed Milk	**63.86 (38)**	**65.92 (36)**	**22.66 (78)**
20% Maltodextrin	**65.92 (36)**	**30.90 (70)**	**25.75 (75)**
8% Sucrose+ 1.5% gelatin+ 10% Skimmed milk	**54.59 (47)**	**73.13 (29)**	**28.84 (72)**
5% lactose + 1.5% gelatin + 1% glycerol	**60.77 (41)**	**77.25 (25)**	**22.66 (78)**
10% Skimmed milk+ 5% glycero 0.1% CaCO3	**62.83 (39)**	**35.02 (66)**	**21.63 (79)**
10% Skimmed milk + 10 ^{0} Brix FOS	**51.50 (50)**	**80.34 (22)**	**24.72 (76)**
10% Maltodextrin + 10 ^{0} Brix FOS	**60.77 (41)**	**45.32 (56)**	**26.78 (74)**
20 ^{0} Brix FOS	**62.83 (39)**	**32.96 (68)**	**56.65 (45)**

* Amounts of cholesterol was measured after 24 hrs incubation at 37^{0}C , MRS broth containing cholesterol was used as a growth media .

Figures in brackets () indicates the % assimilation

7.3.2.4. Effect of cryoprotectants on β-galactosidase activity:

It is well known that probiotics are advocated for use to combat lactose intolerance. In this context it is important that the probiotics cultures retain this property after processing. The results indicate that all the three freezed LAB with all the different cryoprotectants and their combinations retained the β- galactosidase activity to a considerable extent (Table 7.3). Results suggests that freeze drying to be the method of choice, if stable LAB cultures chosen for the study are advocated for use against lactose intolerance.

Table 7.3: β- galactosidase activity of the freeze dried probiotic LAB.

Cryoprotectants	**β- galactosidase activity (+)(blue color colony)**		
	P. acidilactici	*L. salivarius*	*L. plantarum*
Control	+	+	+
Active cells	+	+	+
20% Trehalose	+	+	+
20% Sucrose	+	+	+
20% Lactose	+	+	+
20% Skimmed Milk	+	+	+
20% Maltodextrin	+	+	+
8% Sucrose+ 1.5% gelatin+ 10%Skimmed milk	+	+	+
5% lactose + 1.5% gelatin +1% glycerol	+	+	+
10% Skimmed milk+ 5% glycerol+ 0.1% CaCO3	+	+	+
10% Skimmed milk + 10^{0} Brix FOS	+	+	+
10% Maltodextrin +10^{0} Brix FOS	+	+	+
20^{0} Brix FOS	+	+	+

7.3.3. Growth of LAB cultures at various temperatures:

In order to set the optimal and cold shock temperature, an arrheineus plot of the growth of *P. acidilactici* CFR 2193 was established. Figure 7.5 represents the log of growth rate [k] against the reciprocal of the temperature K [in Kelvins] (Souzo, 1992) (expressed in generation/h) was determined as the slope of semilogarithmic plot of optimal density (OD) at 600nm. The temperatures over which *P. acidilactici* CFR 2193 can grow were found to split into three ranges. An arrheineus zone from 10^0C (critical temperature, T_{crit}) to 27^0C (optimal temperature, T_{opt}) within which the arrheineus energy is constant; a cold shock subrange (below 10^0C) and heat shock subrange (above 41^0C). According to the arrheineus plot, temperatures of 27^0C to 40^0C appears to be optimal for *P. acidilactici* CFR 2193 and hence a temperature of 8^0C, below the critical temperature was chosen for the cold shock experiments.

7.3.4. Cold shock treatment as a function of incubation time:

An experiment was carried out to determine the time required for the expression of cold shock proteins by incubating the bacterial cells (early log phase) at the selected temperature for varying periods of time from 1h-5h. As can be seen from Figure 6, bands corresponding to cold shock protein (CSP) less than 7KD was observed after 2h of incubation, with no further induction of CSP with the increase in incubation time. Based on these results, all the three cultures *L. salivarius CFR 2158, P. acidilactici CFR 2193 & L. plantarum* CFR 2191 were subjected to cold shock treatment at 8^0C for 2 h for all the three organisms. Results clearly indicated that three was an induction of cold shock proteins in all the cultures studied (marked with an arrow).

As discussed earlier freeze dried *L. plantarum* CFR 2191 with different cryoprotectants was found to retain most of the functional properties and hence efforts were not made to induce the cold shock proteins in *L. plantarum* CFR 2191 for further freeze drying experiments.

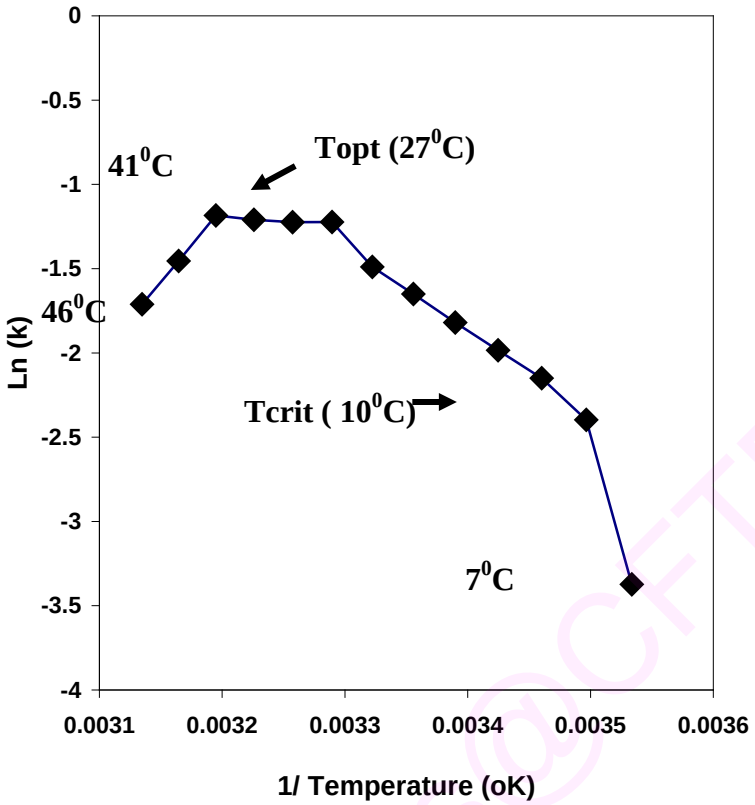
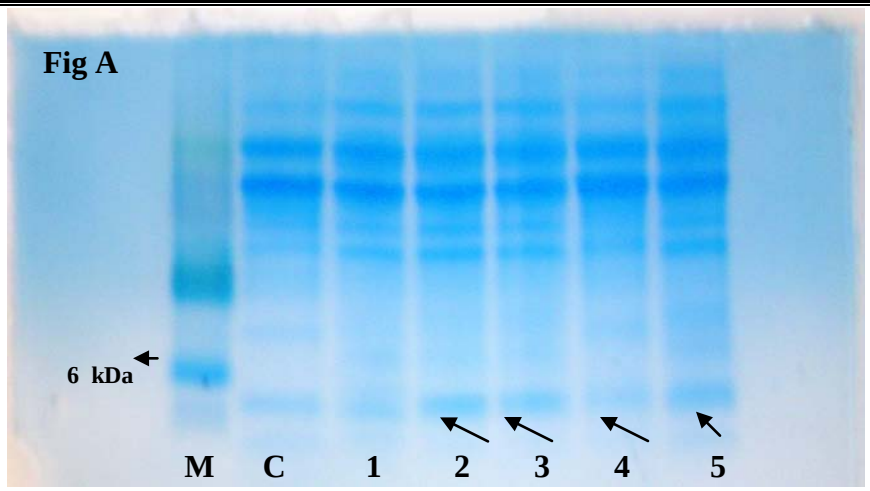


Figure 7.5: Arrhenius plot of the relationship between growth rate (k) and temperature (kelvins) for *P.acidilactici*
T_{opt} Optimal temperature; T_{crit} Critical temperature



a: Induction of Cold Shock proteins at different incubation time (h) at 8^0 C; 15% Tricine SDS-PAGE

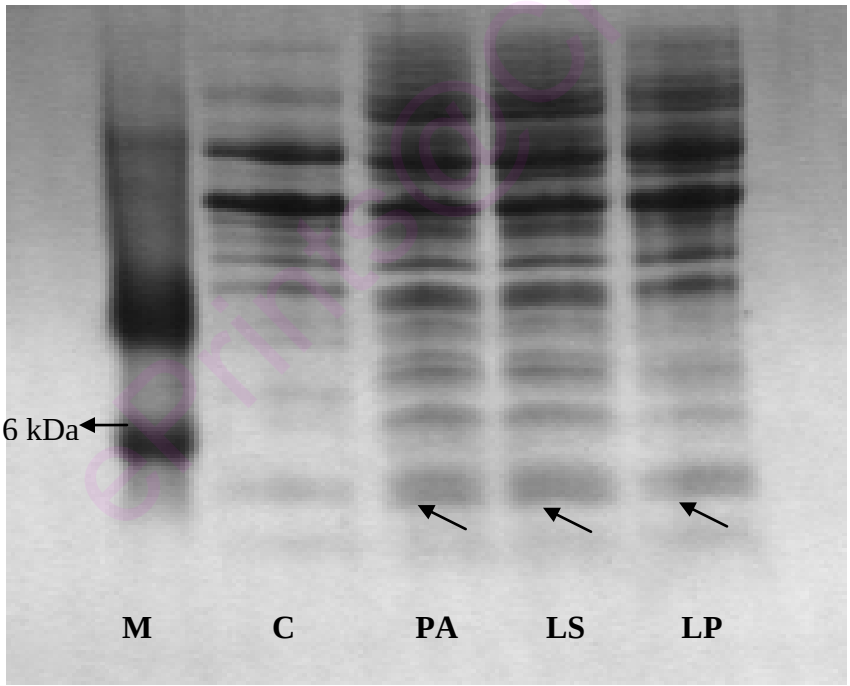


Figure 7.6: b: Induction of Cold Shock proteins in different LAB at 8^0C; 15% Tricine SDS-PAGE,
PA= *P.acidilactici*, LS= *L.salivarius*; LP= *L.plantarum*
M= low molecular weight marker (24kD- 1.2 kD)
C= control without cold shock treatment

7.3.5. Comparative study of retention of functional properties of freeze dried LAB after cold shock treatment :

The cold shock treated *P. acidilactici CFR 2193 and L. salivarius* CFR 2158 were subjected to freeze drying with selected cryoprotectants like lactose, skimmed milk & maltodextrin. Figure 7.7 represents the comparative study of the retention of functional properties of freeze dried *P. acidilactici CFR 2193 & L. salivarius* CFR 2158 after induction of CSPs. In general an increase of 7-8% improvement in the retention of most of the functional properties after the induction of cold shock proteins was noticed.

7.3.6. Stability studies of the freeze dried LAB:

The effect of storage (4^0C) of the three LAB freeze dried with selected cryoprotectants is presented in Figure 7.8. As can be seen from the results, more than 75-89%; 70-76% and 61-67% of the viability was retained after 30 days of storage in case of *L. plantarum* CFR 2191, *L. salivarius* CFR 2158 & *P. acidilactici CFR 2193 respectively* freeze dried LAB with MDX, skimmed milk and lactose respectively. However after 60 days of storage, there was a reduction in the % survival. The % survival was 60-83%; 48-70% and 47-53% correspondingly.

