ISOLATION AND CHARACTERIZATION OF A NATIVE ISOLATE OF *LEUCONOSTOC* FOR FUNCTIONAL ATTRIBUTES

A Thesis Submitted to University of Mysore, Mysore
for the Degree of

DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

By

SHOBHA RANI. P

Under the guidance of

Dr. RENU AGRAWAL

DEPARTMENT OF FOOD MICROBIOLOGY
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE,
MYSORE-20

2008

ACKNOWLEDGEMENT

With great reverence, I extent my deep sense of gratitude to my respected guide and teacher ***Dr. Renu Agrawal*** *for her advice and able guidance, constant supervision, co-operation, inspiration, constructive criticism and novel suggestions throughout the investigation without whose initiative and enthusiasm this study would not be completed. I remain indebted to her for being there for me as a personnel supporter and career builder.*

My thanks are due to ***Dr. Prakash,V.,*** *Director, CFTRI for providing the necessary facilities to carryout research work in the institute.*

I take this opportunity to thank ***Dr. Umesh Kumar S****, Head, Food Microbiology Department, CFTRI for his support and encouragement during the course of the study.*

I sincerely thank ***Dr. Baskaran, V.,*** *Department of Biochemistry and Nutrition,* ***Dr. Manjunath, M.N.,*** *Food Safety and Analytical Quality Control Laboratory and* ***Dr. Ramesh, B.S.,*** *TTBD Department for their suggestions and advice.*

I am also thankful to staff of animal house for their assistance and special thanks to ***Dr. Saibaba, P.,*** *for his help and cooperation during the work.*

I will always cherish my memorable time that I have spent in the lab. I wish to express my sincere thanks to all my Scientists and fellow colleagues in the department both past and present for cooperation and ambient atmosphere all through my research period. ***Prathibha. DV*** *deserves special thanks for having been there all the time as a great source of moral support.*

I had an opportunity to interact with many research fellows and Scientists from various department and they always assisted me every time. I thank all of them.

It is my sincere duty to acknowledge the help rendered by the staff of Central Instrumentation Facilities, computer center, library and administration.

University of Mysore *is greatly acknowledged for the financial support in the form of fellowship.*

Finally I am deeply indebted to my parents and brothers who were a constant source of support and encouragement in this endeavor.

Date:

Place: **Shobha Rani. P**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

SHOBHA RANI. P
Senior Research Fellow
Food Microbiology Department
Central Food Technological Research Institute
Mysore

DECLARATION

I hereby declare that the thesis entitled "***Isolation and characterization of a native isolate of Leuconostoc for functional attributes***" submitted to the **University of Mysore**, Mysore, for the award of Degree of **Doctor of Philosophy** in the faculty of **Microbiology** is the result of work carried out by me under the guidance of **Dr. Renu Agrawal**, Scientist, Department of Food Microbiology, Central Food Technological Research Institute, Mysore.

I further declare that the results of this thesis have not been submitted by me for award of any other degree/diploma to this or any other Universities.

SHOBHA RANI. P
(Candidate)

Date:
Place: Mysore

CERTIFICATE

This is to certify that the thesis entitled "***Isolation and characterization of a native isolate of Leuconostoc for functional attributes"*** submitted to the University of Mysore, Mysore for the award of Degree of **Doctor of Philosophy** in the faculty of **Microbiology** by **Shobha Rani. P** is the result of work carried out by her in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore.

RENU AGRAWAL
(Guide)

Date:
Place: Mysore

ABBREVIATIONS

%	Percent
β	Beta
°C	Degree Celsius
µl	Microliter
µM	MicroMolar
AAS	Atomic Absorption Spectra
AHL	Acyl homoserine lactone
BHSL	Butryl homoserine lactone
BLAST	Basic Local Alignment Search Tool
BSA	Bovin serum albumin
cfu	Colony forming unit
DPPH	1,1-Diphenyl-2-picryl hydrazyl
EDTA	Ethylene diamine tetra acetic acid
EMS	Ethyl methyl sulphonate
g	Gram
GC	Gas chromatograph
h	Hour
HHSL	Hexanoyl homoserine lactone
i.e.	That is
KD	Kilodaltons
kg	kilogram
LAB	Lactic acid bacteria
M	Molarity
mg^{-1}	Per miligram
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Milliliter
mm	Millimeter

Mol. Wt	Molecular Weight
MRS	deMann Rogosa Sharpe media
MS	Mass spectrometry
MTCC	Microbial Type Culture Collection Centre
N	Normality
NaCl	Sodium Chloride
OD	Optical density
ONPG	O-nitrophenyl β-galactopyranoside
PAGE	Poly acrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pH	Negative logarithm of hydrogen ion concentration
ppm	Parts per million
rRNA	Ribosomal RNA
RSM	Response Surface Methodology
RT	Retension time
SDS	Sodium Dodysyl Sulphate
Sec	Second
SEM	Scanning Electron Microscope
TAE	Tris-acetate EDTA buffer
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) amino methane
UV	Ultra Violet
v/v	Volume/Volume
w/v	Weight/Volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ISOLATION AND CHARACTERIZATION OF A NATIVE ISOLATE OF *LEUCONOSTOC* FOR FUNCTIONAL ATTRIBUTES

A Synopsis Submitted to University of Mysore, Mysore
for the Degree of

DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

By

SHOBHA RANI. P

Under the guidance of

Dr. RENU AGRAWAL

DEPARTMENT OF FOOD MICROBIOLOGY
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE,
MYSORE-20

2008

SYNOPSIS

ISOLATION AND CHARACTERIZATION OF A NATIVE ISOLATE OF *LEUCONOSTOC* FOR FUNCTIONAL ATTRIBUTES

INTRODUCTION

During the past several years, the focus of nutritional sciences has shifted from deficiency disease prevention to optimizing health and prevention of chronic diseases. Accordingly the research has encompassed the health effects of bioactive food components. In this regard, probiotic therapy is being used increasingly in humans and veterinary medicine due to their apparent high index of safety and public perception about natural or alternative therapies (Gherty 1995; Sander 1998; FAO Report 2002).

Probiotics are a category of 'nutraceuticals' i.e., viable cultures added to food with the intension of maintaining or improving the nutritional health of consumers. These bacteria favorably alter the intestinal microflora, inhibit growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Malin et al 1996; Haudault et al 1997).

Lactic acid bacteria (LAB) are in the focus of extensive research because of their probiotic nature. One important way in which they affect health of the host is by providing enzymatic activities that improve the utilization of nutrients within the intestine.

Today, probiotic market promises the disease prevention and better health for all as a natural alternative therapy. One of the important challenges to the present day probiotic industry is the eradication of lactose intolerance problem which is quite common all over the world. It is estimated that 70-90% of adults are lactose intolerant (Swegerty 2002), suffering from intestinal discomfort with the symptoms like nausea, cramps and gas (Stiles and Holzapfel 1997). In the present scenario, with increase desire of consumer for natural food products as source of providing nutrition and other desirable benefits, research work towards

the selection of strain with functional properties has become very important especially for treating lactose intolerance.

Considering all these aspects, in the present work a strain of LAB was isolated from milk and milk products, characterized and studied for its probiotic functional attributes to be used in food formulation.

Objectives

1) Isolation and characterization of *Leuconostoc* from milk products
2) Properties of the isolated bacterium in relation to functional significance
3) Colonization of LAB in relation to homoserine lactone

Chapter 1

Isolation and screening of lactic acid bacteria from milk and milk product

The problem for choosing a culture to be used in health promoting probiotic ingredients in food and pharmaceutical preparations was apparent even in the original work of Metchnikoff (1906). Reid (1999) and Sobel (1999) specify certain properties like ability to adhere intestinal cell wall, exclude/ reduce pathogenic microorganisms, produce antimicrobial compound, resist microbicides, non-carcinogenic and non-pathogenic character to be present in the selected strain for their use as probiotic culture.