B. L.salivarius

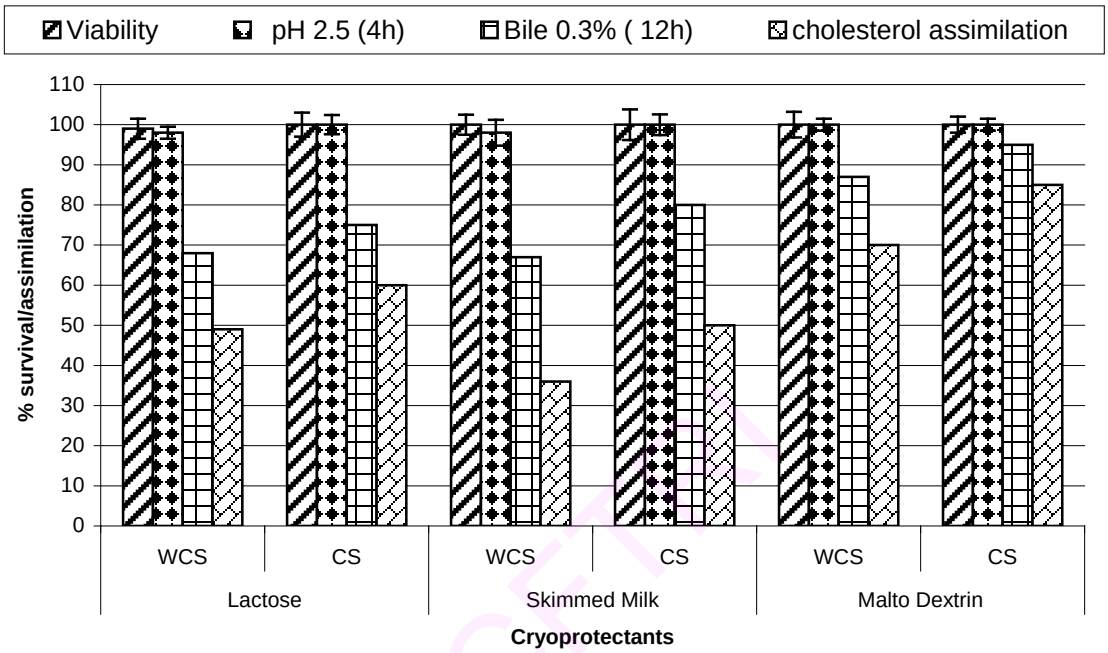


Fig A. *P.acidilactici*

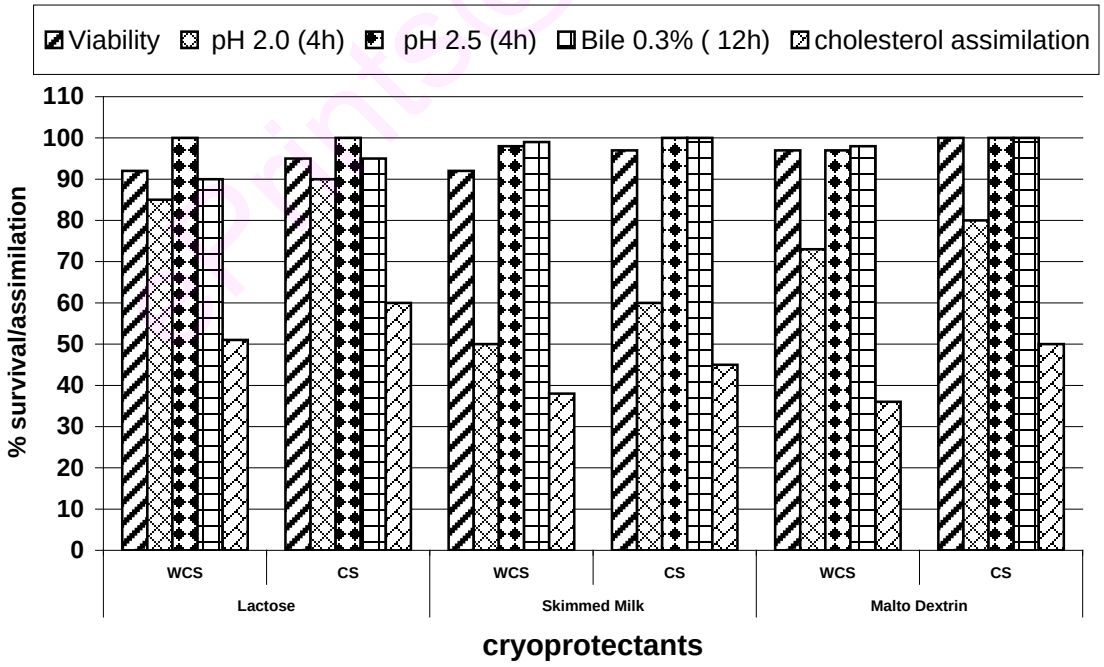
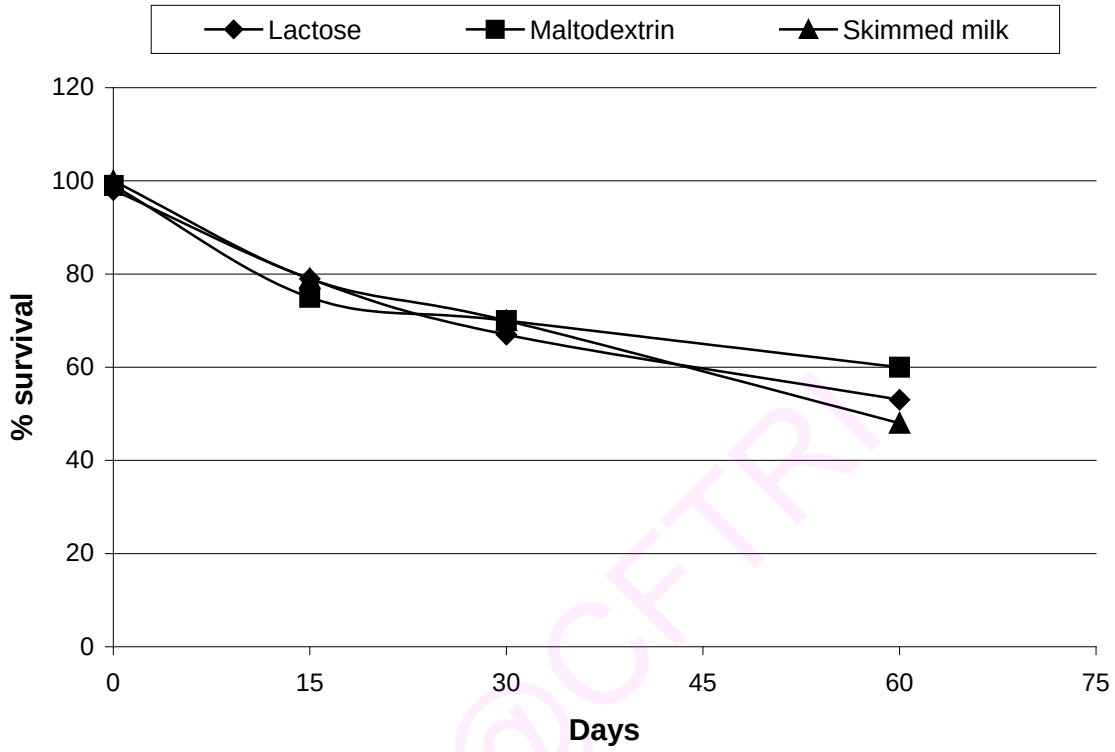
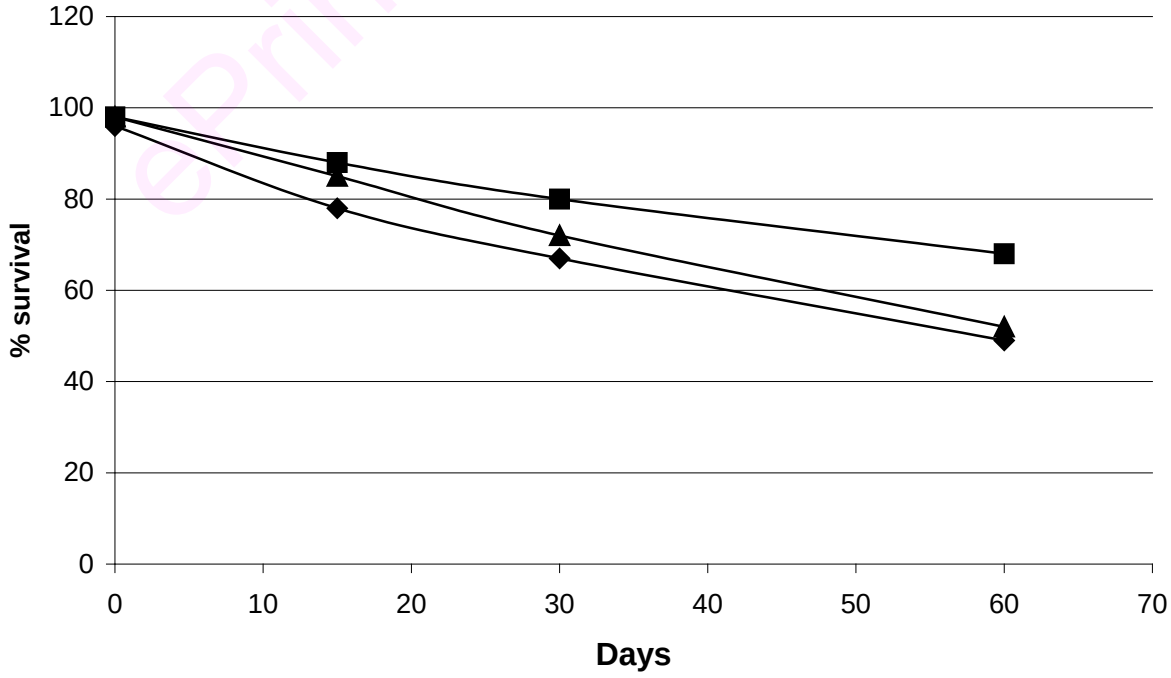


Figure 7.7: Comparative study of retention of functional properties of Freeze dried LAB after Cold shock treatment. WCS: without Cold shock; CS: cold shock treated

A. *P. acidilactici*



B. *L.Salivarius*



C. *L.plantarum*

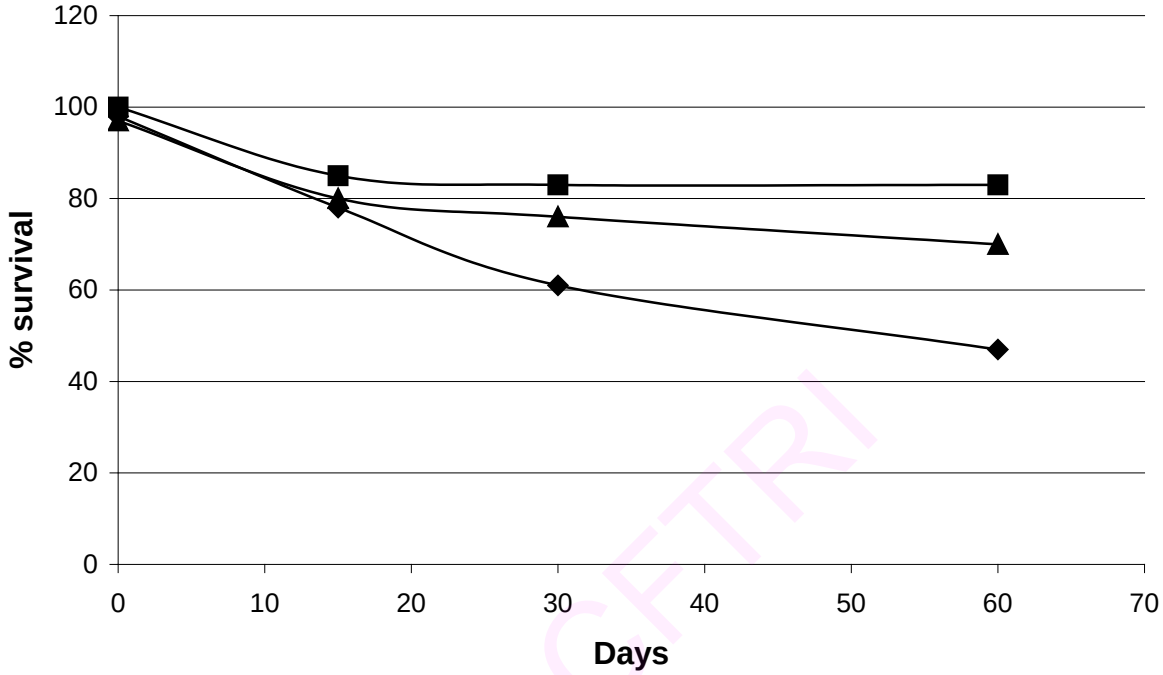


Figure 7.8 : Storage stability of Freeze dried Probiotic lactic acid bacteria for 60 days

7.4. Discussion:

Freeze drying has long been used as a desiccation process for the preservation of solid formulation of bacteria. The present study highlights the effect of cryoprotectants, rehydration media and initial concentration of microorganism on viability and functional properties of freeze dried probiotic LAB. Effects of different combinations (Table 7.1) of cryoprotectant on the protection of the probiotic properties and survival of the probiotic LAB have been studied. when LAB were freeze dried without any cryoprotectants. There was a significant loss in the probiotic properties when water was used as cryoprotectant. This could due to the injury produced by slow cooling , which results in dehydration and consequent solute concentration effects .The probiotic properties are better retained in the presence of some of the cryoprotectants.

Cryo (Freezing) and Lyo (freeze drying) protectants are known to function at several levels. From a kinetic perspective, cryoprotectants such as trehalose promote the formation of amorphous or glassy solids, thereby reducing ice formation, which can be damaging to proteins and cells membrane. Trehalose has been shown to be effective in the formation of glassy slides, and this partly explains its superior cryoprotective ability observed in our studies. [Miller *et. al.* 1998; 1997]. It is also known to interact with lipid bilayers and lower their transition temperature from the liquid crystalline phase to more rigid gel phase thus providing protection from desiccation. Trehalose apparently does this by mimicking the hydrogen bonding behavior of water at a lipid or protein surface [Crowe, 1993a; 1993b]. The protective effect of trehalose has been proved beyond doubt and is established as one of the most widely used cryoprotectants and hence in the present study, trehalose has been used as the control and the effect of other cryoprotectants studied have been compared with that of trehalose. For prolonged storage of starter cultures, Olson [1959] has reported that use of $CaCO_3$ resulted in significant improvement in cell survival, when compared with other various insoluble buffer salts added to the cryoprotective medium. It has also been showed that the combination of $CaCO_3$ with skimmed milk and glycerol resulted in the 85% survival after freeze drying. Based on these reports, $CaCO_3$ with skimmed milk was also examined in the present study.

Synbiotics are another class of products that have the goodness of both pre and probiotics (Roberfroid, *et. al.*, 2000). Efforts were made to prepare freeze dried LAB with fructooligosaccharides , a well known prebiotic (Sangeetha, *et. al.*, 2005). As discussed earlier some of the key functional properties like viability, bile and acid tolerance were moderately retained. However the resultant freeze dried powder was highly hygroscopic and was forming clumps. A combination of skimmed milk, MDX with FOS was used with an idea to bring down the hygroscopicity of the resultant powder and to get a more uniform free flowing freeze dried powder. However the efforts were not successful.

It is equally important that the processed LAB cultures either freeze dried or spray dried need to possess some acceptable physicochemical properties. One such important characteristic is the free flowing powder form without clumping and much hygroscopicity (Table 7.1). As can be seen from the results, the sample were free flowing when freeze dried with lactose, trehalose, skimmed milk and MDX but not other samples. This aspect needs further investigations. Based on the above results obtained in this study, it can be concluded that lactose, MDX and skimmed milk can effectively be used as cryoprotectants for the preparations of freeze dried *L. plantarum CFR 2191, L. salivarius CFR 2158 & P. acidilactici* CFR 2193 with high viability coupled with a high degree of retention of most of the functional properties.

It is well known that a number of bacteria including LAB react to a quick changes in temperature by the production of a set of β- barrel, small molecular weight proteins referred to as cold shock proteins (CSPs). A major cold shock protein (CSP) is reported to be rapidly induced in LAB upon a shift from 37 to 10^0C . This peptide may act as antifreeze protein (Phadtare and Inouye, 2001; Thammavongs, *et. al.*, 1996). These cold shock proteins seem to play a key role in the adaptations of the bacterial cultures to various stresses such as cold temperature, stationary phase or nutritional deprivation, and also during growth under optimal conditions (Weber and Marahiel, 2002; Yamanaka, *et. al.*, 2001; 1998; Graumann and Marahiel, 1999; Graumann, *et. al.*, 1997;]. These low

molecular weight cold shock proteins were also induced in all the three probiotic LAB when subjected to cold shock at 8^0C for 2h.

As can been seen from the results, about 8 to 10% increase in cell viability and also in the retention of functional properties was observed when LAB were freeze dried after cold shock treatment at 8^0C for 2h in comparison with that of freeze dried LAB without cold shock treatment.. This indicates that cold shock treatment prior to the freeze drying of probiotic lactic acid bacteria may have beneficial effects.

7.5. Conclusion:

The results presented in this chapter indicates that lactose, skimmed milk and maltodextrin can be used as potent cryopreservatives during freeze drying of three probiotic LAB chosen for the study. Results also indicate that these cryoprotectants not only protect the viability, but also the functional properties of the probiotic LAB. Retention of probiotic properties in freeze dried LAB is also essential, when used in pharma and neutraceutical industries. Freeze dried LAB with maltodextrin showed significant stability than those freeze dried with other cryoprotectants when stored at 4^0C for a period of 60 days. The important observation is that MDX can effectively be used as a cryoprotectant for the preparation of shelf stable probiotic LAB. The advantage of using MDX is that it is very cost effective and widely available. The resultant freeze dried LAB also exhibited the desired functional properties in addition to high degree of viability.

Summary and highlights of the investigation:

The primary role of diet is to provide enough nutrients to fulfill metabolic requirements; recent discoveries support the hypothesis that, beyond nutrition in the conventional sense, diet may modulate various functions in the body. Diet, stress, and modern medical practices have been implicated as factors capable of exerting an influence on human health and nutrition. The development of probiotics during the past decade has signaled an important advance in the food industry transferring it towards the development of functional foods. Probiotic enriched foods, are one of the major subset of the functional foods. The demand for health foods fully justifies the efforts of the various researchers in understanding the physiological effects of food components and their health benefits.

Probiotic products represent a strong and growing area within the group of functional foods and intense research efforts are under way to develop dairy products incorporated with probiotic organisms such as *Lactobacillus* and *Bifidobacterium* species. For successful development of food products containing probiotics, a thorough knowledge of the abilities of the microorganism to survive the manufacturing and storage condition of the product is required. To be beneficial to the host, dietary cultures must reach the gastrointestinal tract (the target organ) in significant numbers, thus survival under harsh conditions, including acid in the stomach and bile in the small intestine become a key prerequisite.

Criteria for the selection of potent probiotic bacteria for the dairy and pharmaceutical application are very critical. Probiotic bacteria selected for commercial use in foods and therapeutics must retain the characteristics for which they were originally selected. These include characteristics for growth and survival during manufacture and after consumption, transit through the stomach and small intestine. Importantly, probiotics must retain the characteristics which lead to the beneficial health effects. Consequently, it is necessary to test the stability of these characteristics during manufacture and storage and to ensure that they are retained in different types of food.

The work presented in the thesis describes the possibilities of selection of potent probiotic cultures and the preparation of stable spray and freeze dried lactic acid bacteria powder for neutraceutical and therapeutical application

The objectives of the thesis:

- To examine the effect of additives, cultural conditions and pretreatment on the viability and functional properties of lactic acid bacteria.
- To develop suitable preservation techniques for lactic acid bacteria to be used as probiotics.

The contents of the thesis has been organized and presented in 6 different chapters

Chapter 1 : Introduction

Importance of lactic acid bacteria, concept of probiotics, functional properties, their beneficial health effects and application of lactic acid bacteria are presented in the introductory chapter, Scope of the investigation is presented at the end of the chapter.

Chapter 2: Review of literature

A through review of relevant literature cited on the topic of investigation for the past decade is presented in this chapter. Current research trends in the area of probiotic and its application is also presented.

Chapter 3 : Isolation of probiotic lactic acid bacteria from *kanjika*

This chapter details the isolation of lactic acid bacteria and screening of lactic acid bacteria for potent probiotic properties. Lactic acid bacteria were isolated from the *Kanjika, an Ayruvedic* lactic acid fermented product, prepared in our laboratory. Some of the important probiotic properties such as, tolerance to low pH, high bile salt concentration up to 1%, antimicrobial activity against food borne pathogens, β-galactosidase and phytase activity, antibiotic susceptibility and cholesterol lowering capacity of the isolates were evaluated**.** Out of the 17 isolates**,** 6 isolates K3a, K1a, K23c, K7a, K7b and K4a belonging to lactobacillus genera showed more than 90% survival at pH 2.0 and pH 2.5 after 4 h of incubation. All the isolates except K7b exhibited tolerance

up to 1% bile salt concentration after 24 h incubation. Isolates like K3a, K1a, K7a, K7b and K4a were tested positive for β-galactosidase activity. Isolates other than K4a were found to be capable of assimilating cholesterol in the range of 30 to 50%. All isolates were tested positive for phytase activity after 12 h incubation by agar spot method.

Chapter 4: Phenotypic & genotypic characterization of probiotic lactic acid bacteria

Over the past decade, the probiotic field has exploded with a number of new cultures, each exhibiting a variety of benefits. Rational selection and design of probiotics remains an important challenge and will require a platform of basic information about the physiology and genetics of candidate strains relevant to their intestinal roles, functional activities, and interactions with other resident microflora. In this context, in addition to the morphological and biochemical characterization, molecular characterization assumes greater importance. 16S rDNA gene sequences characterization was undertaken for the identification of Lactic acid bacteria (LAB) isolated at different periods during the preparation of Kanjika, an Ayruvedic lactic acid fermentation product. Total DNA isolation, optimization of PCR conditions for the amplification of the 16S rDNA gene and cloning of PCR product for the sequencing was carried out. The sequenced data was subjected to BLAST analysis and phylogenic study. Potent probiotic isolates; K3a, K7b and K23c were identified as *Lactobacillus plantarum* CFR 2191, *Lactobacillus fermentum* CFR 2192 and *Pediococcus acidilactici* CFR 2193, respectively.

Chapter 5: In-vitro evaluation of lactic acid bacteria (from culture collection) for potent probiotic properties.

It is important to objectively seek, cause and effect relationships for many potential and currently investigated probiotic species and strain combinations. In this context 13 lactic acid bacteria (LAB) were evaluated for their probiotic properties. Out of the 13 LAB cultures, *L. plantarum* B-4496 and *L. salivarius* CFR-2158 showed more than 70% survival at pH 2.0, 82% and 90% survival at pH 2.5 and 80% survival at 0.3% bile salt. *L. casei* NCIM-2586, *L. helveticus* B-4526, *L. acidophilus* B-4495 and

L. salivarius CFR-2158 were found to significantly reduce the cholesterol to 90.50%, 85.59%, 84.6% and 81.33% in the presence of 0.3% of bile salt respectively. The above LAB cultures also showed β- galactosidase activity and good antibacterial activity against Enterotoxigenic *E. coli* (ETEC) which is the main culprit in causing traveller's diarrhea. Among the cultures evaluated *L. plantarum* B-4496 and *L. salivarius* CFR-2158 exhibited potent functional properties. Scope exits for the application of the two cultures for the preparation of a potent probiotic supplements for food and dairy industries.

Chapter 6: Spray drying of probiotic lactic acid bacteria

Spray drying is one of the most common down stream processing step used for the preparation of stable microbial culture in particular probiotic cultures. The preparation of concentrated starter culture via spray drying is of practical importance to dairy industries. An effort has been made to study the effect of spray drying on the viability and retention of key probiotic properties like acid and bile tolerance , cholesterol assimilation of the three potent probiotic cultures *viz*.**,** *Lactobacillus plantarum, Pediococcus acidilactici,* and *Lactobacillus salivarius.* Maltodextrin (MDX) and non fat skimmed milk (NFSM) were used as carriers. Viability of three spray dried cultures was more than 95% at all the concentration of cell suspension (1%, 3% & 5%), with 10% NFSM was used as the carrier. The three probiotic LAB exhibited more than 97% survival, when 1% cell suspension was spray dried with 10% MDX. Effect of different cell concentration (1, 3 and 5% w/v) on the functional properties of spray dried cultures was also studied. All the three lactic acid bacterial cultures retained more than 90% survival and key functional properties when spray dried with MDX. No significant improvement in viability and retention of probiotic properties were noticed in case of cultures subjected to heat shock treatment. The results highlight that spray drying of LAB cultures with MDX as a carrier may be a cost effective way of producing large quantities of a few probiotic cultures with functional properties for neutraceutical application.

Chapter 7: Freeze drying of probiotic lactic acid bacteria

Freeze-drying is one the most widely used techniques for preserving foods and biological materials. Many factors have been reported to influence the survival of lactic acid bacteria during freeze-drying. This chapter details the effect of cryoprotectants, cold shock treatment on probiotic properties and stability of freeze dried probiotic lactic acid bacteria like *Pediococcus acidilactici* CFR 2193; *Lactobacillus plantarum* CFR 2191 which were isolated and well characterized with 16S rDNA method and *Lactobacillus salivarius* CFR 2158 from the departmental culture collection center of CFTRI. Different combinations of carbohydrates were evaluated for their cryoprotectant ability during freeze drying. Effect of cryoprotectants on probiotic properties like pH, bile tolerance, β-galactosidase and cholesterol assimilation of the freeze dried probiotic lactic acid bacteria were evaluated. Lactose, skimmed milk and maltodextrin were found to be good cryoprotectants for the retention of the properties as well as viability during freeze drying. There was an increase in 6-8% both in viability and probiotic properties when lactic acid bacteria were subjected to cold shock treatment and freeze dried in comparison with that of the probiotic LAB, freeze dried without subjecting them to cold shock treatment. Storage stability of the freeze dried lactic acid bacteria with the three chosen cryoprotectants was carried out for 60 days at 4^0C. *Lactobacillus plantarum* CFR 2191, *Pediococcus acidilactici* CFR 2193 and *Lactobacillus salivarius* CFR 2158 freeze dried with maltodextrin retained 83%, 60% & 68% of viability respectively in comparison with that of the initial values.

The thesis has focuses on the selection of potent probiotic lactic acid bacteria on an '*in-vitro*' criteria from different lactic acid bacterial isolates obtained from Aryuvedic fermented product. The selected probiotic bacteria were subjected to drying processes, like freeze drying and spray drying in the presence of different drying medium. The optimized drying medium was selected on the basis of protection of both viability and functional properties of the probiotic lactic acid bacteria. The probiotic bacteria were

further subjected to stress treatment (heat shock and cold shock) for the improvement of the protection of the probiotic properties during processing. The storage stability of the spray dried/freeze dried LAB powders obtained under the optimized process conditions was studied. In this thesis some of the key problems in the preparation of stable Spray and freeze dried potent probiotic lactic acid bacteria has been addressed. It is hoped that the work carried out would further help in assessing their probiotic properties in animals models. It is further recommended that in depth '*In-vivo'* studies to evaluate the probiotic properties of the isolated potent cultures are carried out. This would widen the scope for varied applications on a commercial scale.

Chapter – 9

References

Referencas::

Abd El gawad, I. A., Metwally, M. M., El Nockrashy, S. A. and Ahmed, K. E. (1989) Spray drying of lactic acid cultures. II the effect of culture conditions and storage on microorganism survival. *Egypt J. Dairy Sci.*, **17**, 273-281.

Acharya Sidh Nandan Misra. (1993) Abhinav Bhaisajyakalpana Vijnan. Chapter: Sandhana Kalpana, Choukhanba, Sur Bharali, and Publication Varanasi., pp 268.

Adamowicz, M., Kelley, P. M. and Nickerson, K. W. **(**1991) Detergent (sodium dodecyl sulfate) shock proteins in *Escherichia coli*. *Journal Bacteriology*, **173:** 229–233.

Adlerberth, I., Ahrne, S., Johansson, M.-L., Molin, G., Hanson, L.A. and Wold, A.E., (1996) A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Appl. Environ. Microbiol.*, **62:** 2244–2251.

Aiba, Y., Suzuki, N., Kabir, A.M., Takagi, A. and Koga, Y. (1998) Lactic acid mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. *Am. J. Gastroenterol.*, **93:** 2097-2101.

Alm, L. (1982) Effect of fermentation on B-vitamin content of milk in Sweden. *J. Dairy Sci.*, **65:** 353–359.

Angelis, M. D., Cagno, R.D., Huet, C., Crecchio, C., Fox, P. F. and Gobbetti, M. (2004). Heat shock response in Lactobacillus plantarum. *Appl. Environ. Microbiol.*, **70:** 1336-1346.

Baird-Parker, A. C. (1980) Organic acids, in Microbial ecology of foods. Silliker, J. H., Elliott, R. P., Baird-Parker, A. C., Bryan, F. L., Christian, J. H. B., Clark, D. S., Olson, J. C., and Roberts, T. R. (Eds.), Academic Press, New York, pp 126–135.

Barbara Mombelli and Maria Rita Gismondo. (2000) The use of probiotics in medical practice. *Int. J. Antimicrobiol. Agents.*, **16**: 531–536.

Baronofsky, J. J., Schreurs, W. J. and Kashket. E. R. (1985) Uncoupling by acetic acid limits growth of and acetogenesis by Clostridium thermoaceticum. *Appl. Environ. Microbiol.*, **48:**1134–1139.

Beachey, E.H. (1981) Bacterial adherence: Adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J. Infect. Dis.*, **143 (3):** 325-345

Beal, C., Fonseca, F. and Corrieu, G. (2001) Resistance to freezing and frozen storage of *Streptococcus thermophilus* is related to membrane fatty acid composition. *J. Dairy Sci.*, **84:** 2347-2356 .

Begley, M., Gahan, C.G. and Hill, C. (2002) Bile stress response in *Listeria monocytogenes* LO28: Adaptation, cross-protection, and identification of genetic loci involved in bile resistance, *Appl. Environ.Microbiol.* **68:** 6005–6012.

Beijernick, M. W. (1901) Anhaufungsversuche miUreumbakterien. Ureumspaltung durch Urease and durch Katabolismus. *Zentrabl. Bakteriol. Parasitenkd. Infektionskr. Hyg. II Abt*, **7:** 33–61.

Benno Y, Mitsuoka, T and Kanazawa, K (1991) Human faecal flora in health and colon cancer. *Acta Chirurgica Scand.* **521:** 15–23.

Bergey's Manual of Systemiic Bacteriology. Vol 2 . Peter, H. A., Sneath, Mair, N.S., Sharpe, M.E., Holt, J.G. (Eds.). Williams & wilkins publishers , waverly Press, P 1208-1234

Bernet, M.F., Brassart, D., Neeser, J.-R. and Servin, A.L. (1994) Lactobacillus acidophilus LA-1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut*, **35:** 483–489.

Bernet, M.F., Brassart, D., Neeser, J.R. and Servin, A.L., (1993) Adhesion of human *bifidobacterial* strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. *Appl. Environ. Microbiol.*, **59:** 4121–4128.

Berny, J. F. and Hennebert, G. L. (1991) Viability and stability of yeast cells and filamentous fungus spores during freeze- drying: effects of protectants and cooling rates. *Mycologia,* **83:** 805-815.

Blum, S. and Reniero, R. (2000) Adhesion of selected *Lactobacillus* strains to enterocyte-like Caco-2 cells *in vitro*: a critical evaluation of reliability of *in vitro* adhesion assays. 4th Workshop, Demonstration of Nutritional Functionality of Probiotic Foods, Rovaniemi, Finland, February 25–28.

Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S.D. and Sorokin, A. (2001) The complete genome sequence of the lactic acid bacterium *Lactococcus lactis ssp. lactis* IL1403. *Genome Res.*, **11:** 731–753.

Brashears, M. M. and Gilliland, S. E. (1995) Survival during frozen and subsequent refrigerated storage of *Lactobacillus acidophilus* cells as influenced by the growth phase. *J. Dairy Sci.*, **78**: 2326-2335.

Brennan, M., Wanismail, B., Johnson, M.C. and Ray, B. (1986) Celluar damage in dried *Lactobacillus acidophilus*. *J. Food Prot.*, **49:** 47-53.

Broadbent, J. R. and Lin, C. (1999) Effect of heat shock or cold shock treatment on the resistance of *Lactococcus lactis* to freezing and lyophilization. *Cryobiology*, **39**: 88-102.

Buydens, P. and Debeucklaere, S. (1996) Efficacy of SF 68 in the treatment of acute diarrhea: a placebo controlled trial. *Scand. J. Gastroenterol.*, **31:** 887–891.

Campbell-Platt, G. (1994) Fermented foods- a world perspective. *Food Res. Int.*, **27:** 253–257.