Now a days, a consensus is emerging for selection criteria of LAB to achieve positive probiotic effect (Collins et al 1998). Therefore, in the present work a screening procedure is performed to select a potential probiotic culture with an ultimate aim of using the culture in functional food for beneficial effect.

In the present work, 45 isolates were screened for basic LAB characteristics from milk and milk products. As the most critical characteristics of probiotic strain are tolerance gastrointestinal condition (Ammor and Mayo 2007), the isolated strains were initially screened for their resistance in this harsh environment and further adapted to grow at low pH and high bile salt concentration. The strains were further analyzed for their survival under simulated gastrointestinal condition. The strains that were able to survive under

such environment were characterized and identified through biochemical assays and molecular techniques. The identified cultures *Leuconostoc mesenteroides* (Lsr-$1_{(W)}$) and *Lactobacillus plantarum* (Lsr-$12_{(Cu)}$) were further studied for their resistance to digestive enzymes pepsin and trypsin. *Leuconostoc mesenteroides* (Lsr-$1_{(W)}$) that was better resistant to these enzymes was selected for further studies and was **coded as PLsr-$12_{(Cu)}$**.

Chapter 2

Probiotic functional properties of culture isolate

Probiotics have health promoting effects including inhibition of pathogens, antimutagenic, anticarcinogenic activity, prevention of diarrhea, stimulation of immune response and ability to reduce serum cholesterol levels (Tannock 1999). The development of new applications such as life vaccines and probiotic foods reinforces the need for these characteristics.

In this regard, the selected culture of *Leuconostoc mesenteroides* (PLsr-$1_{(W)}$) was evaluated for its functional properties. According to the data obtained, the culture PLsr-$1_{(W)}$ shows antimicrobial activity against 7 toxic food pathogens such as *E. coli, S. typhi, S. dysenteriae, P. aeroginosa, V. cholerae, Y. enterocolitica* and *S. aureus*. The isolate was found resistant to 3 antibiotics tested. The inhibitory activity of intracellular cell free extract of culture to ascorbate autooxidation, ferrous ion chelating ability and scavenging ability of oxygen radical represent the antioxidative property of culture isolate. The culture was also able to assimilate 28 µg/ml of cholesterol from media which shows the anticholesterol activity of the strain. Analysis of *β*-galactosidase in culture indicate the ability of the culture to hydrolyze lactose into simple sugar for easy absorption. From the results of cell surface hydrophobicity and intestinal adherence test, the adhesion ability of the culture was confirmed. Analysis of S-layer adhesion protein by SDS-PAGE showed a prominent protein band of 60 KD. Volatile compounds analyzed by GC and GCMS of culture extract confirmed the presence of therapeutically important compounds. Stress response of PLsr-$1_{(W)}$ to low pH and high bile salt indicate the increased content of stress

proteins and membrane fatty acids (saturated and unsaturated). All these properties make the present isolate a potent probiotic and will stand out as a natural cure to many diseases.

Chapter 3

***Leuconostoc* as a source for *β*-galactosidase enzyme**

Lactose is a non-reducing disaccharide which provides almost half the total energy required by infants. In presence of lactase/ *β*-galactosidase, lactose gets hydrolyzed into galactose and glucose for easy absorption. But in persons deficient in lactase show symptoms of intestinal discomfort known as lactose intolerance (Sieber et al 1997). Hence studies were carried out to enhance the enzyme activity in the present culture isolate.

Leuconostoc mesenteroides PLsr-$1_{(W)}$ that had the ability to survive the harsh conditions of GIT was studied for its ability to increase lactose tolerance. Primarily strain was improved by UV irradiation and chemical mutagenesis for enhanced enzyme activity. UV mutant strain showed 2 folds higher activity than the parent strain and hence was selected for all further studies (**mutant strain was coded as M7-PLsr-$1_{(W)}$**). Cell permeabilization method was optimized for maximum release of enzyme. Response surface methodology (RSM) studies were undertaken for optimization of chemical and physical parameter. The enzyme produced under optimum condition of pH 7.5 with 1.25 % lactose was partially purified and studied for kinetics. The results indicate a 25 fold increase in the activity of partially purified enzyme as compared to the crude extract.

Chapter 4

Enhancement of culture shelf life on storage

LAB in food biotechnology or for any probiotic formulations are strictly concerned for the preservation techniques employed to ensure stable culture in terms of viability and bacterial metabolism. It is very important to use suitable technology or process to enhance and maintain the viability of the culture on storage for a beneficial effect.

As the present isolate M7-PLsr-$1_{(W)}$ is very important culture due to its characteristic multifunctional probiotic properties, it becomes necessary to keep the culture viable for a longer time. Hence the aim of the present work was to preserve the culture for longer shelf life. In this regard, the culture was subjected to different preservation techniques i.e., oven, spray, vacuum and freeze drying. The viability and resistance of culture to these methods was tested and it was found that the culture showed maximum survival to freeze drying condition. The viability under freeze drying condition was further enhanced with supplementation of different cryoprotective agents (PEG, lactose and sucrose). Enhanced membrane fatty acid composition and cellular protein confirmed the adoptive nature of the culture to freeze drying.

In the present work, we also report the viability of the freeze dried culture for 6 months. It showed resistance to low pH (2.0) and high bile salt (4%). Even after 6 months of freeze drying, culture showed antimicrobial activity against 6 toxic food pathogens. Data determines that the culture retains the *β*-galactosidase enzyme even after 6 months of storage.

The above study determines the capability of culture to remain viable even after 6 months of storage. It also shows the importance of cryoprotectants in enhancing the viability and beneficial attributes of culture during storage. This determines the capability of the culture to be utilized in the form of capsules or any functional food.

Chapter 5

Functional food with *Leuconostoc*: a native isolate

The demand for probiotic foods is increasing all over the world reflecting the heightened awareness among the public relationship between diet and health. Fermented dairy products are the most widely used food vehicles for these probiotic bacteria because of their healthy image. During the past twenty years there has been a tremendous increase in the world wide sales of cultured products containing probiotic bacteria because of their health effects (Ostlie 2005; Maltila-Sandholm 1999).

In this regard, the culture M7-PLsr-$1_{(W)}$ that has the potent probiotic properties was used as starter culture in the preparation of fermented milk beverage. The product was analyzed to be rich in protein, fat, sugar and minerals like iron, zinc and magnesium. Hence the viability of culture in the product was further enhanced with supplementation of different adjuvants (tryptone, casein hydrolysate, cysteine hydrochloride and ascorbic acid). The results conclude that the culture maintained maximum viability on supplementation of tryptone (100 mg/L) after 5 days of storage at 4°C. Fatty acid composition of the product also confirmed the nutritional property of the product.

Chapter 6

Preservation of fermented milk over shelf storage

Spoilage causes a significant loss to dairy industries and also disease outbreaks. In this concern, research work was carried out to investigate and preserve fermented milk beverage prepared with M7-PLsr-$1_{(W)}$.

In this regard, the functional fermented milk beverage prepared by M7-PLsr-$1_{(W)}$ was studied for the predominant bacterial cultures responsible for spoilage of the product. *Pseudomonas sp* was identified as dominant spoilage bacteria and through TLC, GC and GCMS, the signal molecule for spoilage was investigated as hexanoyl homoserine lactone (HHSL).