Canducci, F., Armuzzi, A., Cremonini, F., Cammarota, G., Bartolozzi, F., Pola, P., Gasbarrini, G. and Gasbarrini, A. (2000) A lyophilized and inactivated culture of *Lactobacillus acidophilus* increases *Helicobacter pylori* eradication rates. *Aliment Pharmacol. Ther.* **14:**1625–1629.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X. and Gibbs, P. (2004) Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freezedried *Lactobacillus delbrueckii ssp. bulgaricus*. *Biotechnol. Prog.*, **20:** 248–254.

Castro, H. P., Teixeira, P.M. and Kieby, R. (1995) Storage of lyophilized cultures of *Lactobacillus bulgaricus* under different relative humidities and atmospheres. *Appl. Microbol. Biol.*, **44**: 172-176.

Champagne, C. P., Gardner, N., Brochu, E. and Beaulieu, Y. (1991) The freeze-drying of lactic acid bacteria. A review. *Canadian Institute Sci. Technol. J.*, **24**: 118-128.

Charteris W. P., Kelly, P. M., Morelli, L. and Kevin Collins. (2000) Effect of conjugated Bile salts on Antibiotic susceptibility of Bile salt tolerant lactobacillus and Bifidobacterium isolates. *J. Food Prot.*, **63**: 1369-1376.

Charteris, W.P., Kelly, P. M., Morelli, L. and Collins, J.K. (1998a) Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacilli* and *Bifidobacterium* species in the upper human gastrointestinal tract. *J. Appl. Microbiol.*, **84:** 759–768.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. (1998b) Antibiotic susceptibility of potentially probiotic lactobacillus species. *J. Food Prot.*, **61:** 1636-1643.

Chateau, N., Deschamps, A.M. and Sassi, H. (1993) Heterogeneity of bile salts resistance in the *Lactobacillus* isolates of a probiotic consortium, *Lett. Appl.Microbiol.*, **18:** 42–44.

Chauviere, G., Coconnier, M. H., Kerneis, S., darfeuille- Michaud, A., Joly, B. and Servin, A.L. (1992a). Competitive exclusion of diarrheagenic *Escherichia coli* (ETEC) form hman enterocyyte- like Caco2 cells by heat-killed *Lactobaciilus, FEMS microbial. Lett.*, **91:** 213-218.

Chauviere, G., Coconnier, M.H., Kerneis, S., Fourniat, J. and Servin, A.L. (1992b). Adhesion of human *Lactobacillus acidophilus* strain LB to human entrocyte-like Caco2 cells. *J. Gen. Microbiol.*, **138:** 1689-1696.

Chou, L. and Weimer, B. (1999). Isolation and characterization of acid and bile tolerance isolates from strains of *lactobacillus acidophilus*. *J. dairy Sci.*, **82:** 23-31.

Chung, C.T., Niemela, S.L. and Miller, R.H. (1989) One-step preparation of competent *E.coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci.*, **86:** 2172-2175.

Chung, H.S., Kim, Y.B., Chun, S.L. and Ji, G.E. (1999) Screening and selection of acid and bile resistant bifidobacteria. *Int. J. Food Microbio.*,**47 :** 25– 32.

Clark, P.A. and Martin, J.H. (1994) Selection of bifidobacteria for use as dietary adjuncts in cultured dairy foods: III. Tolerance to simulated bile concentrations of human small intestines. *Cult. Dairy Prod. J.*, **29:** 18– 21.

Clarridge III, J.E., (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious disease. *Clin. Microbiol. Rev.* **17**: 840–862.

Cleland, D., Krader, P., McCree, C., Tang, J. and Emerson. D. (2004) Glycine betaine as a cryoprotectant for prokaryotes, *J. Microbiol. Methods,* **58**: 31-38.

Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.L. (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.,* **71:** 1 – 20.

Coconnier, M. H., Lievin, V., Hemery, E. and Servin. A. L. (1998) Antagonistic activity against *Helicobacter* infection in vitro and in vivo by the human *Lactobacillus acidophilus* strain LB. *Appl. Environ. Microbiol.* **64:** 4573–4580.

Collins, J.K., Thornton, G. and Sullivan, G.O. (1998) Selection of probiotic strains for human application. *Int. Dairy J.,* **8:** 487– 490.

Colombel, J. F., Cortot, A., Neut, C. and Romond, C. (1987) Yogurt with *Bifidobacterium longum* reduces erythromycininduced gastrointestinal effects. *Lancet*, **2:** 43-45.

Conrad, P. B., Miller, D. P., Cielenski, R. P. and De-Pablo. J. J. (2000) Stabilization and Preservation of *Lactobacillus acidophilus* in Saccharide Matrices. *Cryobiology*, **41:** 17-24.

Conway, P.L., Gorbach, S.L. and Goldin, B.R. (1987) Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J. Dairy Sci.*, **70:** 1 –12.

Coolins, M.D. and Gibson, G.R. (1999). Probiotics, probiotics and synbiotics: approaches for modulating the microbial ecology of gut. *Am. J. Clin. Nutr.*, **69:** 1052S-1057S.

Corcoran, B.M., Stanton, C., Fitzgerald, G.F. and Ross, R.P. (2005) Survival of probiotic *lactobacilli* in acidic environments is enhanced in the presence of metabolizable sugars. *Appl. Environ Microbiol.*, **71:** 3060-3067.

Corsetti, A., Lavermicocca, P., Morea, M., Baruzzi, F., Tosti, N. and Gobbetti, M. (2001). Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species *Triticum durum and Triticum aestivum*) sourdoughs of Southern Italy. Int. J. Food Microbiol., **64:** 95–104.

Corthier, G. and Renault, P. (1998) Future directions for research on biotherapeutic agents: Contribution of genetic approaches on lactic acid bacteria. In: Biotherapeutic Agents and infectious Diseases, Elmer, G. W., McFarland, L. and Surawicz, C. (Eds.), Humana Press, Totowa, New Jersis. pp 269-304.

Costa, E., Usall, J., Teixido, N., Garcia, N. and Vinas, I. (2000) Effect of protective agants, rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain CPA-2 subjected to freeze-drying. *J. Appl. Microbiol.*, **89**: 793-800.

Crowe, J. H., Crowe, L. M., Oliver, A. E., Yevetkova, N., Wolkers, W. and Tablin, F. (2001) The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. *Cryobiology*, **43:** 89-105.

Crowe, J. H., Crowe, L. M. and Carpenter, J. F. (1993) Preserving dry biomaterials: the eater replacement hypothesis. Part 1, *Biopharm.*, **6:** 28-32.

Crowe, J. H., Crowe, L. M. and Carpenter, J. F. (1993) Preserving dry biomaterials: the eater replacement hypothesis. Part 2, *Biopharm.*, **6:** 40-43.

Crowe, J. H.,Crowe, L. M., Carpenter, J. F., Rudolph, A. S., Wistrom, C. A., Spargo, B. J. and Anchordoguy, T. J. (1998). Interactions of sugars with membranes. *Biochem. Biophys. Acta*, **947:** 367-384.

Cummings, J. H. and Branch, W. J. (1986) Fermentation and the production of short chain fatty acids in the human large intestine. In: Dietary fiber: basic and clinical aspects, Vahouny, G. and Kritchevsky, D.(Eds.), Plenum Press, New York and London, pp131–149.

D'Souza, A. L., Rajkumar, C., Cooke, J., and Bulpitt, C. J. (2002) Probiotics in prevention of antibiotic-associated diarrhoea: meta analysis. *Brit. Med. J.*, **324:** 1361–1364.

Daemen, A. L. H. and Van der Stege, H. J. (1982) The destruction of enzymes and bacteria during the spray drying of milk and whey. 2. The effect of the drying conditions. *Neth. Milk Dairy J.* **36:** 211-229.

Dahiya, R. S. and Speck, M. L. (1968) Hydrogen peroxide formation by *lactobacilli* and its effect on *Staphylococcus aureus*. *J. Dairy Sci.,* **51(10):** 1068–1072.

Dahl, T. A., Midden, W. R., and Hartman, P. E. (1989) Comparison of killing of Gram-negative and Gram-positive bacteria by pure singlet oxygen. *J. Bacteriol.,* **171:** 2188–2194.

De Boever, P., Wouters, R., Verscaheve, L., Berckmans, P., Schoeters, G. and Verstraete, W. (2000) Protective effect of the bile salt hydrolase-active Lactobacillus reuteri against bile salt cytotoxicity, *Appl. Microbiol. Biotechnol.*, **53:** 709–714.

De Roos, N. M. And Katan, M.B. (2000) Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. *Am. J. Clin. Nutr.,* **71:** 405-4011.

De Ruyter, P.G.G.A., Kuipers, O.P. and de Vos, W.M. (1996) Controlled gene expression systems for *Lactococcus lactis* with the foodgrade inducer nisin. *Appl. Environ. Microbiol.* **62:** 3662–3667.

De Smet, I., Van Hoorde, L., De Saeyer, N., Van de Woestyme, M. and Vestraete, W. (1994) *In vitro* study of bile salt hydrolase (BSH) activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of lowering through enhanced BSH activity, *Microbiol. Ecol. Health Dis.,* **7**: 315–329.

De Smet, I., van Hoorde, L., vande Woestyne, M., Christiaens, H. and Verstraete, W. **(**1995) Significance of bile-salt hydrolytic activities of *lactobacilli*. *J. Appl. Bacteriol.,* **79:** 292-301.

De Urraza, P. and De Antoni, G. (1997) Induced cryotolerance of *lactobacillus delbrueckii subsp. Bulgaricus* LBB by preincubation at suboptimal temperatures with fermentable sugar. *Cryobiology*, **35:** 159-164.

Deeth, H. C. and Tomine, A. Y. (1981) Yogurt: nutritive and therapeutic aspects. *J. Food Protect.*, **44:** 78–86.

Derzelle, S., Hallet, B., Francis, K. P., Delcour, J. and Hols, P. (2000) Changes in cspL, cspP, and cspC mRNA abundance as a function of cold shock and growth phase in *Lactobacillus plantarum*. *J. Bacteriol.*, **182**: 5105-5113.

Desmond, C., Fitzgerald, G., Stanton, C. and Ross, P. (2004) Improved stress tolerance of GroESL-overproducing Lactococcus lactis and probiotic Lactobacillus paracasei NFBC338. *Appl. Environ. Microbiol.* **70:** 5929–5936.

Dodd, H. M. and Gasson, M. J. (1994). Bacteriocins of lactic acid bacteria. In: Genetics and biotechnology of lactic acid bacteria, Gasson, M.J, ad de Vos W.M. (Eds.), Blackie Academic and Professional, Glasgow. pp211-251.

Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzgerald, G., Daly, C., Kiely, B., Quigley, E.M.M., O'Sullivan, G.C., Shanahan, F. and Kevin, J. (1999) Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie van Leeuwenhoek* ,**76:** 279– 292.

Eili´S O' Sullivan and Se´amus condon, (1997) Intracellular pH Is a Major Factor in the Induction of Tolerance to Acid and Other Stresses in *Lactococcus lactis* . *Appl. Envirol. Microbiol.*, **63:** 4210-4215.

Elina Ronka, Erja Malinen, Maria Saarela, Merja Rinta-Koski, Johannes Aarnikunnasa, Airi Palva. (2003) Probiotic and milk technological properties of *Lactobacillus brevis* . *Int. J. Food Microbiol.*, **83:** 63– 74.

El-Ziney, M. and Debevere, J. (1998) The effect of reutirin on *Listeria monocytogenes* and *Escherichia coli* O157:H7 in milk and cottage cheese. *J. Food Prot.,* **61:** 1275-1280.

Ennahar, S. and Deschamps, N. (2000) Anti-Listeria effect of enterocin A, produced by cheese-isolated *Enterococcus faecium* EFM01, relative to other bacteriocins from lactic acid bacteria. *J. Applied Microbiol.,* **88:** 449– 457.

Erika Isolauri, Yelda Sütas, Pasi Kankaanpää, Heikki Arvilommi, and Seppo Salminen. (2001) Probiotics: effects on immunity. *Am J Clin Nutr.,* **73:** 444S–450S.

Espina, F. and Packard, V.S. (1979) Survival of Lactobacillus acidophilus in a spray drying process. *J. Food Prot.,* **42:** 149-152.

Famularo, G., Moretti, S., Marcellini, S. and De Simone, C. (1997) Stimulation of immunity by probiotics. In: Probiotics 2: Applications and Practical Aspects, Fuller, R., (Ed.), Chapman and Hall, London, UK. pp. 133–161.

Felley, C. P., Corthesy-Theulaz, I., Rivero, J. L., Sipponen, P., Kaufmann, M., Bauerfeind, P., Wiesel, P. H., Brassart, D., Pfeifer, A., Blum, A. L. and Michetti, P. (2001) Favourable effect of an acidified milk (LC -1) on *Helicobacter pylori* gastritis in man. *Eur. J. Gastroenterol. Hepatol.* **13:** 25–29.

Fernandes, C. F. and Shahani, K. M. (1989) Lactose intolerance and its modulation with lactobacilli and other microbial supplements. *J. Applied Nutr.,* **1:** 50-64.

Fernandes, C. F., Shahani, K. M. and Amer, M.A. **(**1987) Therapeutic role of dietary lactobacilli and *Lactobacillus* fermented dairy products. *FEMS Microbiological Review*, **46:** 343-356.

Finlay, B. B. and Falkow, S. (1997). Common themes in micro- bial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.,* **61:** 136-169.

Flahaut, S., Frere, J., Boutibonnes, P. and Auffray, Y. (1996) Comparison of the bile salts and sodium dodecylsulfate stress responses in Enterococcus faecalis, *Appl. Environ. Microbiol.,* **62:** 2416–2420.

Floch, M.H., Binder, H.J., Filburn, B. and Gershengoren, W. (1972) The effect of bile acids on intestinal microflora. *Am. J. Clin. Nutr.,* **25:** 1418– 1426.

Fredricks, D.N. and Relman, D.A. (1996) Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin. Microbiol. Rev.* **9:** 18–33.

Friend, B.A. and Shahani, K.M. (1984) Nutritional and therapeutic aspects of *Lactobacilli*. *J. Appl. Nutr.,* **36:** 125-153.

Fu, W.-Y. and Etzel, M.R. (1995). Spray drying of *Lactococcus lactis ssp. Lactic streptococci. J. Food Sci.,* **60:** 805-809.

Fukushima, M., Yamada, A., Endo, T. and Nakano, M. **(**1999) Effects of a mixture of organisms, *Lactobacillus acidophilus* or *Streptococcus faecalis* on delta6-desaturase activity in the livers of rats fed a fat- and cholesterol-enriched diet. *Nutr.,* **15:** 373-378.

Fuller, R. (1991) Probiotics in human medicine. *Gut,* **32:** 439-442.

Fuller, R. (1993) Probiotic foods - current use and future developments, *IFI NR.*, **3:** 23–26.