This culture releases HHSL and forms a quorum when high cell density is reached. Inhibition of bacterial growth in this biofilm by using biocides, antibiotic and bacteriophages has many obstacles such as cell permeability, specificity and efficacy in mode of delivery. Hence the natural furanones which are non-toxic are used in present study because of its small size and ease of delivery. Because of its structural similarity they specifically interfere with signal molecule without any adverse effect on the beneficial bacterial consortia. Results show that 2(5H)-furanone tested was having better inhibitory activity against *Pseudomonas* than bromofuranone. This was also in concurrence with reduction in rhamnolipid content, reduced motility and exoprotease enzyme activity. Using 2(5H)-furanone about 5-6 log of *Pseudomonas* culture was reduced in the fermented beverage.

Chapter 7

In-vivo studies using *Leuconostoc* for functional attributes

The health benefits described for probiotic lactic acid bacteria make them a good agent for preparation of functional food and hence a number of bacterial strains are being identified and incorporation into these foods. As more probiotic organisms are discovered, it is important to carefully document the efficacy of the strain for its potential application and safety.

Looking into the probiotic characteristic of the present isolate M7-PLsr-$1_{(W)}$ of being resistant to GIT condition, adherence ability, antimicrobial, β-galactosidase activity and anticholesterol activity through *in-vitro* assays the present study was aimed to assess its safety and functions in *in-vivo* model using albino rats.

According to the result there was no deleterious effect on probiotic feeding for 3 months. No bacterial translocation was observed and hence it is likely to be safe for human consumption. Increase in the general body weight and serum urea concentration provides a potential proof for the health promoting effect of the culture. High LAB count in feces and ceacum shows the ability of the culture to resist the GIT condition and adhere to exert beneficial effect. Results of cholesterol assay confirmed the anticholesterol effect of the culture. The ability of the isolate to protect against pathogens was determined by decrease in *E. coli* count.

Lactose intolerance, a clinical problem associated with unpleasant abdominal discomfort is due to undigested lactose. According to a survey, about 70-80% of the world population are lactose intolerant (Swagerty 2002). Innovative approaches have been tried as alternative to antibiotics in treating lactose intolerance because of the growing antibiotic resistance problem. Alternative methods such as exogenous β-galactosidase administration in functional foods or in pharmaceutical preparation is advised but the draw back is the inactivation of the enzyme in gastrointestinal transit. The other approach is gene therapy or exclusion of milk and dairy products with lactose from diet which may cause nutritional disadvantages.

Considering this, the present culture M7-PLsr-$1_{(W)}$ was tested for its ability to reduce lactose intolerance problem by *in-vivo* experiments. Single dose study and long term (3 months) experiments were carried out with albino Wister rats. From the study, the effective dose of culture was determined to be 10^8 cfu/ml. Disappearance of diarrhea in lactose intolerant induced rats after culture feeding confirmed the positive impact of culture in treating lactose intolerance.

From the report presented here, the culture M7-PLsr-$1_{(W)}$ was found to have probiotic characters in terms of resistance to GIT, antimicrobial activity, anticholesterol activity and adherence ability. The culture was also able to reduce the symptoms of lactose intolerance and hence can be used as an alternative source to treat the problem.

Achievement of the work

The culture is native, isolated in laboratory and shows potential probiotic characteristics along with high β-galactosidase activity. The *in-vivo* experiments conducted with albino Wister rats conclude the potential probiotic functional properties and its importance in reducing lactose intolerance problem. The culture preserved by freeze drying shows viability even after 6 months of storage. The fermented milk beverage prepared with the present isolate is having a high nutritive value so that it can be used by all ages for its beneficial effects. This fermented milk beverage was preserved over a storage time by interrupting the signal molecules produced by of spoilage bacteria using 2(5H)-furanone which is a natural compound produced by an algae and known to be safe.

Social and scientific relevance

With the rise in the consumer's awareness of individual health, nutrition and well being, the interest and demand for value added foods and beverages has expanded. Although some companies have marketed the probiotic products, they are either expensive or are specified for one specific cure. Most of these products

are not indigenous in our country and the viable count of the culture is questionable or not to the mark as labeled.

Today WHO and Indian five year plans both have a common priority to replace antibiotics with natural means of cure. In this regard, the present isolate having probiotic functional properties promises to be as a source of natural alternative cure. The fermented beverage prepared by *Leuconostoc mesenteroides* (M7-PLsr-$1_{(W)}$) has many functional attributes.

References

1. Ammor MS, Mayo B (2007). Slection criteria for LAB to be used as functional starter cultures in dry sausage production: an update. *Meat Sci* 76, 138-146.
2. Collins JK, Thornton G, Sullivan GO (1998). Selection of probiotic strains for human applications. *Int Dairy J* 8, 487-490.
3. FAO Expert Report 2002. evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. American Cordoba Park Hotel, Cordeba. Argentina.
4. Gherty J (1995). American dairy science association foundation, lecture. *J Dairy Sci* 78(7): 1401
5. Haudault S, Lieven V, Bernet-Camard MF, Servin AL (1997). Antagonistic activity exerted *in vitro* and *in vivo* by *L. casei* (strain GG) against *S. typhimurium* C5 infection. *Appl Environ Microbiol* 63, 513-518.
6. Malin M, Verronen P, Mykkanen H, Salminene S, Isolauri E (1996). Increased bacterial urease activity in feces in juvenile chronic arthritis evidence of altered intestinal microflora? *Br J Rheumatol* 35, 689-694.
7. Mattila-Sandholm T, Matto J, Saarela M (1999) Lactic acid bacteria with health claims – interactions and interference with gastrointestinal flora. *Int Dairy J* 9, 25–35.
8. Metchnikoff E (1906). The prolongation of life. G.P. Putnam's Sons, New York.

9. Ostlie HM, Treimo J, Narvhus JA (2005). Effect of temperature on growth and metabolism of probiotic bacteria in milk. 15(10), 989-997.
10. Reid, G., 1999. The scientific basis for probiotic strains of *Lactobacillus*. *Appl Environ Microbiol* 65, 3763–3766.
11. Sanders ME (1998). Over view of functional foods: emphasis on probiotic bacteria. *Int Dairy J* 8, 341-347
12. Sieber R, Stransky M, de Vrese M (1997). Lactose intolerance and consumption of milk and milk products. *Z Emahrungswiss* 36(4), 375-393
13. Stiles ME, Holzapfel WH (1997). Lactic acid bacteria of foods and other their current taxonomy. *Int J Food Microbiol* 36, 1-29.
14. Swagerty, DL, Walling AD, Klein RM (2002). Lactose intolerance. *American Fan Physician* 65(9), 1845-1850
15. Tannock, GW (1999). Probiotic: A critical review, Horizon Scientific Press, Norfolk England.

Signature of the candidate
Date:

Signature of the guide
Date:

Review of Literature

REVIEW OF LITERATURE

INTRODUCTION

1. Lactic acid bacteria: characterization, classification and importance

Lactic acid bacteria (LAB) are nutritionally fastidious microorganisms that have been used to ferment or culture foods for atleast 4000 years. All over the world they are used particularly in the preparation of fermented milk products including yogurt, cheese, butter, buttermilk and kefir. They refer to a large group of beneficial bacteria that produce lactic acid as their major metabolic end product. They produce characteristic flavor and aroma compounds like acetaldehyde and diacetyl.

1.1. Classification of lactic acid bacteria

Lactic acid bacteria belong to the phylum *Firmicutes*, which share the property of being gram positive (Fooks et al., 1999) that ferment carbohydrates into energy and lactic acid (Jay, 2000). Depending on the metabolic pathway they are classified into homofermentative and heterofermentative cultures (Caplice and Fitzgerald, 1999; Kuipers et al., 2000).

Schleifer et al. (1991) have classified lactic acid bacteria into four genera based on sugar fermentation and growth at specific temperatures. This includes *Lactobacillus* (rod shaped), *Streptococcus* (homofermentative), facultative anaerobic cocci, *betacoccus* and *tetracoccus*. Later, Carr et al. (2002) and Frank et al. (2002) grouped them into four important genera namely *Streptococcus*, *Pediococcus*, *Leuconostoc* and *Lactobacillus*.

At present *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are the recognized genera of lactic acid bacteria (Stiles and Holzapfel, 1997; Ercolini et al., 2001; Holzapfel et al., 2001).

Streptococcus, *Pediococcus* and *Lactobacilli* are homofermentative species that produces 2 moles of lactic acid for each mole of glucose consumed as their metabolic product. They possess enzyme aldolase and hexose isomerase but lack phosphoketolase, so it uses the Embden-Meyernhoff (EM) glycolytic pathway for converting glucose to fructose-1,6 diphosphate. In heterofermentative species, the key enzyme fructose-1,6 diphosphate aldolase is absent and so they possess an alternative glycolytic pathway, where in the glucose is converted to 6-phosphogluconate that gets decarboxylated into pentose resulting in lactic acid and ethanol and/or acetate (Blackwood and Blakley, 1960; Holzapfel and Wood, 1998). *Leuconostoc* and *Weissella* belong to this group (Jay, 1992).

The taxonomy of LAB is based on comparative 16srRNA sequence analysis. Molecular techniques, especially polymerase chain reaction (PCR) based methods, such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse field gel electrophoresis (PFGE) are regarded as important techniques for specific characterization and detection of LAB strains (Gevers et al., 2001). Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) of 16srRNA gene have shown to be powerful approaches in determining and monitoring bacterial community (Cocconcelli et al., 1997; Zoetendal et al., 1998). Methods such as DNA-DNA hybridization (Yaeshima et al., 1996), genus specific and species-specific probes (Hensiek et al., 1992; Timisjarvi and Alatossava, 1997), 16s and 23s intergeneric spacer region sequencing (Bourget et al., 1996) and ribotyping (Ning et al., 1997) have also been used for identification and characterization of LAB strain.

1.2. Habitat and characterization of lactic acid bacteria

LAB are typically fastidious and require a variety of amino acids, Vitamins, purine and pyrimidine bases for their growth (Calderon et al 2001). Although they are mesophilic, some can grow below 5°C and others at temperature as high as 45°C. Usually most of the LAB cultures grow at pH

6.0-6.5, but some can also grow in acidic pH (3.2) and others in alkaline pH (9.6) (Jay, 2000). Some of the characteristic features of lactic acid bacteria are presented in the table 1.

Heterofermentative bacteria especially *Leuconostoc spp* appear to be common in plant materials like vegetables and roots. In refrigerated products generally *Streptobacteria* predominates whereas *thermobacteria* are commonly found in products of higher temperature (Kitchell and Shaw, 1995; Franz and Holy, 1996; Samelis et al., 2000; Holm et al., 2004). LAB occur naturally in fermented food and have been detected in soil, water, manure and sewage (Holzapfel et al., 2001). They are the normal intestinal microflora in human (Boris et al., 1998; Reid, 2001; Schrezenmeir and deVrese, 2001) and in animals (Fujisawa and Mitsuoka, 1996; Klijn et al., 1995).

1.3. Importance of lactic acid bacteria

Without understanding the scientific basis people are using lactic acid bacteria to produce fermented foods with characteristic flavors and texture. These LAB also help our normal gut bacteria to function more efficiently. The world wide market for these products continues to increase in response to the demands of an increasingly health conscious public. Lactic acid bacteria are therefore excellent ambassadors for an often maligned microbial world. With growing interest in self-care linked between diet and health, the market of food that promotes health beyond basic nutrition has become stronger and is flourishing all over the world.

Table 1: Characteristic features of lactic acid bacteria

Type	Species	Shape	Growth temperature (°C)			Lactate isomer	Citrate metabolism	Galactose metabolism	NH_3 from arginine	Salt inhibition (%)	Important metabolic product
			10	40	45						
Mesophilic	*Lactococcus lactic subsp lactis*	Cocci	+	+	-	L(+)	-	+	+	4.0-6.5	Lactate
	Lactococcus lactis subsp lactis (cit^+)	Cocci	+	+	-	L(+)	+	+	+	4.0-6.5	Lactate, diacetyl, CO_2
	Lactococcus lactis lactis subsp cremoris	Cocci	+	-	-	L(+)	-	+	-	2-4	Lactate
	Leuconostoc mesenteroides subsp cremoris	Cocci	+	-	-	D(-)	+	+	-	2-4	Lactate, diacetyl, CO_2
	Leuconostoc lactis	Cocci	+	-	-	D(-)	+	+	-	2-4	Lactate, diacetyl, CO_2
Thermophilic	*Streptococcus thermophilus*	Cocci	-	+	+	L(+)	-	-	-	<2	Lactate acetaldehyde
	Lactobacillus bulgaricuss	Rods	-	+	+	D(-)	-	-	-	<2	Lactate acetaldehyde
	Lactobacillus helveticus	Rods	-	+	+	DL	-	+	-	<2	Lactate
	Lactobacillus lactis	Rods	-	+	+	D(-)	-	-	+/-	2-4	Lactate

Source: Stanley, G (1998) Cheeses. In: Microbiology of fermented foods. Eds. Brian JB Wood, Vol. 1(2), Blackie Academic and Professional, New York, pp 263-307.

2. Lactic acid bacteria as probiotics

2.1. Probiotics: Definitions and History

LAB are regarded as a major group of probiotic bacteria (Collins et al., 1998; Tannock, 1998; Schrezenmeir and deVrese, 2001). There is a long history of health claims concerning LAB in food. In a Persian version of the Old Testament (Genesis 18:8) it states that "Abraham owed his longevity to the consumption of sour milk". In 76 BC, the Roman historian Plinius recommended the administration of fermented milk products for treating gastroenteritis (Bottazzi, 1983).

The term 'probiotic', meaning "for life", is derived from the Greek language. As early as 1906, Tissier noted that significant stool colonization with *Bifidobacteria sp* was protective against diarrhea in children. Metchnikoff (1908) suggested that long, healthy life of Bulgarian peasants results from the consumption of fermented milk products. It was Ferdinand Vergin (Vergin, 1954) a German scientist who introduced the term 'probiotic' in an article entitled "Anti-Und Probiotika", wherein he compared the harmful effects of antibiotics and the beneficial ("Probiotic") effects of the lactic acid bacteria. Later in 1965, Lilley and Stillwell described it as "substances secreted by one microorganism which stimulates the growth of another".

In 1971, Sperti applied the term probiotic as "tissue extracts that stimulate microbial growth". Parker (1974) defined it as "organisms and substances which contribute to intestinal microbial balance". In 1992, Fuller redefined it as "a live microbial feed supplement which beneficially affects the host animals by improving its intestinal microbial balance". Further, Havenaar and Huis in't Veld et al. (1992) broadened the definition as "a viable mono or mixed culture of microorganisms which are given to animals or human for its beneficial effect in improving the properties of indigenous microflora".