Gade, J. and Thorn, P. (1989) Paraghurt for patients with irritable bowel syndrome. *Scan J Prim Health Care*, **7:** 23-26.

Gagne, J., Roy, D. and Gauthier, S. F. (1993) Production of frozen and freeze-dried concentrates of *Bifidobacterium infantis* by membrane filtration. *Milchwissenschaft*, **48:** 501-505.

Gardiner, G. E., O'Sullivan, E., Kelly, J., Auty, M.A.E., Fitzgerald, G.F., Collins, J.K., Ross, R.P. and Stanton, C. (2000) Comparative survival rates of human-derived probiotic lactobacillus paracasei and L. salivarius strains during heat treatment and spray drying. *Appl. Environ. Microbiol.*, **66**: 2605-2612.

Gasson, M. J. and Fitzgerald, G. F. (1994) Gene transfer systems and transposition. In: Genetics and biotechnology of lactic acid bacteria, Gasson, M. and de Vos, W. M. (Eds.), Blackie Academic & Professional, New York, pp 1–44.

GastroNet, (2001) Your digestive system, downloaded from http://www.gastro.net.au/frame_digestive.html .

German, B., Eduardo, J. S., Reniero, R., Mollet, B., Pfeiffer, A. and Neeser, J. R. (1999) The development of functional foods: Lessons form the gut. *Tibtech*, **17:** 492-499.

Gerritse, K., Posno, M., Schellekens, M. M., Boersma, W. J. and Claassen, E. (1990) Oral administration of TNP-Lactobacillus conjugates in mice: a model for evaluation of mucosal and systemic immune responses and memory formation elicited by transformed lactobacilli. *Res. Microbiol.*, **141:** 955–962.

Gibson, G. R., Saavedra, J. M., Macfarlane, S. and Macfarlane, G. T. (1997) Probiotics and intestinal infections. In: Probiotics 2: Applications and practical aspects, Fuller, R. (Ed.),Chapman & Hall, New York, pp 10–39.

Gilliland, S. E. and Kim, H. S. (1984) Effect of viable starter culture bacteria in yogurt on lactose utilization in humans. *J. Dairy Sci.*, **67:** 1–6.

Gilliland, S. E. and Lara, R. C. (1988) Influence of storage at freezing and subsequent refrigeration temperature on β-galactosidase activity of *Lactobacillus acidophilus*. *Appl. Environ. Microbiol*., **54:** 898-902.

Gilliland, S. E. and Speck**,** M. L. **(**1977) Deconjugation of bile acids by intestinal lactobacilli. *Appl. Environ.l Microbiol.,* **33:** 15–18.

Gilliland, S.E. (1976) Preparation and storage of concentrated cultures of lacti streptococci. *J.Dairy Sci.,* **60:** 805-807.

Gilliland, S.E. (1989) Acidophillus milk products: A review of potential benefits to consumer. *J. Dairy Sci.,* **72:** 2483-2494.

Gilliland, S.E. and Speck, M.L. (1977) Deconjugation of bile acids by intestinal *lactobacilli*. *Appl. Environ. Microbiol.,* **33**: 15–18.

Gilliland, S.E., Nelson, C.R. and Maxwell, C. (1985) Assimilation of cholesterol by *Lactobacillus acidophilus. Appl. Environ. Microbiol.,* **49:** 377-381.

Gilliland, S.E., Speck, M. L. and Nauyok, G. F. (1978) Influence of consuming nonfermented milk containing lactobacillus acidophilus on fecal flora of healthy males. *J. Dairy Sci.,* **61:** 1-5.

Gilliland, S.E., Staley, T.E. and Bush, L.J. (1984) Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *J.Dairy Sci.,* **67:** 3045-3051.

Gismondo, M. R., Drago, L. and Lombardi, A. (1999) Review of probiotics available to modify gastrointestinal flora. *Int. J. Antimicrobial Agents.,* **12:** 287-292.

Goldin, B.R. and Gorbach, S.L. (1992). Probiotics for humans. In: Probiotics, the Scientific Basis, Fuller R. (Ed.), Chapman & Hall, London, pp. 355–376.

Goldin, B.R., Gorbach, S. L., Saxelin, M., Barakat, S., Gaulthieri, L. and Salminen, S. (1992) Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. *Dig. Dis. Sci.,* **37:** 121–128.

Gomes, A.M.P. and Malcata, F.X. (1999). *Bifidobactrium spp*. and *Lactobacillus acidophilus*: biologica, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trend Food Sci. Technol.,* **10***:* 139-157.

Gomez Zavaglia, A., Disalvo, E. A. and De Antoni, G. L. (2000) Fatty acid composition and freeze-thaw resistance in lactobacilli. *J. Dairy res.,* **67**: 241-247.

Gonzlez, C. J., Encinas, J. P., Garcia-Lopez, M. L. and Otero, A. (2000) Characterization and identification of lactic acid bacteria from freshwater fishes. *Food Microbiol.,* 17: 383–391.

Goodson, M. and Rowbury, R. J. (1989) Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sublethal acid pH. *Lett. Appl. Microbiol.,* **8:**77–79.

Gorbach, S. L. (1990) Lactic acid bacteria and human health. *Ann. Med.,* **22:** 37–41.

Gouesbert, G., Jan, G. and Boyaval, P. (2002) Two-dimensional electrophoresis study of *Lactobacillus delbrueckii* subsp. *Bulgaeicus thermotolerance*. *Appl. Environ. Microbiol*., **68:** 1055-1063.

Gousebert, G., Jan, G. and Boyaval, P. (2001) *Lactobacillus delbruecki* subsp. *Bulgaricus* thermotolerance. *Lait,* **81:** 301-309.

Graumann, P. and Marahiel, M. A. (1999) Cold shock proteins CspB and CspC are major stationary phase induced protein in *B. subtilis*. *Arch. Microbiol.,* **171:** 135-138.

Graumann, P., Schroder, K., Schmid, R. and Marahiel, M.A. (1996) Cold shock stress-induces proteins in *Bacillus subtilis*. *J. bacterial.*, **178:** 4611-4619.

Graumann, P., Wendrich, T. M., Weber, M. H. W., Schroder, K. and Marahiel, M. A. (1997) A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperature. *Mol. Microbiol.,* **25:** 741-756.

Green, J. L. and Angell, C. A. (1989) Phase relations and vitrification in saccharide-water solutions and the trehalose anomaly. *J. Phys. Chem.,* **93:** 2880-2882.

Greene, J. D. and Klaenhammer, T. R. (1994) Factors involved in adherence of lactobacilli to human Caco-2 cells. *Appl. Environm. Microbiol.*, **60:** 4487-4494.

Greenwald, P., Clifford, C. K. & Milner, J. A. (2001) Diet and cancer prevention. *Eur. J. Cancer,* **37:** 948–965.

Guandalini, S., Pensabene, L., Zikri, M. A., Dias, J. A., Casali, L.G., Hoekstra, H., Kolacek, S., Massar, K., Micetic-Turk, D. and Papadopoulou, A. (2000) *Lactobacillus GG* administered in oral rehydration solution to children with acute diarrhea: a multicenter European study. *J. Pediatr. Gastroenterol. Nutr.,* **30:** 54–60.

Guiseppe Famularo, Claudo De Simone, Vandana Pandey, Adita Ranjan Sahu and Giovanni Minisola. (2005) Probiotic lactobacilli: An innovative tool to correct the malabsorption syndrome of vegetarians?" *Medical Hypotheses.,* **65:** 1132–1135

Gupta, P., Andrew, H., Kirschner, B. S. and Guandalini, S. (2000) Is *Lactobacillus GG* helpful in children with Crohn's disease? Results of a preliminary, open-label study. *J. Ped. Gastroenterol. Nutr.* **31:** 453–457.

Guslandi, M., Mezzi, G., Sorghi, M. and Testoni, P.A. (2000) *Saccharomyces boulardii* in maintenance treatment of Crohn' s disease. *Digestive dis. Sci.,* **45:** 1462-1464.

Ha, C. L., Lee, J. H., Zhou, H. R., Ustunol, Z. and Pestka, J. J. (1999) Effects of yogurt ingestion on mucosal and systemic cytokine gene expression in the mouse. *J. Food Prot.,* **62:** 181–188.

Hamilton-Miller J.M.T. (2001) A review of clinical trials of probiotics in the management of inflammatory bowel diesease. *Infect. Dis.Rev.,* **3:** 83–87.

Hammes, W. P. and Tichaczek, P. S. (1994) The potential of lactic acid bacteria for the production of safe and wholesome food. *Z. Lebensm. Unters. Forsch.,* **198:** 193–201.

Havenaar, R. and Huis in't Veld, J. H. (1992) Probiotics, a general view. In:The lactic acid brcteria Vol 1: " the lactic acid bacteria in health and disease" Wood, B.J.B. (Ed.), Elsevier, Amsterdam, pp1-200.

Havenaar, R., Brink, B. and Huis in't Veld, J. H. J. (1992) Selection of strains for probiotic use. In: Probiotics, the scientific basis, Fuller, R. (Ed.), Chapman and Hall, London, pp209–224.

Hilliam, M. (1998) Functional foods in Europe. The World of Ingredients March/April: pp 45–7.

Hirayama, K. and Rafter, J. (2000) The role of probiotic bacteria in cancer prevention. *Microbes Infect.,* **2:** 681-686.

Hoektra, F. A., Wolkers, W.F., Buitink, J., Golovina, E. A., crowe, J.H. and Crowe, L.M. (1997) Membrane stabilization in the dry state.*Comp. Biochem. Physiol.,* **17:** 335-341.

Hols, P., Kleerebezem, M., Schank, A.N., Ferain, T., Hugenholtz, J., Delcour, J. & de Vos, W.M. (1999a) Conversion of Lactococcus lactis from homolactic to homoalanine fermentation through metabolic engineering. *Nat. Biotechnol.,* **17:** 588–592.

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

Hugenholtz, J., Hols, P., Starrenburg, M.J.C., de Vos, W.M. & Kleerebezem, M. (2000) Metabolic engineering of *Lactococcus lactis* leading to high diacetyl production. *Appl. Environ. Microbiol.,* **66:** 4112–4114.

Hughes, D. B. and Hoover, D. G. (1991) *Bifidobacteria*: their potential for use in American diary products. *Food Technol.,* **April:**74–83.

Huijghebaert, S. M., Mertens, J. A. and Eyssen**,** H. J. **(**1982) Isolation of a bile salt sulfatase-producing *Clostridium* strain from rat intestinal microflora. *Appl. Enviro. Microbiol.,* **43:** 185–192.

Hull, R. R., Conway, P. L. and Evans, A. J. (1992) Probiotic foods- a new opportunity. *Food Australia,* **44:** 112–113.

Hurst, A. (1981) Nisin. *Adv. Appl. Microbiol.*, **27:** 85–123.

Hyronimus, B., Le Marrec, C., Hadj Sassi, A. and Deschamps, A. (2000) Acid and bile tolerance of spore-forming lactic acid bacteria. *Int. J. Food Microbiol.,* **61:** 193-197.

Ibrahim, S.A. and Bezkorovainy, A. (1993) Survival of bifidobacteria in the presence of bile salt. *Journal of the Science of Food and Agriculture,* **62:** 351– 354.

Ingram, M., Ottoway, F. J. H. and Coppock,J. B. M. (1956) The preservative action of acid substances in food. *Chem. Ind.,* **42:** 1154–1165.

Ishibashi, N. and Shimamura, S. (1993) *Bifidobacteria*: research and development in Japan. *Food Technol.,* **June:** 126–135.

Isolauri, E., Juntunen, M., Rautanen, T., Sillanaukee, P. and Koivula, T. (1991) A human *Lactobacillus* strain (*Lactobacillus casei* sp. strain GG) promotes recovery from acute diarrhea in children. *Pediatrics,* **88:** 90–97.

Isolauri, E., Majamaa, H., Arvola, T., Rantala, I., Virtanen, E. and Arvillomi, H. (1993) *Lactobacillus casei* strain GG reverses intestinal premeablity induced by cow milk in suckling rats. *Gastroentrology,* **105:** 1643-1650.

Isolauri, E., Sutas, Y., Kankaanpaa, P., Arvilommi, H. and Salminen, S. (2001) Probiotics: effects on immunity. *Am. J. Clin. Nutr.,* **73:** 444S-450S.

Jack, R. W., Tagg, J. R. and Ray, B. (1995). Bacteriocins of gram positive bacteria *Microbiol. Rev.,* **59:** 171-200.

Jacobsen, C. N., Rosenfeldt Neilsen, V., Hayford, A. E., Møller, P. L., Michaelsen, K. F., Pærregaard, A., Sandstro¨m, B., Tvede, M. and Jakobsen, M. **(**1999) Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Appl. Environ. Microbiol.,* **65:** 4949–4956.

Janda, M.J. and Abbott, S.L. (2002) Bacterial identification for publication: when is enough enough? *J. Clin. Microbiol.,* **40:** 1887–1891.

Janne Kerovuo, Marko Lauraeus, Paivi Nurminen, Nisse Kalkkinen and Juha Apajalahti, (1998) "Isolation, Characterization, Molecular Gene Cloning, and Sequencing of a Novel Phytase from *Bacillus subtilis*". *Appl. Environ. Microbial.,* **June:** 2079-2085.

Jin, L.Z., Ho, Y.W., Abdullah, N. and Jalaludin, S. (1998) Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. *Letters in Applied Microbiology.,* **27:** 183-185.

Johan Scnurer and Jesper Magnusson. (2005) Antifungal lactic acid bacteria as biopreservatives. *Trends in food science & Technology*, **16:** 70-78.

Johnson, J. A. C. and Etzel, M.R. (1995) Properties of *Lactobacillus helveticus* CNRZ-32 attenuated by spray drying, freeze drying or freezing. *J. Dairy Sci.* **78:** 761-768.

Johnson, L.R. (1977) Regulation of gastric secretion. Gastrointestinal Physiology, Mosby C.V. (Ed.), Saint Louis, pp. 62–69.

Kabir, A.M., Aiba,Y., Takagi, A., Kamiya, S., Miwa, T. and Koga, Y. (1997) Prevention of *Helicobacter pylori* infection by *lactobacilli* in a gnotobiotic murine model. *Gut.,* **41:** 49-55.

Kaila, A.M., Isolauri, E., Soppi, E., Virtanen, E., Laine, S. and Arvilommi, H. (1992) Enhancement of the circulating antibody secreting cell response in human diarrhea by human *Lactobacillus* strain. *Ped.Res.,* **32:** 141-144.

Kao, C. T. and Frazier, W. (1996). Effect of lactic acid bacteria on growth of *Staphylococcus aureus*. *Appl. Microbiol.,* **14:** 251-255.

Karasova, P., Spiwok, V., Mala, S., Kralova, B. and Russell, N.J. (2002) β-galactosidase activity in psychrotrophic microorganisums and their potential use in food industry. *Czech J. food Sci.,* **20:** 43-47.

Kashket, E. R. (1987) Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. *FEMS Microbiol. Rev.* **16:** 233–244.

Kashket, S. and Kashket, E. R. (1985) Dissipation of the proton motive force in oral *streptococci* by fluoride. *Infect. Immun.* **48:**19–22.

Katelaris, P.H., Salam, I. and Farthing, M. J. G. (1995) *Lactobacilli* to prevent traveler's diarrhea? *N. Engl. J. Med.,* **333:** 1360–1361.

Kato, I., Yokokura, T. and Mutai, M. (1983) Macrophage activation by *Lactobacillus* casei in mice. *Microbiology & Immunology*, **27:** 611-618.

Kearney, L., Upton, M. and McLaughlin, A. (1990) Enhancing the viability of *Lactobacillus plantarum* inoculum by immobilizing the cells in calcium-alginate beads incorporating cryoprotectants. *Appl. Environ. Microbiol.,* **10**: 3112-3116.

Keele, C.A. and Neil, E. (1965) Secretion of digestive juices. Samson Wright's Applied Physiology. Oxford Univ. Press, London, pp. 353–363.

Keiko Shirai, Revah-Moiseev, S., Garcia-Garcia-Garibay M. and Valerie M. Marshall. (1994) "Ability of some strains of lactic acid bacteria to degrade phytic acid" *Letters in Appl. Bacteriol.,* **19:** 366-369.

Kilara, A. and Shahani, K. M. (1975) Lactase activity of cultured and acidified dairy products. *J. Dairy Sci.,* **59:** 2031–2035.

Kim, S.S., & Bhowmilk, S.R. (1990) Survival of lactic acid bacteria during spray drying of plain yogurt. *J. Food Sci.,* **55:** 1008-1010.

Kim, W. S. and Dunn, N. W. (1997) Identification of a cold shock gene in lactic acid bacteria and the effect of cold shock on cryotolerance. *Curr. Microbiol.,* **35:** 59-63.

Kirjavainen, P.V., Ouwehand, A.C., Isolauri, E. and Salminen, S. (1998) The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiol. Lett.* **167:** 185–189.

Klaenhammer, T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews*, **12:** 39–86.

Klaver, F.A.M. and Van Der Meer, R. **(**1993) The assumed assimilation of cholesterol by *Lactobacillus* and *Bifidobacterium bifidum* is due to their bile salt-deconjugation activity. *Appl. Enviro. Microbiol.,* **59**: 1120-1124.

Klebanoff, S. J., Clem, W. H. and Luebke, R. G. (1966) The peroxidase-thiocyanate-hydrogen peroxide antimicrobial system. *Biochim. Biophys. Acta,* **117(1):** 63–72.

Kleerebezem, M and Hugenholtz, J. (2003) Metabolic pathway engineering in lactic acid bacteria. *Curr Opin Biotechnol.,* **14:** 232-237.

Kleerebezem, M., Beerthuyzen, M.M., Vaughan, E.E., de Vos, W.M. & Kuipers, O.P. (1997) Controlled gene expression systems for lactic acid bacteria: transferable

nisin inducible expression cassettes for *Lactococcus*, *Leuconostoc* and *Lactobacillus* spp. *Appl. Environ. Microbiol.*, **63:** 4581–4584.

Knorr, D. (1998). Technology aspects related to microorganisms in functional foods. *Trends food Sci. Technol.*, **9**, 295-306.

Kolars, J. C., Levitt, M. D., Aouji, M. and Savaiano, D. A. (1984) Yogurt- an autodigesting source of lactose. *N. Engl. J. Med.*, **310(1):** 1–3.

Kong, S. and Davison, A. J.(1980) The role of interactions between O2, H2O2, OH, e– and O2–in free radical damage to biological systems. *Arch. Biochem. Biophys.*, **204:** 13–29.

Kroll, R. G. and Patchett, R. A. (1992) Induced acid tolerance in *Listeria monocytogenes. Lett. Appl. Microbiol.* 14: 224–227.

Kruis, W., Schutz, E., Fric, P., Fixa, B., Judmaier, G. and Stolte, M. (1997) Double-blind comparison of an oral *Escherichia coli* preparation and mesalazine in maintainingremission of ulcerative colitis. *Aliment Pharmacol Ther.*, **11:** 853:858.

Kuan-Yuan Wang, Shui-Nin Li, Chiang-Shin Liu, Daw-Shyong Perng, Yu-Chung Su, Deng-Chyang Wu,Chang-Ming Jan, Chun-Huang Lai, Tsu-Nai Wang and Wen-Ming Wang. (2004) Effects of ingesting *Lactobacillus*- and *Bifidobacterium*-containing yogurt in subjects with colonized *Helicobacter pylori. Am. J. Clin. Nutr.*, **80:** 737– 741.

Kuipers, O.P., de Ruyter, P.G.G.A., Kleerebezem, M. & de Vos, W.M. (1998) Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotech.*, **64:** 15–21.

Lankaputhra, W.E.V. and Shah, N.P. (1995) Survival of Lactobacillus acidophilus and Bifidobacterium spp. in the presence of acid and bile salts. *Cult. Dairy Prod. J.*, **30:** 2 –7.

Lan-szu chou, and Bart weimer. (1999) Isolation and Characterization of acid and Bile-tolerant isolates from Strains of *Lactobacillus acidophilus*. *J.Diary Sci.*, **82:** 23-26.

Le Vay, D. (1988) . 13 Digestion. Human Anatomy Physiology, 3rd ed. Hodder & Stoughton, (Ed.), Sevenoaks, Kent, Teach Yourself. pp. 231– 233.

Leavitt, J., Barrett, J. C., Crawford, B. D. and Tso, P. O. P. (1978) Butyric acid suppression on the *in vitro* neoplastic state of Syrian hamster cells, *Nature*, **271:** 262.

Lee, Y.-K. and Salminen, S. (1995) The coming of age of probiotics. *Trends in Food Sci. Tech.,* **6:** 241-245.

Lee, Y.W., Roh, W.S. and Kim, J.G. **(**1992) Benefits of fermented milk in rats fed by hypercholesterolemic diet (II). *Korean Journal of Food Hygiene,* **7:** 123-135.

Lehto, E. M. and Salminene, S. (1997). Adhesion of two Lactobacillus strains, one *Lactococcus* and one *Propionibacterium* strain to cultured human intestinal Caco-2 cell line. *Biosci. Microflora* , **16:** 13-17.

Leim, I. T., Steinkraus, K. H. and Cronk, T. C. (1977) Production of vitamin B-12 in tempeh, a fermented soybean food. *Appl. Environ. Microbiol.,* **34:** 773–776.

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H. and Crowe, L. M. (1995) Trehalose and sucrose protect both membranes and protein in intact bacteia during drying. *Appl. Environ. Microbiol.,* **61:** 3592-3597.

Lilley, D. M., Stellwel, R. H. (1965). Probiotics. Growth promoting factors produced by microorganisms. *Science,* **147:** 747-748.

Lin, M.-Y., Savaiano, D. and Harlander, S. (1991) Influence of nonfermented dairy products containing bacterial starter cultures on lactose maldigestion in humans. *J. Dairy Sci.*, **74:** 87–95.

Lister, J. (1873) A further contribution to the natural history of bacteria and the germ theory of fermentative changes. *Quart. J. Microsc. Sci.,* **13:** 380–408.

Mallett, A. K., Bearne, C. A. and Rowland, I. R. (1989) The influence of incubation pH on the activity of rat and human gut flora enzymes. *J. Appl. Bacteriol.,* **66:** 433–437.

Mamaeva, P. (1956) Dry Koumiss culture. *Dairy Sci. Abstr.,* **18:** 639.

Mann, G.V. and Spoerry, A. (1974) Studies of a surfactant and cholesterolemia in the Massai. *Am. J. Clin. Nutr.*, **27:** 464- 469.

Maria De Angelis, Giovanna Gallo, Maria Rosaria Corbo, Paul, L.H., McSweeney, Michele Faccia, Marinella Fiovine and Marco Gobbetti. (2003) "Phytase activity in sourdough lactic acid bacteria: purification and characterization of phytase from *Lactobacillus sanfranciscensis* CB1". *Int.J.Food Microbiol.,* **87:**259-270.

Marin, M. L., Tejada-Simon, M. V., Lee, J. H., Murtha, J., Ustunol, Z. and Pestka, J. J. (1998) Stimulation of cytokine production in clonal macrophage and T-cell

models by *Streptococcus thermophilus*: comparison with *Bifidobacterium* sp. and *Lactobacillus bulgaricus*. *J. Food Prot.*, **61:** 859–864.

Marteau, P., Flourie, B., Pochart, P., Chastang, C., Desjeux, J. F. and Rambaud, J. C. **(**1990) Effect of the microbial lactase (EC 3.2.2.23) activity in yoghurt on the intestinal absorption of lactose: an *in vivo* study in lactase deficient humans. *Br. J. Nutr.,* **64:** 71–79.

Marteau, P.R., Vrese de, M., Cellier, C.J. and Schrezenmeir, J. (2001) Protection from gastrointestinal diseases with the use of probiotics. *Am. J. Clin. Nutr.,* **73:** 430S-436S.

Martini, M. C., Lerebours, E.C., Lin, W. J., Harlander, S. K., Berrada, N.M., Antoine, J.M. and Savaiano, D.A. (1991) Strains and species of lactic acid bacteria in fermented milks (yogurts): effect on in vivo lactose digestion. *Am. J. Clin. Nutr.*, **54:** 1041-1046.

Martini, M. C., Kukielka, D. and Savaiano, D. A. **(**1991) Lactose digestion from yogurt: influence of a meal and additional lactose. *Am. J. Clin. Nutr*. **53:** 1253–1258.

Mastretta, E., Longo, P., Laccisaglia, A., Balbo, L., Russo, R., Mazzaccara, A. and Gianino, P. (2002) Effect of *Lactobacillus* GG and breast-feeding in the prevention of rotavirus nosocomial infection. *J. Pediatr. Gastroenterol. Nutr.*, **35:** 527–531.

Matsuzaki, T., Yamazaki, R., Hashimoto, S. and Yokokura, T. (1998) The effect of oral feeding of Lactobacillus casei strain Shirota on immunoglobulin E production in mice. *J. Dairy Sci.*, **81:** 48–53.

Mattila-Sandholm, T., Blum,S., Collins, J.K., Crittenden,R., De Vos, W., Dunne, C., Fonden, R., Grenov, G., Isolauri, E., Kiely, B., Marteau, P., Morelli, L., Ouwehand, A., Reniero, R., Saarela, M., Salminen, S., Saxelin, M., Schiffrin, E., Shanahan, F., Vaughan, E. and Von wright, A. (1999) Probiotics: towards demonstrating efficacy. *Trends Food Sci. Technol.*, **10:** 393-399.

Mauriello, G., Aponte, M., Andolfi, R., Moschetti, G. and Villani, F. (1999) Spray drying of bacteriocin producing lactic acid bacteria. *J. Food Prot.*, **62**: 773-777.

McDonald, L. C., Fleming, H. P. and Hassan, H. M. (1990) Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum. Appl. Environ. Microbiol.* **56:** 2120–2124.

McFarland, L. V., Surawicz, C.M., Greenberg, R. N., Elmer, G.W., Moyer, K. A., Melcher, S. A., Bowen, K. E. and Cox, J. L. (1995) Prevention of beta-lactam-

associated diarrhea by Saccharomyces boulardii compared with placebo. *Am. J. Gastroenterol.*, 90, 439–448.

McKay, L. L. (1985) Roles of plasmids in starter cultures, in Bacterial Starter Cultures for Foods, Gilliland, S. E., (Ed.), CRC Press Inc., Boca Raton, Florida, 159–174.

McKay, L.L. and Baldwin, A.B. (1990). Applications of biotechnology: present and future improvements in lactic acid bacteria. *Fed. Er. Microbiol. Soc. Rev.*, **87:** 3.

Mercenier, A., Pouwels, P. H. and Chassy, B. M. (1994) Genetic engineering of *lactobacilli*, *leuconostocs* and *Streptococcus thermophilus*. In: Genetics and biotechnology of lactic acid bacteria, Gasson, M. J. and DeVos, W. M. (Eds.), Blackie Academic & Professional, New York, pp 253–293.

Metchnikoff, E. (1907) The Prolongation of Life. *Heinemann*, London,

Metchnikoff, E. (1908) The prolongation of life. Putnam's Sons: New york.

Metwally, M. M., Abd El-Eawad, I. A., El-Nochrashy, S. A. and Ahmad K. E. (1989) Spray- drying of lactic acid cultures I; the effect of spray-drying conditions on the survival of microorganisums. *Egypt. J. Dairy Sci.* , **17:** 35-43.

Michetti, P., Dorta, G., Wiesel, P. H., Brassart, D., Verdu, E., Herranz, M., Felley, C., Porta, N., Rouvet, M., Blum, A. L. and Corthesy-Theulaz, I.(1999) Effect of whey-based culture supernatant of *Lactobacillus acidophilus (johnsonii)* La1 on *Helicobacter pylori* infection in humans. *Digestion*, **60:** 203–209.

Midolo, P.D., Lambert, J. R., Hull, R., Luo, f. and Grayson, M.L.(1995) *In vitro* inhibition of *Helicobacter pylori* NCTC 11637 by organic acids and Lactic acid bacteria. *J. Appl. Bacteriol.*, **79:** 475-479.

Miettinen, M., Matikainen, S., Vuopio-Varkila, J., Pirhonen, J., Varkila, K., Kuri-moto, M. and Julkunen, I. (1998) *Lactobacilli* and *streptococci* induce interleukin-12 (IL-12), IL-18, and gamma interferon production in human peripheral blood mononuclear cells. *Infect. Immun.* **66:** 6058–6062.

Mikelsaar, M, Malnder, R. and Sepp, E. (1998) Lactic acid microflora in the human microbial ecosystem and its development. In: Lactic Acid Bacteria: Microbiology and Functional Aspects, Sasminen, S. and von Wright, A. (Eds.), 2nd Edn., Marcel Dekker, New York. pp 279-342

Miller, D. P., Anderson, R. E. and De Pablo, J. J. (1998) The stabilization of lactate dehydrogenase following freeze thawing and vacuum-drying of trehalose and borate. *Pharm. Res.*, **15:** 1215-1221.

Miller, D. P., De Pablo, J. J. and Corti, H. R. (1997) Thermophysical properties of trehalose and its concentrated aqueous soluctions. *Pharm. Res.* **14:** 578-590.

Mishra, V. and Prasad D.N. (2005) Application of in vitro methods for selection of *Lactobacillus casei* strains as potential probiotics. *Int. J. Food Microbiol.*, **103:** 109–115.