Salminen (1996) defined it as "a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host". Further Schaafsma (1996) described it as "oral probiotics are

microorganisms which upon ingestion in certain numbers exert health effects beyond inherent basic nutrition". Ouwehand et al. (2002) have defined probiotics as "non-pathogenic microorganisms that when ingested in certain number exerts a positive influence on the host physiology and health beyond inherent general nutrition"

The joint FAO/WHO (2002) have proposed a general definition that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host".

2.2. Probiotics: Essential characteristic requirements

Many scientific publications and review articles has listed a series of essential requirements in the screening of microorganisms for the probiotic value. The list of essential requirements based on theoretical consideration included the following (Guarner, 2005; Rashid et al., 2007; Maurad and Merien, 2008).

1) Human origin (as a token of safety for human use)
2) Resistance to gastric acidity and bile toxicity (for good survival during gastrointestinal transit)
3) Adhesion to gut epithelial cells (for successful colonization)
4) Production of antimicrobial substances or bacteriocins (for pathogenic antagonism)
5) Ability to modulate immune responses.

2.3. Probiotic: Mechanism of action

Stress in modern day life has disrupted the homeostasis in the gut through change in dietary pattern and eating habits. Another contributory factor includes the consumption of pharmaceutical compounds, in particular antibiotics that destroy bacteria creating an imbalance in the gut microflora. This leads to a number of diseases. Hence there is an increasing demand for food products that support health beyond providing basic nutrition.

Consumption of probiotics is known to help in balancing the normal intestinal microflora. It is also understood that each probiotic strain is independent of the genera and species and is unique in their properties. The exact manner in which probiotics affect is uncertain. However some mechanisms have been speculated (Hatcher and Lambrecht, 1993; Ouwehand, 1998; Jacobsen et al., 1999; Boirivant and Strober, 2007; Allan, 2008)

1) Biochemical effect
    - ❖ Through production of bacteriocins
    - ❖ Short chain fatty acids with antagonistic effect
    - ❖ Creation of unfavorable environment for pathogens by reduction of pH
    - ❖ Production of antimicrobial compounds including organic acids, hydrogen peroxide and diacetyl that inhibit growth of spoilage organisms
2) Competition for nutrients
3) Immune clearance
    - ❖ By surface Ig A attachment to mucosal membrane, adherence of enteropathogens has been limited
    - ❖ Stimulation of cell mediated response by increasing macrophage phagocytic activity.
4) Attachment
    - ❖ Blockage of adherence of enteropathogens by occupying the niche of the intestinal mucosa.

2.4. Probiotic: Criteria for selection

The selection of lactic acid bacterial culture to be used as a probiotic source depends on the host specificity and colonization ability. It should be generally regarded as safe (GRAS status) with antimicrobial activity and other desirable metabolic activities for beneficial effect (Canducci et al., 2000; Sanders, 2003; Reid et al., 2003). Criteria should also include sensory characteristics and technological suitability for general acceptance

(O'Sullivan et al., 2002; Charteris et al., 1998). The market of a probiotic strain requires a well designed, double blind, placebo controlled host specific studies with animal and humans model. Culture also should resist to technological processes, with respect to viability and activity throughout processing (Dunne et al., 2001). Each potential probiotic strain must be documented independently, without extrapolating any data from closely related strains and employing only well defined strains in the trials.

Safety assessment of the strain is a very important criteria (Saarela et al., 2002). Intrinsic properties of a strain, its interactions *in-vivo* with the host and its pharmacokinetics should also be accounted for commercial approval of the strain (Marteau et al., 1993; Pelletier et al., 1996; Saxelin, 1996).

2.4.1. Survival within the gastrointestinal tract

To survive passage through the gastrointestinal condition, probiotic strains must tolerate the acidic and protease rich environment of stomach and also bile rich environment of the intestine (Tuomola et al., 2001). It is observed that there is an intraspecies variation in the mechanism to tolerate low pH and high bile concentration between the potential probiotic strains (Lee and Salminen, 1995; Lee and Wong, 1998). Lorca and Font de Valdez (2001) have described that acid tolerance in *Lactobacillus acidophilus* is mediated by membrane ATPases. Bile resistance appears to be mediated by bile salt hydrolase activity (De Boever et al., 2000) or by deconjugation of bile salts (Ahn et al., 2003; Ashar and Prajapathi, 1998). Duc et al. (2004) have reported a probiotic *Bacillus sp* that can survive in the gastrointestinal transit because they are in the form of spores. Report says that the culture in the presence of milk or other food products show significantly higher resistance to GIT conditions (Saxelin et al., 1999). For example *Saccharomyces boulardii* has shown better survival in presence of dietary fibers (Elmer et al., 1999).

2.4.2. Adhesion/ cell surface properties

The ability to adhere to the intestinal mucosa is one of the important criteria for the culture to be used as probiotic because adhesion is considered a prerequisite for colonization and also important for stimulation of immune system (Alander et al., 1997; Tuomola et al., 2001). Mechanisms of adherence involves receptor specific binding and hydrophobic interaction which can be measured by salt aggregation test (SAT), contact angle and adhesion to hydrocarbons (Strus et al., 2001; Wojnicz and Jankowski, 2007). LAB also expresses binding to extracellular matrix like collagen, fibronectin and vitronectin (Aleljung et al., 1994, Howard et al., 2000; Lorca et al., 2002). Cell surface proteins have also been shown in various LAB to mediate adhesion to mucus (Kirjavainen et al., 1998; Roos and Jonsson, 2002). Probiotic culture needs to adhere to the mucosa atleast temporarily, colonize the ileum where they are thought to exert their beneficial effects (Goldin and Gorbach, 1992; Ouwehand et al., 2002; Ouwehand and Salminen, 2003). Therefore probiotic starter strains should be screened for adherence and persistence in the human GIT conditions.

2.4.3. Tolerance to digestive enzymes

Probiotics after getting entry through oesophagus and stomach have to pass through duodenum where it is constantly washed with bile acids along with a number of digestive enzymes like trypsin, pepsin, rennin and lipases (Olejnik et al., 2005). These enzymes may adversely affect microbial physiology and metabolism. Though not much work has been done in this regard, the detrimental effects of the enzymes are some important aspects to be concerned (Zhou et al., 2007).

2.4.4. Resistance towards intestinal pathogens

The concept of microbial antagonism is very well known and refers to the inhibition of other microorganisms by competition for nutrients or production of microbial metabolites (Hugas, 1998; Makras and deVyust,

2006). Probiotic cultures that are claimed for the beneficial effect have to resist and also competitively exclude the pathogenic microflora of the intestine. *Lactobacillus sp* are known to inhibit enteropathogenic *E. coli* adherence *in-vitro* by inducing intestinal mucin gene expression (Mark et al., 1999).

2.5. Probiotic functional characteristics

2.5.1. Antibiotic resistance

Regular use of antibiotics causes depletion of vitamins resulting in gut microbial imbalance. This causes excessive loss of calcium, magnesium and potassium ions from the body, thus creating an environment which is more susceptible for proliferation of opportunistic microbes causing abnormal health to the host. Thus it is an essential characteristic of probiotic culture to be resistant to common antibiotics so that it can proliferate in the gut and maintain microbial balance thereby reducing opportunistic microbes. Antibiotic resistance has been reported in a large number of lactic acid bacterial strains (Kozlova et al., 1992; Teuber et al., 1999; Strompfova et al., 2004; Mathur and Singh, 2005; Herreros et al., 2005; D'Aimmo et al., 2007).

2.5.2. Immunomodulatory activity

Nutritional interventions that can enhance immunity have been highlighted for the potential to offer benefits to human health (Scrimshaw and San Giovanni, 1997). In this regard probiotics are playing a very important role. Administration of probiotic strains causes a range of specific and non-specific host immune responses (Schiffrin et al., 1997; Gill et al., 2001; Gill and Rutherfurd, 2001). These include enhancement of phagocytic activity of peripheral blood leukocytes and natural killer cell activity (Fernandes and Shahani, 1990; Solis Pereyra and Lemonnier, 1993; Schiffrin et al., 1997). Some of the bacterial moieties with immunomodulatory activities are peptidoglycans, lipoteichoic acid and endotoxic lipopolysaccharide (Standiford et al., 1994). *Lactobacillus rhamnosus* HN001 and

Bifidobacterium sp are shown to enhance immune function on the development and progression of autoimmune thyroiditis and are safe in hosts with immume dysfunction (Gill et al., 2000). Earlier reports have shown that a large number of LAB are potent modulators of immune function in children, adults and elderly (Kaila et al., 1992; Schiffrin et al., 1995; Kishi et al., 1996; Donnet Hughes et al., 1999; Arunachalam et al., 2000; Esther et al., 2007).

2.5.3. Antigenotoxicity/ Antitumerogenic activity/ Anticarcinogenic activity

A large number of reports support for the beneficial effect of probiotics against tumor/ malignance. Administration of *Lactobacillus sp* and *Lactococcus sp* have shown to suppress bacterial enzyme activity such as *β*-glucoronidase, urease, fecal glycocholate, nitroreductase and azoreductase (Lidbeck et al., 1991; Aso et al., 1995; Spanhaak et al., 1998; Gorbach, 2000). Abdelali et al. (1995) have suggested that antigenotoxic property of lactic acid bacteria ingested with foods could be promising in preventing the effect of food related mutagens and can potentially reduce the risk of cancer. Fermented milk product consumption reduces the diet-associated risk of carcinogenesis either by reduction of carcinogen itself or by reducing the enzymes that causes the conversion of pre-carcinogens to carcinogens (Challa et al., 1997; Reddy, 1998; Femia et al., 2002).

The probable mechanisms suggested for the action of LAB are summarized below (O'Sullivan, 1992).

1) Suppress growth of intestinal microflora incriminated in producing putative carcinogen.
2) Produce antitumerogenic/ antimutagenic compounds.
3) Alter the physiological condition (such as pH).
4) Affect the metabolic activity of intestinal flora.
5) Reduce the levels of fecal bacterial enzymes such as *β*-glucoronidase, nitroreductase and azoreductase which are considered to be factors in the carcinogenic process.

6) Metabolically convert, degrade and absorb the carcinogenic compound.
7) Stimulate host immune system.
8) Assimilate and detoxify the dietary, endogenous and toxic compound generated by intestinal flora.
9) Deconjugate bile acids, which are considered to have a role in the carcinogenic process.

An antitumor effect has been reported by oral intake of LAB (Hirayama and Rafter, 2000) wherein it has been suggested that the effect may be mediated by production of antimutagens. Some studies have shown that LAB can bind to mutagens and detoxify, thus reducing the risk of mutagenesis (Lankaputra and Shah, 1998; Knasmuller et al., 2001; Vorobjeva and Abilev, 2002). Probiotic mechanism involved in prevention of cancer may be through binding of carcinogens, stimulation of protective enzymes, increasing immune response and production of metabolite that affect bacterial enzymes (Burns and Rowland, 2000).

A large number of reports support for the beneficial effect of probiotics against malignance (Marteau et al., 1990; Penner et al., 2005). They reduce the activity of nitroreductase and increase *β*-glucosidase enzyme, which release flavonoides that have antimutagenic, antioxidative and immune stimulatory effects (Stoner and Mukhtar, 1995). Piotrowska and Zakowska (2005) have shown the potential effect of LAB in reduction of carcinogenic/ genotoxic effect on consumption of food contaminated by ochratoxin A.

Aberrant crypts of putative pre-neoplastic lesions are known to reduce by probiotic feeding (Marotta et al., 2003). Pool-Zobel et al. (1993) have demonstrated the antimutagenic activity of *Lactobacillus casei* and *Lactococcus lactis*. Using the single cell microgel electrophoresis they have investigated the ability of these LAB cultures to inhibit DNA damage caused by treatment of carcinogens like N-nitroso compounds, N-methyl-N-nitro-N-nitroguanide and N-methyl nitroso urea heterocylic amines and aflatoxin B_1.

2.5.4. Antagonistic activity

The property of antagonism is very important in probiotic culture for their use as therapeutic or prophylactic agent. Table 2 represents the metabolic products of these probiotic cultures and their mode of action to reduce pathogenic growth. They maintain healthy balance of intestinal microflora and produce lactic acid, hydrogen peroxide and acetic acid as antimicrobial compounds (Schnurer and Magnusson, 2005). Klingberg et al. (2006) have shown that *L. pentosus* and *L. plantarum* inhibits the growth of *Listera monocytogenes, Escherichia coli, Salmonella typhimurium, Bacillus cereus, Shigella flexneri* and *Yersinia enterocolitica.* The inhibitory activity of LAB can be attributed to the creation of hostile environment for food borne pathogens and spoilage organisms in food. Even though several mechanisms are elucidated for such effects, the net effect in terms of better food preservation or suppression of intestinal microflora is the result of more than one mechanism operative against food borne pathogens.

Table 2: Antagonistic activity caused by lactic acid bacteria

Metabolic product	**Mode of action**
Carbon dioxide	Inhibits decarboxylation Reduces membrane permeability
Diacetyl	Interacts with arginine binding proteins
Hydrogen peroxide/ lactoperoxidases	Oxidizes basic protein
Lactic acid	Undissociated lactic acid penetrates the membrane, lowers intracellular pH and interferes with metabolic processes
Bacteriocins	Affect membrane DNA synthesis Protein synthesis

It is known that LAB produces certain proteinaceous substances known as “bacteriocins” which act like antibiotics for inhibition of pathogens. In the last few years, LAB has attracted special attention because of its ability to produce a variety of bacteriocins (Table 3).

Table 3 : Bacteriocins of lactic acid bacteria

Bacteriocins	Producer	Inhibitory spectrum
Fermenticin	*Lactobacillus fermenti*	*Lactobacilli spp*
Plantacin B Plantaricin	*Lactobacillus plantarum*	*Lactobacillus plantarum, Lactobacillus sakei*
Lactocidin	*Lactobacillus acidophilus*	*Broad antibiotic spectrum (gram positive and gram negative)*
Acidphilin	*Lactobacillus acidophilus*	*Spore formers, Salmonella sp, E. coli, S. aureus, Pseudomonas sp*
Acidolin	*Lactobacillus acidophilus*	*Spore formers, Enteropathogens*
Protein	*Lactobacillus acidophilus*	*B. subtilis, S. aureus, E. coli, Salmonella sp.*
Reuterin	*Lactobacillus reuteri*	*Salmonella sp, Shigella sp, Clostridia sp, Staphylococcus sp, Listeria sp, Candida sp, Trypanosoma sp*
Nisin	*Lactococcus lactis*	*Bacillus sp, Clostridia sp, Micrococci, S. aureus*
Lactococcin I	*Lactococcus lactis supsp cremoris*	*Clostridia sp*
Lactoccin	*Lactococcus lactis supsp lactis*	*S. aureus, B.cereus, S. typhi*
Mesenterocin	*Leuconostoc mesenteroides*	*L. monocytogenes, B. linens, E. faecalis, P. pentosaceus*
Leucocin S	*Leuconostoc paramesenteorides*	*L. monocytogenes, S. aureus, A. hydrophilic, Y. enterocolitica*
Carnocin	*Leuconostoc carnosum*	*Enterococci, Carnobacteria, Listeria sp*
Pediocin AcH	*Pediococcus acidolactici*	*S. aureus, P. putida, L. monocytogenes, C. perfringens*
Pediocin A	*Pediococcus pentosaceus*	*S. aureus, C. botulinum, C. perfringens*

Source: Dave JM and Prajapathi JB (1994) Lactic acid bacteria as antibacterials against food borne pathogens and food spoilage organisms. In: Microbes for better living Ed: Sankaran R, Manja KS, Proceedings of MICON-INTL-94, Conference Secretariat, DFRL, Mysore.pp 361-367.