Misra, A.K. and Kuila, R.K. (1991) Intensified growth of *Bifidobacterium* and preparation of *Bifidobacterium bifidum* for a dietary adjunct. *Cult. Dairy Prod. J.*, **26:** 4-6

Miyamoto-Shinohara, Y., Imaizumi, T., Sukenobe, J., Murakami, Y., Kawamura, S. and Komatsu, Y. (2000) Survival rate of microbes after freeze-drying and long-term storage. *Cryobiology*, **41**: 251-255.

Modler, H. W., McKellar, R. C. and Yaguchi, M. (1990) *Bifidobacteria* and bifidogenic factors. *Can. Inst. Food Sci. Techol.*, **23:** 29–41.

Mora, D., Fortina, M.G., Parini, C., Daffonchio, D. and Manachini, P. L. (2000) Genomic subpopulation within the species *Pediococcus acidilactici* detected by multilocus typing analysis: relationships between pediocin AcH/PA-1 producing and non-producing strains. *Microbiol.*, **146:** 2027-2038.

Morice, M., Bracquart, P. and Linden, G. (1992) Colonial variation and freeze-thew resistance of Streptococcus thermophilus. *J. Dairy Sci.*, **75**: 1197-1203.

Morris, J. G. (1979) Nature of oxygen toxicity in anaerobic microorganisms, In: Strategies of microbial life in extreme environments, Shilo, M. (Ed.), Verlag Chemi, Berlin, pp 149–162.

Moser, S.A. and Savage, D.C. (2001) Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. *Appl. Environ. Microbiol.*, **67:** 3476–3480.

Muscettola, M., Massai, L., Tanganelli, C. and Grasso, G. (1994) Effects of lactobacilli on interferon production in young and aged mice. *Ann. N.Y. Acad. Sci.*, **717:** 226–232.

Nader de Macias, M. E., Apella, M. C., Romero, N. C., Gonzalez, S. N. and Oliver, G. (1992) Inhibition of *Shegella sonnei* by *lactobacillus casei* and *L. acidophilus. J. Appl. Bacteriol.*, **73:** 407-411.

Nanji, A. A., Khettry, U. and Sadrzadeh, S. M. H. (1994) *Lactobacillus* feeding reduces endotoxemia and severity of experimental alcoholic liver (disease). *Proc. Soc. Exp. Biol. Med.*, **205:** 243-247.

Nes, I. F., Diep, D. B., Havarstein, L. S., Bruberg, M. B., Eijsink, V. and Holo, H. (1996) Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leewenhoek,* **70:** 113–128.

Neumann, E., Oliveira, M. A., Cabral, C. M., Moura, L. N., Nicoli, J. R., Vieira, E. C., Cara, D. C., Podoprigora, G. I. and Vieira, L. Q. (1998) Monoassociation with *Lactobacillus acidophilus* UFV-H2b20 stimulates the immune defense mechanisms of germfree mice. *Braz. J. Med. Biol. Res.*, **31:** 1565–1573.

Nicaise, P., Gleizes, A., Forestier, F., Quero, A. M. and Labarre, C. (1993) Influence of intestinal bacterial flora on cytokine (IL -1, IL-6 and TNF-alpha) production by mouse peritoneal macrophages. *Eur. Cytokine Netw.*, **4:** 133–138.

Noh, D. O. and Gilliland, S. E. (1993). Influence of bile on cellular integrity and galactosidase activity of *L. acidophilus. J. Dairy Sci.*, **76:** 1253-1259.

Noh, D. O., Kim, S. H. and Gilliland**,** S. E. **(**1997) Incorporation of cholesterol into the cellular membrane of *Lactobacillus acidophilus* ATCC 43121. *J. Dairy Sci.,* **80:** 3107–3113.

O'Driscoll, B., Gahan, C. G. M. and Hill, C. (1996) Adaptive acid tolerance in *Listeria monocytogenes*: isolation of an acid tolerant mutant which demonstrates increased virulence. *Appl. Environ. Microbiol.* **62:**1693–1698.

O'Halloran, S., Feeney, M., Morrissey, D., Murphy, L., Thornton, G., Shanahan,F., O'Sullivan, G. C. and Collins, J. K. (1997). Adhesion of potential probiotic bacteria to human epithelial cell lines. Poster in conference: Functional Foods: Designer Foods for the Future, Cork, Ireland

Oconnier, M.-H., Klaenhammer, T.R., Kerneis, S., Bernet, M.-F. and Servin, A.L.(1992) Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. *Appl. Environ. Microbiol.* **58:** 2034– 2039.

Ogawa, M., Shimizu, K., Nornoto,K., Takhashi, M., Watanuki, M., Tanaka, R., Tanaka, T., Harnabata, T., Yarnasaki, S. and Takeda, Y. (2001) Protective effect of *Lactobacellus casei* strain Shirota on shiga toxin-producing *Escherichia coli* O157-H7 infection in infant rabbits. *Infect immune,* **69:** 1101-1108.

Oksanen, P.J., Salminen, S. and Saxelin, M. (1990) Prevention of traveler's diarrhea by *Lactobacillus GG*. *Ann. Med.* **22:**53-60.

Olimpia Pepe, Giuseppe Blaiotta, Marilena Anastasio, Giancarlo Moschetti, Danilo Ercolini, and Francesco Villani. (2004) Technological and Molecular Diversity of *Lactobacillus plantarum* Strains Isolated from Naturally Fermented Sourdoughs. *System. Appl. Microbiol.,* **27:** 443–453.

Olson, H. C. (1959) Preservation of lactic acid bacteria. *J. Dairy Sci.,* **42:** 388.

Ouwehand, A. C., Kirjavainen, P. V., Gronlund, M. M., Isolauri, E. and Salminen, S. J. (1999). Adhesion of probiotic microorganisms to intestinal mucs. *Int. Dairy J.*, **9:** 623-630.

Ouwehand, A. C., Niemi, P. and Salminen, S. J. (1999). The normal fecal microflora does not affect the adhesion of probiotic bacteria *in vitro*. *FEMS Microbiol. Lett.*, **177:** 35-38.

Ouwehand, A.C., Salminen, S. and Isolauri, E. (2002) Probiotics: an overview of beneficial effects. *Anton. Leeuwen.,* **82:** 279–289.

Panoff, J. M., Legrand, S., Thammavongs, B. and Boutibonnes, P. (1994) The cold shock response in *Lactococcus lactis* subsp. Lactis. *Curr. Microbiol.*, **29**: 213-216.

Panoff, J. M., Thammavongs, B., Gueguen, M. and Boutibonnes, P. (1998) Cold stress responses in mesophilic bacteria. *Cryobiology*, **36:** 75-83.

Panoff, J., Thammavongs, B., Laplace, J., Hartke, A., Boutibonnes, P. and Auffray, Y. (1995) Cryotolerance and cold adaptation in *Lactococcus lactis* subsp. *lactis IL* 1403. *Cryobiology*, **32**: 516-520.

Pant, A. R., Graham, S.M., Allen, S. J., Harikul, S., Sabchareon, A., Cuevas, L. and Hart, C. A. (1996) *Lactobacillus GG* and acute diarrhoea in young children in the tropics. *J. Trop. Pediatr.*, **42:** 16–25.

Pelto, L., Isolauri, E., Lilius, E. M., Nuutila, J. and Salminen, S. (1998) Probiotic bacteria down-regulate the milk-induced inflammatory response in milk -hypersensitive subjects but have an immunostimulatory effect in healthy subjects. *Clin. Exp. Allergy* **28:** 1474–1479.

Perdigon, G., Alvarez, S. and Pesce de Ruiz Holgado, A. (1991) Immunoadjuvant activity of oral *Lactobacillus casei*: influence of dose on the secretory immune

response and protective capacity in intestinal infections. *J. Dairy Res.*, **58:** 485–496.

Perdigon, G., Alvarez, S., Rachid, M., Aguero, G. and Gobbato, N. (1995) Immune system stimulation by probiotics. *J. Dairy Sci.*, **78:** 1597–1606.

Peter, G. and Reichart, O. (2001) The effect of growth phase, cryoprotectants and freezing rates on the survival of selected microorganisms during freezing and thawing. *Acta Aliment Hung.*, **30:** 89-97.

Phadtare, S. and Inouye, M. (2001) Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in *Escherichia coli*. *J. Bacteriol.*, **183:** 1205-1214.

Piard, J. C. and Desmazeaud, M. (1992) Inhibiting factors produced by lactic acid bacteria. II. Bacteriocins and other antibacterial substances. *Lait*, **71:** 525–541.

Pocard, J. A., Smith, L. T., Smith, G. M. and Le Rudulier, D. (1994) A prominent role for glucosylglycerol in the adaptation of pseudomonas mendocina SKB70 to osmotic stress. *J. Bacteriol.*, **176:** 6877-6884.

Pochart, P., Dewit, O., Desieux, J. F. and Bourlioux, P. (1989) Viable starter cultures, beta-galactosidase activity, and lactose in duodenum after yoghurt ingestion in lactase-deficient humans. *Am. J. Clin. Nutr.*, **49:** 828–831.

Poirier, I., Marechal, P. A. and Garvais, P. (1998) Survie des bacteries lactiques soumises a la deshydratation: importance de la cinetique de deshydratation. *Lait.*, **78:** 173-180.

Porubean, R.S. and Sellars, R.L. (1975). Spay drying of yogurt and related cultures. *J. Dairy Sci.*, **58:** 787.

Potts, M. (1994) Desiccation tolerance of prokaryotes. *Microbiol. Rev.*, **58:** 755-805.

Prasad, J., McJarrow, P. and Gopal, P. (2003) Heat and osmotic stress responses of probiotic Lactobacillus rhamnosus HN001 (DR20) in relation to viability after drying. *Appl. Environ. Microbiol*., **69**: 917-925.

Pringle, M. J. and Chapman, D. (1981) Biomembrane structure and effects of temperature. In: "Effects of low temperature on biological systems", Morris G. J. and Clark, A. (Eds.), Academic Press, New York, P. 21.

Raoult, D., Fournier, P.-E. and Drancourt, M. (2004) What does the future hold for clinical microbiology. *Nat. Rev.,* **2:** 151–159.

Rasic, J. L. and Kurmann, J. A. (1983) Bifidobacteria and their role. In: Experientia supplementum, Vol. 39, Birkhauser Verlag, Basel.

Ray, B. and Daeschel, M. (1992). Food biopreservatives of microbial origin. In: Boca Raton, CRC Press, Florida: pp 3-11

Reddy, K. P., Shahani, K. M. and Kulkarni, S. M. (1976) B-complex vitamins in cultured andacidified yogurt. *J. Dairy Sci.*, **59:** 191–195.

Rhodes, D.J.(1993) Formulation of biocontrol agents. In: Exploitation of Microorganisms, Jones,D. G. (Ed.), Chapman & Hall Press, London. pp.411-439.

Riley, M.A. and Wertz, J.E. (2002) Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimica,* **84:** 357–364.

Roberfroid, M. B. (2000) Prebiotics and probiotics: are they functional foods?. *Am. J. Clin. Nutr.,* **71**: 1682S-1687S.

Roberfroid, M.B. (1998) Prebiotics and synbiotics: concepts and nutritional properties. Br *J. Nutr.*, **80:** S197–S202.

Rodrı´guez, J.M., Martı´nez, M.I., Horn, N. and Dodd, H.M. (2003) Heterologous production of bacteriocins by lactic acid bacteria. *Int. J. Food Microbiol.,* **80:** 101– 116.

Rogers, L.A. (1914). The preparation of dried cultures. *J. Infect. Dis.,* **14:** 100.

Rombeau, J. L., Kripke, S. A. and Settle, R. G. (1990) Short-chain fatty acids. Production, absorption, metabolism and intestinal effects. In: Dietary fiber: chemistry, physiology, and health effects, Kritchevsky, D., et al., (Ed.) , Plenum Press, New York and London, pp 317–337.

Rossello-Mora, R. and Amann, R. (2001) The species concept for prokaryotes. *FEMS Microbiol. Rev.,* **25:** 39–67.

Saavedra, J. M. (1995) Microbes to fight microbes: a not so novel approach to controlling diarrheal disease. *J. Pediatr. Gastroenterol. Nutr.*, **21(2):** 125–129.

Saavedra, J. M. (1999). Probiotics plus antibiotics. Regulating our bacterial envinorment. *J. Pediater.*, **135:** 535-537.

Saavedra, J.M., Bauman, N. A., Oung, I., Perman, J. A. and Yolken, R. H. (1994) Feeding of Bifidobacterium bifidum and Streptococcus thermophilus to infants in hospital for prevention of diarrhea and shedding of rotavirus. *Lancet,* **344:** 1046–1049.

Salminen, S., Isolauri, E. and Salminen, E. (1996). Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. *Antonie van Leeuwenhoek.* **70:** 347-358.

Sambrook, J. and Russell, D.W. (2001) Molecular cloning: A laboratory manual, 3rd edition cold spring harbor laboratory, cold spring Harbor, NY.

Sanders, M. E. and In't Veld, J. H. (1999) Bringing a probiotic-containing functional food to the market: Microbiological, product, regulatory and labeling issues. In Proc. 6th Symp. Lactic Acid Bacteria: Genetics, Metabolisms and Applications. Konings, W. N., Kuipers, O. P. and Huis in't Veld, J. H. J. (Eds.), Kluwer Academic Publishers, Amsterdam. pp 293–315.

Sanders, M. E. (1993) Healthful attributes of bacteria in yogurt, Contemporary Nutr., vol. 18: General Mills, Minneapolis, MN, pp.1–2.

Sanders, M. E. (1994) Lactic acid bacteria as promoters of human health. In: Functional foods: designer foods, pharmafoods, nutraceuticals, Goldberg, I. (Eds.), Chapman & Hall, NewYork, pp294–322.

Sanders, M. E. (1999). Probiotics. *Food Technology,* **53:** 67-77.

Sanders, M.E. (1998) Development of consumer probiotics for the U.S. market. *Br. J. Nutr.,* **80:** S213–S218.

Sanders, M.E. (1993) Summary of the conclusions from a consensus panal of experts on the attributes on lactic cultures: significance to fluid milk products containing cultures. *J. Diary Sci.,* **76:** 1819-1828.

Sangeetha, P. T., Ramesh ,M. N. and Prapulla, S. G. (2005) Recent trends in the microbial production analysis and application of frutooligosaccharide. *Trends in food Sci. Technol.,* **16:** 442-457.

Sato, K. (1984). Enhancement of host resistance against *Listeria* infections by *lactobacillus casei*: role of macrophages. *Infect. Immun.* , **44:** 445-451.

Savage, D. C. **(**1992) Gastrointestinal microbial ecology: possible modes of action of direct-fed microbials in animal production—a review of the literature, In: Direct-fed microbials in animal production. (National Feed Ingredients Association, West Des Moines, Iowa), pp 11–81.

Savage, D. C., Lundeen, S. G. and O'Connor, L. T. **(**1995) Mechanisms by which indigenous microorganisms colonise epithelial surfaces as a reservoir of the lumenal microflora in the gastrointestinal tract. *Microecolloids and Therapeutics*, **21:** 27–36.