2.5.6. Cholesterol lowering capacity

Hypercholesterol has been linked with increased risk for coronary heart diseases. The use of probiotics to reduce this risk seems very attractive, especially if consumed as a part of a normal daily diet. Several human studies have suggested that dairy fermented products with certain strains of probiotic bacteria are able to lower the cholesterol level (Larsen et al., 2000; Sindhu and Khetarpaul, 2003; Parvez et al., 2006). De Smet et al. (1998) conducted an experiment in hypercholesterolemic pigs and showed a significant reduction of serum cholesterol level after administration of *Lactobacillus reuteri*. A number of mechanisms have been proposed for this action of probiotic bacteria. These include physiological action of short chain fatty acid fermentation, bile acid deconjugation and cholesterol assimilation by bacteria (Klaver and Vander Meer, 1993; Mercenier et al., 2002). Probiotics are known to ferment carbohydrates and produce short chain fatty acids in the intestine which inhibits cholesterol synthesis in liver and redistributes cholesterol from plasma to the liver. Individual strains can deconjugate bile salts and hamper absorption of cholesterol from the gut (Gilliland et al., 1985; De Boever et al., 2000; Doncheva et al., 2002; Ahn et al., 2003). Pereira and Gibson (2002) have proved that probiotics along with prebiotics have a potential to decrease serum lipid levels.

2.5.7. Antioxidative activity

The importance of reactive oxygen species in biology and medicine is evident because of their strong relationship with phenomenon such as aging and disease process (Cao et al., 1995). It is well known that free radicals and reactive oxygen (ROS) are continuously been produced in living organisms. As a result, defense mechanisms have evolved to deactivate these free radicals and repair the damage caused by their reactivity. Free radical scavenging properties of starter cultures are very useful in many food manufacturing industries. The probiotic cultures provide beneficial effect to the consumer by releasing antioxidants during the growth in the intestinal tract (Virtanen et al., 2007).

2.5.8. Enzymatic activity

LAB cultures are known to produce large number of enzymes for their survival and to impart beneficial effect. For rapid microbial growth and as precursors for aroma development in baked foods they are equipped with proteolytic system. This system is composed of cell envelop associated proteinase, peptidase transferase and intracellular peptidase for hydrolysis of protein and amino acids (Di Cagno et al., 2004). Aminopeptidase in particular, could reduce the amount of proline rich gliadin peptides in baked foods, which are known to elicit immune response in celiac disease (Gallo et al., 2005).

β-glucosidases, a major group of glycosyl hydrolase enzymes catalyze the selective cleavage of β-1,4-glycosidic linkages that play an important role in several biological pathways (Yan et al., 1998). Phytase, a phytate degrading enzyme is present in LAB that catalyzes the stepwise hydrolysis of phytic acid to myo-inositol via penta to mono phosphates (Lopez et al., 2000; Reale et al., 2004). Phytic acids are regarded as antinutritional compound since it chelates proteins, aminoacids and divalent cations such as Ca^{2+}, Fe^{2+}, Mg^{2+} and Zn^{2+} preventing their absorption by the intestinal mucosa (De Angelis et al., 2003). So phytase is very important enzyme in LAB when used in any functional food prepared with cereal grains. Urease, another important enzyme is known to protect microorganisms from harmful effect of acidic condition by increasing the environmental pH through conversion of urea into ammonia and CO_2 (Vande Guchte et al., 2002).

2.5.9. Nutraceutical property

LAB are the ideal factories for the production of nutraceutical compounds (Hugenholtz and Smid, 2002). Fermentation of food with LAB has been shown to increase niacin, riboflavin and folic acid content of yogurt, bifidus milk and kefir. Vitamin B_{12} and Vitamin B_6 have found to increase in

cottage cheese and cheddar cheese (Shahani and Chandan, 1979; Alm, 1982; Sauer et al., 1998). In addition to nutrient synthesis LAB improves digestibility of some available food supplements such as protein and fat (Friend and Shahani, 1984). Short chain fatty acids such as lactic, propionic and butyric acid produced by these cultures help in maintaining an appropriate pH and protect against pathological changes in the colonic mucosa. Burgess et al. (2004) have overexpressed a metabolic gene in *Lactobacillus lactis* for production of vitamins. *Lactobacillus plantarum* has been designed for excess production of folate that is known to maintain normal plasma homocysteine level in cognitive function and to provide protection against cancer (Sybesma et al., 2003; Jagerstad et al., 2004).

2.5.10. Probiotic as a source of fats and Fatty acids

Role of fats and fatty acids are well-known in human nutrition. Fats contained in foodstuffs provide substantial amount of energy for humans. Fatty acids are basic building blocks of the lipids in dairy products. In their free form they make significant contributions to the flavor of fermented foods. They also act as precursors for the formation of other aroma components, such as esters, aldehydes, alcohols, and ketones (Kinsella and Hwang, 1976; Scott, 1981). Analysis of the fatty acid profile which gives dairy products their particular organoleptic properties can be regarded as an index that can be very helpful in characterizing the functional properties of the foods. These fatty acids are also known to be therapeutically very important. Studies with animal models have demonstrated that conjugated linolenic acid consumption inhibits the initiation of carcinogenesis and tumorigenesis (Ip et al., 1991; Devery et al., 2001; Pariza et al., 2001) reduces body fat content and increases muscle mass (Chin et al., 1992; Akahoshi et al., 2002), decreases atherosclerosis (Lee et al., 1994; Nicolosi et al., 1997), improves hyperinsulinemia (Houseknecht et al., 1998), enhances the immune system

(Cook et al., 1993; Miller et al., 1994) and alters the low-density lipoprotein/high-density lipoprotein cholesterol ratio (Lee et al., 1994). Inoculation of *L. acidophilus* into skim milk medium was effective in promoting conjugated linolenic acid (CLA) formation (Lin, 2000). Dahi, an Indian equivalent of yoghurt is found to contain more CLA than the raw material (Aneja and Murthi, 1990).

Recent studies have shown that components of fermented dairy foods such as LAB cultures, dairy proteins, and dairy fats (including sphingomyelin, ether lipids, fatty acids such as oleic acid, palmitic acid, palmitoleic and conjugated linoleic acids) have antimutagenic and anticarcinogenic properties (Lidbeck et al., 1992; McIntosh et al., 1995).

2.5.11. Probiotic: Medical importance

Table 4 represents the LAB used in treatment of large number of diseases. Fuller (1999) has reviewed the uses of these products in animal husbandry. Mechanism of action of these LAB are summarized in Table 5. These LAB cultures constitute an integral part of healthy intestinal flora and are involved in host metabolism (Denev, 2006). LAB along with other gut microbiota ferments various substrates like lactose, biogenic amines and allergic compounds into short chain fatty acids and other organic acids and gases (Gibson and Fuller, 2000; Jay, 2000). LAB are known to synthesize enzymes, vitamins, antioxidants and bacteriocins (Knorr, 1998). All these properties contribute for the detoxification of foreign substances entering the body (Salminen, 1990).