Savaianao, D.A. and Lewitt, M.D. (1987) Milk intolerance and microbe- containing dairy foods. *Journal of Dairy Science*, **70:** 397-406.

Saxelin, M., Pessi, T. and Salminen, S. (1995). Fecal recovery following oral administration of *Lactobacillus strain GG* (ATCC 53103) in gelatine capsules to healthy volunteers. *Int. J. Food Microbiol.* **25:** 199-203.

Schillinger, U. and Lucke, F.-K. (1987) Identification of lactobacilli from meat and meat products. *Food microbiology.,* **4,** 199-208.

Schmidt, E.J., Boswell, J.S., Walsh, J.P., Schellenberg, M.M., Winter, T.W., Li, C., Allman, C.W. and Savage, P.V. (2001) Activities of cholic acid-derived antimicrobial agents against multidrug-resistant bacteria, *J. Antimicrob. Chemother*, **47:** 671–674.

Searchy R. L. and Bergquist, L. M. (1960) "New colour reaction for the quatification of serum cholesterol". *Clinical Chimica Acta,* **59:** 192-199.

Seher Demirer, M.D., Semih Aydýntug, M.D., Belma Aslým, M.D., Ilknur Kepenekci, M.D., Neriman Sengül, M.D., Oya Evirgen, M.D., Devran Gerceker, M.D.,Meltem Nalca Andrieu, M.D., Cemal Ulusoy, M.D. and SercinKarahüseyinoglu, M.D. (2006) Effects of probiotics on radiation-induced intestinal injury in rats. *Nutrition.,* **22:** 179-186.

Shahani, K. M. and Chandan, R. C. (1979) Nutritional and healthful aspects of cultured and culture-containing dairy foods. *J. Dairy Sci.,* **62:** 1685–1694.

Shaper, A.G., Jones, K.W., Jones, M. and Kyobe, J. (1963) Serum lipids in three nomadic tribes of northern Kenya. *Am. J. Clin. Nutr.,* **13:** 135–146.

Shermak, M. A., Saavedra, J. M., Jackson, T. L., Huang, S. S., Bayless, T. M. and Perman, J. A. (1995) Effect of yogurt on symptoms and kinetics of hydrogen production in lactosemalabsorbing children. *Am. J. Clin. Nutr.*, **62:** 1003–1006.

Shornikova, A.V., Casas, I., Mykkanen, H., Salo, E. and Vesikari, T. (1997) Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis. *Ped. Infect. Dis. J.*, **16:** 1103-1107.

Siitonen, S., Vapaatalo, H., Salminen, S., Gordin, A., Saxelin, M., Wikberg, R. and Kirkkola, A. L. (1990). Effects of *Lactobacillus GG* yoghurt in prevention of antibiotic associated diarrhea. *Ann. Med.* **22:** 57-59.

Silva, M., Jacobus, N. V., Deneke, C. and Goebach, S. (1987). Antimicrobial substance from a Human lactobacillus strain. *Antimicro. Agentis Chemother.,* **31:** 231-233.

Smid, E. J, van Enckevort, F. J, Wegkamp, A., Boekhorst, J., Molenaar, D., Hugenholtz, J., Siezen, R.J. and Teusink, B. (2005) Metabolic models for rational improvement of lactic acid bacteria as cell factories. *J. Appl. Microbiol.* **98:**1326-1331.

Smith, T. (1995) The digestive system. The Human Body. Ken Fin Books, Collingwood, pp. 150– 173.

Soomro, A. H., Masud, T. and Kiran anwaar. (2002). Role of Lactic acid bacteria (LAB) in Food preservation and Human health – A review. *Pakistan journal of Nutrition* **1:** 20-24.

Souzo, H. (1992) Freeze drying of microorganisum, Encyclopedia of microbiology, vol.2, Academic press, New York.

Speck, M. L., Dobrogosz, W. J. and Casas, I. A. (1993) *Lactobacillus reuteri* in food supplementation. *Food Technol.,* **90:** 92–94.

Sreeramulu, G., Srinivasa, D.S., Nand, K., and Joseph, R. (1996) "*Lactobacillus amylovorous* as a phytase producer in submerged culture". *Letters in Applied Bacteriology.,* **23:** 385-388.

Stevens, C. E. and Hume, I. D. **(**1995) Comparative physiology of the vertebrate digestive system, United Kingdom , Cambridge: Cambridge University Press. pp-245–246.

Sugita, T., and Togawa, M. (1994) Efficacy of *Lactobacillus* preparation Biolactis powder in children with rotavirus enteritis. *Jpn. J. Pediatr.,* **47:** 2755-2762.

Surawicz, C.M., Elmer, G.W., Speelman, P., McFarland, L. V., Chinn, J. and van Belle, G. (1989) Prevention of antibiotic associated diarrhea by *Saccharomyces boulardii*. A prospective study. *Gastroenterology,* **96:** 981–988.

Svensson, U. (1999) Industrial perspectives, In Probiotics: A critical review. Tannock, G.W. (Eds.), Wymondham, UK: Horizon scientific Press.

Sybesma, W., Burgess, C., Starrenburg, M., van Sinderen, D. and Hugenholtz, J. (2004) Multivitamin production in *Lactococcus lactis* using metabolic engineering. *Metab. Eng.,* **6:** 109-15.

Szajewska, H., Kotowska, M., Mrukowicz, J. Z., Armanska, M. and Mikolajczyk, W. (2001) Efficacy of *Lactobacillus GG* in prevention of nosocomial diarrhea in infants. *J. Pediatr.,* **138:** 361–365.

Tahri, K., Grill, J. P. and Schneider, F. (1997) Involvement of trihydroxyconjugated bile salts in cholesterol assimilation by bifidobacteria. *Current Microbiology* , **34:** 79–84.

Tahri, K., Grill, J. P. and Schneider, F. (1996) Bifidobacteria strains' behaviour toward cholesterol: coprecipitation with bile salts and assimilation. *Current Microbiology,* **33:** 187–193.

Tamime, A. Y., Marshall, V. M. E. and Robinson, R. K. (1995) Microbiological and technical aspects of milks fermented by Bifidobacteria. *J. Dairy Res.,* **62:** 151–187.

Tamine, A.Y. (1981). Microbiology of starter cultures". In: Dairy Microbiology, Vol. II, R.K. Robinson, (Eds). Pergamon Press, New York, NY. pp113

Tamura, N., Norimoto, M., Yoshida, K., Hirayama, C. and Nakai, R. (1983). Alteration of fecal bacterial flora following oral admininstation of bifidobacterial preparation. *Gastroenterol. Jpn.,* **18:** 17-24.

Tannock, G. (1981). Microbial interference in the gastrointestinal tract. *Asean J. Clin. Sci.,* **2:** 2-34.

Tannock, G. W. (1992) The lactic microflora of pigs, mice and rats. In: The lactic acid bacteria, vol. 1, Wood, B. J. B. (Eds.), The lactic acid bacteria in health and disease. London: Elsevier Applied Science, pp-21–48.

Tannock, G. W., Dashkevicz, M. P. and Feighner, S. D. (1989) Lactobacilli and bile salt hydrolase in the murine intestinal tract. *Appl. Environ. Microbiol.,* **55:** 1848–1851.

Teixeira, P., Castro, H. and Kirby, R. (1995) Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*. *J. Appl. Bacteriol.* **78:** 456-462.

Teixeira, P., Castro, H., Mohacsi- Farkas, C. and Kieby, R.(1997) Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. *J. Appl. Microbiol.*, **83**: 219-226.

Teixeira, P.C., Castro, M.H., Malcata, F.X. and Kirby, R.M. (1995) Survival of Lactobacillus delbrueckii ssp. Bulgaricus following spray drying. *J. Dairy Sci.*, **78**: 1025-1031.

Tejada-Simon, M. V., Lee, J. H., Ustunol, Z. and Pestka, J. J. (1999a) Ingestion of yogurt containing Lactobacillus acidophilus and fidobacterium to potentiate immunoglobulin A responses to cholera toxin in mice . *J. Dairy Sci.*, **82:** 649–660.

Tejada-Simon, M. V., Ustunol, Z. and Pestka, J. J. (1999b) Effects of lactic acid bacteria ingestion of basal cytokine mRNA and immunoglobulin levels in the mouse. *J. Food Prot.*, **62:** 287–291.

Ten Brink, B. and Konings, W. N. (1982) Electrochemical proton gradient and lactate concentration gradient in *Streptococcus cremoris* cells grown in batch culture. *J. Bacteriol.*, **162:**383–390.

Thammavongs, B., Corroler, D., Panoff, J. –M., Auffray, Y. and Boutibonnes, P. (1996) Physiological response of Entrococcus faecalis JH2- 2 to cold shock: growth at low temperature and freezing/thawing challenge. *Lett. Appl. Microbiol.*, **23:** 398-402.

Tissier, H. (1905) Taxonomy and ecology of Bifidobacteria. *Bifidobacteria Microflora*, **3:** 11–28.

Tuomola, E. and Salminen, S.J. (1998) Adhesion of some probiotic and dairy Lactobacillus strains to Caco-2 cell cultures. *Int. J. Food Microbiol.* **41:** 45–51.

Tuomola, E. M. and Salminen, S. J. (1998). Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *Int. J. Food Microbiol.*, **41:** 45-51.

Tuomola, E.M., Ouwehand, A.C. and Salminen, S.J. (1999) Human ileostomy glycoproteins as a model for small intestinal mucus to investigate adhesion of probiotics. *Lett. Appl. Microbiol.*, **28:** 159–163.

Usman, H. A. (1999) Bile tolerance, taurocholate deconjugation, and binding of cholesterol by *Lactobacillus gasseri* strains. *Journal of Dairy Science*, **82:** 243–248.

Van Eldere, J., Robben, J., De Pauw, G., Merckx, R. and Eyssen, H. **(**1988) Isolation and identification of intestinal steroid-desulfating bacteria from rats and humans. *Applied Environmental Microbiology* ,**54:** 2112–2117.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. and Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.*, **60:** 407–438.

Vaughan, E. E. and Moller, B. (1999). Probiotics in the new millennium. *Nahrung* **43:** 148-153.

Walker, D. K. and Gilliland, S. E. (1993) Relationships among bile tolerance, bile salt deconjugation, and assimilation of cholesterol by *Lactobacillus acidophilus*. *J. Dairy Sci.*, **76:** 956-961.

Weber, M. H. W. and Marahiel, M. A. (2002) Coping with the cold. *Phil. Trans. R. Soc. Lond.*, **B 357**: 895-907.

Whitaker, R. D. and Batt, C. A. (1991) Characteization of the heat shock response in *Lactobacillus lactis* subsp. *Lactis*. *Appl. Environ. Microbiol.,* **57:** 1408-1412.

Wolf, B. W., Garleb, K. A., Ataya, D. G. and Casas, I. A. (1995) Safety and tolerance of *Lactobacillus reuteri* in healthy adult male subjects. *Micro. Ecol. Hlth. Dis.,* **8:** 41–50.

Yamanaka, K., Fang, L. and Inouye. M. (1998) The CspA family in Escherichia coli: multiple gene duplication for stress adaptation. *Mol. Microbiol.*, **27:** 247-255.

Yamanaka, K., Zheng, W., Crooke, E., Wang, Y. H. and Inouye, M. (2001) CspD, a novel DNA replication inhibitor induced during the stationary phase in *Escherichia coli*. *Mol. Microbiol.*, **39:** 1572-1584.

Yeong, S., Ji-Young Lee, P., Young-Suk Kim, & Dong-Hwa Shin. (2002) Isolation and Characterization of Lactic Aicd Bacteria from Feces of Newborn Baby and from Dongchimi. *Journal of Agricultural Food Chemistry*, **50:** 2531-2536.

Young, J. (1996) Functional foods: strategies for successful product development. FT management report. London: Pearson Professional Publishers.

Zarate, G., Perez-Chaia, A., Gonzalez, S. and Oliver, G. (2000) Viability and β-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. *J. Food Prot.,* **63:** 1214– 1221.

List of publications

Published:

1. **K. B. Praveen Kumar Reddy,** P. Raghavendra, B. Girish Kumar, M.C.Misra and S.G.Prapulla. Screening of probiotic properties of lactic acid bacteria isolated from Kanjika, an ayruvedic lactic acid fermented product: an *in-vitro* evaluation. **Journal of General Applied Microbiology,** 53, 207-213, (2007).

In Press:

1. **K. B. Praveen Kumar Reddy** and S.G. Prapulla. In-vitro evaluation of lactic acid bacteria as potent probiotics for nutraceutical applications. **International Journal of probiotic and prebiotic,** 2, (2007).

Communicated:

1. **K. B. Praveen Kumar Reddy,** A. N. Madhu and S. G. Prapulla. Comparative survival and evaluation of functional probiotic properties of spray dried Lactobacillus strains isolated from Kanjika . **International Dairy Journal.**

2. **K. B. Praveen Kumar Reddy,** Sharda Prasad Awasthi and S. G. Prapulla. Role of cryoprotectants on the viability and functional properties of probiotic lactic acid bacteria during freeze drying. **Cryobiology**

Under preparation:

1. **K. B. Praveen Kumar Reddy,** V. Badarinath , Prakash M. Halami and S.G.Prapulla. Molecular Characterization and evaluation of Probiotic properties of the lactic acid bacteria isolated from Kanjika.

Papers presented in Symposia/ Conference:

1. **Praveen Kumar Reddy** and S.G.Prapulla. Probiotic Lactic Acid bacteria for Neutraceutical application: Selection criteria. SYMBIO 2007, Held at Chennai, Jan 19-20, 2007. [***Awarded first prize***]

2. **Praveen Kumar Reddy**, Sharda Prasad Awasthi and S. G. Prapulla. Effect of cryoprotectants on the probiotic properties of *Pediococcus acidilactici* during freeze drying . Presented in SYMBIO 2007, Held at Chennai, Jan19-20, 2007.

3. **Praveen Kumar Reddy**, A. N. Madhu and S. G. Prapulla. Effect of spray-drying on the viability and probiotic properties of *Lactobacillus salivarius*. Presented in SYMBIO 2007, Held at Chennai, Jan 19-20, 2007.

4. **Praveen Kumar Reddy**, Prakash M. Halami, and S. G. Prapulla. Molecular Characterization and evaluation of Probiotic properties of the *Pediococcus acidilactici* (K23c) isolated from Kanjika. National symposium on Biotechnology, Held at Bangalore, Feb 17-18, 2007.

5. **Praveen Kumar Reddy** and M.C.Misra. Effect of spray-drying on viability and functional properties of probiotic lactic acid bacteria. Presented in SBC, Held at CDRI Lucknow, Nov 7-10, 2005.

6. **Praveen Kumar Reddy**, B. Girish Kumar, and M.C. Misra. Production of Kanjika - an ayruvedic lactic acid based fermented product. SBC, Held at CDRI Lucknow, Nov 7-10, 2005.