Table 4: The protective effect of LAB in human and animal health

Medical target	Example strains	Reference
Prevent food allergy	*L. rhamnosus GG*	Sutas et al., 1996
Overcome lactose intolerance	*L. acidophilus*	Gilliland et al., 1984
Prevent diarrhea	*LAB, L. ehamnosus GG, L. acidophilus LB*	Fooks et al., 1999; Heyman, 2000; Simakachorn et al., 2000; Sanders, 2003
Reduce intestinal disorder	*L. rhamnosus GG*	Gionchetti et al., 2000; Kuisma et al., 2003
Suppress H. pylori	*L. acidophilus*	Canducci et al., 2000
Treat crohn's disease, ulcerative colitis and IBD	*L. rhamnosus GG, B. infantis* UCC 35624	Gupta et al., 2000; Von Von Wright et al., 2002; Marteau et al., 2002
Anticarcinogenic activity	*L. acidophilus*	Goldin, 1990; Hirayma and Rafter, 2000
Treat coronary heart disease and anticholesterlaemic effects	*L. acidophilus*	Schaafsma et al., 1998
Control urinary tract infection and vaginosis	*L. rhamnosus GG, L. rhamnosus GR*-1	Kontiokari et al., 2001; Reid, 2001
Prevent kidney stones	*L. acidophilus, L. plantarum, L. brevis, S. thermophilus, B. infantis*	Campieri et al., 2001
Treat atopic disease	*L. rhamnosus GG*	Kalliomaki et al., 2001
Prevent caries formation	*L. rhamnosus GG*	Nase et al., 2001
Protect against tetanus toxin	*L. plantarum*	Grangette et al., 2001
Treat chronic fatigue syndrome	*LAB*	Logan et al., 2003
Inhibit bovine mastitis	*L. lactis DPC 3147*	Ryan et al., 1998
Growth promotion in animals	*L. brevis C10*	Jin et al., 1998
Inhibit enteropathogens in small intestine of animals	*L. acidophilus LA1*	Bernet-Camard et al., 1997

Table 5 : Action of probiotic culture

Action	Mechanism
Alteration of intestinal condition to be less favorable for pathogens	❖ Alteration of toxin binding sites ❖ Gut flora alteration ❖ Adherence to intestinal mucosa ❖ Preventing pathogen adherence ❖ Competition for nutrients
Improvement of immune system	❖ Strengthening of non-specific defense against infection ❖ Increased phagocytic activity of white blood cells ❖ Increased serum Ig A after attenuated *Salmonella typhimurium* challenge
Reduction of inflammatory or allergic reactions	❖ Restoration of homeostasis of immune system ❖ Regulation of cytokine synthesis ❖ Prevention of antigen translocation into blood stream
Anticolon cancer effect	❖ Mutagen binding ❖ Carcinogen deactivation ❖ Alteration of activity of colonic microbes ❖ Immune response ❖ Influence on secondary bile salt concentration
Blood lipids, heart disease	❖ Assimilation of cholesterol ❖ Alteration of activity of bile salt hydrolase ❖ Antioxidative effect
Antihypertensive effect	❖ Peptidase action on milk results in antihypertensive tripeptides ❖ Cell wall components act as angiotensin converting enzyme inhibitors
Urogenital infections	❖ Adhesion to urinary and vaginal tract cells ❖ Competitive exclusion ❖ Inhibitor production (H_2O_2, biosurfactants).
Infection caused by *Helicobacter pylori*	❖ Competitive exclusion ❖ Lactic acid production ❖ Decrease urease activity of *Helicobacter pylori*
Regulation of gut motility	❖ Constipation
Alleviation of lactose intolerance	❖ Bacterial β-galactosidase enzyme acts on lactose
Positive influence on intestinal flora	❖ Influences activity of overgrowth microbial flora ❖ Decreases toxic metabolite production ❖ Antibacterial characteristics
Prevention of intestinal tact infection	❖ Adjuvants effect: increasing antibody production ❖ Stimulation of the systemic/ secretory immune response ❖ Competitive exclusion

Source: Sander and Huis In't Veld, 1999; Sanders, 1998; Sanders, 2001; Mercenier et al., 2002; Fiocchi, 2006.

2.5.11.1. Protection against gastrointestinal tract problem

Gastroenteritis is caused by viral, bacterial or parasitic infections. They also cause acute diarrhea, *Clostridium difficili* infections, ulcerative colitis, crohn's disease, traveller's diarrhea, *Helicobacter* infection, pouchitis and other inflammatory bowel diseases. A large number of reviews (Saavedra, 1995; Elmer, 1996; Michetti et al., 1999; Felley et al., 2001) focus on their preventive action by probiotic LAB. A large number of reports also determined that probiotic therapy shortened the length of acute diarrheal illness (Pedone et al., 1999; Pedone et al., 2000; Huang et al., 2002; Weizman et al., 2005). LAB have been found to control intestinal disorders partially due to antibodies like Ig G, IgA and IgM for enhancing immune responses (Kimura et al., 1997; Grangette et al., 2001). Some strains of LAB can translocate across the intestinal mucosa influencing systemic immune system (Cross, 2002). Characteristic adherence of the culture to mucosal surface contributes to their efficacy of being probiotic, since adherent strains confer competitive advantage that is important for the maintenance of balanced gastrointestinal microflora. Antibiotic associated colitis is known to be reduced by administration of the probiotic *Lactobacillus GG* (Vanderhoof et al., 1999; D'Souza et al., 2002).

Inflammatory bowel disease (IBD): Inflammatory bowel disease (IBD) mainly like Crohn's disease and Ulcerative colitis are chronic inflammation of terminal ileum. It is caused due to three pathogenic factors i.e., genetic susceptibility, immune dysregulation and environmental triggering events (Shanahan, 2004). A few experimental studies support probiotic LAB for the treatment of IBD. Administration of probiotic culture is known to restore epithelial barrier function with reduction of TNF α and INF-X secretion (Madsen et al 2001), inhibition of proteosome and activation of NF-KB (Petrof et al., 2004). Probiotic DNA is known to induce anti-inflammatory effect by signaling via TLR 9 (Rachmilewitz et al., 2004). They also activate anti-apoptotic A Kt/protein kinase B and inhibit activation of pro-apoptotic

P38/ mitogen activation protein kinase (Yan and Polk, 2002) which resulted in inhibition of cytokine induced apoptosis.

Irritable bowel syndrome (IBS): Irritable bowel syndrome (IBS) is a common functional disorder of lower intestine affecting both adults and children. It is associated with abdominal distension, diarrhea, constipation, bloating and urgency to defecate. Earlier reports have shown that this syndrome can be reduced with administration of probiotic culture (Brigidi et al., 2001; Sen et al., 2002; Kim et al., 2003).

Traveller's diarrhea: A number of specific strains, including *Lactobacillus GG, Lactobacillus reuteri, Saccharomyces boulardii, Bifidobacteria sp* and others have been shown to have significant benefit for diarrhea. In a study of 820 travellers, the diarrhea rate was 40% for control compared with 24% in *Lactobacillus GG* treated ones (Oksanen et al., 1990). In another study in a hospital based travel and immunization centre, an overall incidence averaged 7.4% for placebo and 3.9% for probiotic (*Lactobacillus GG*) group (Hilton, 1997).

2.5.11.2. Management of atopic disease and allergy

Atopic disease such as atopic eczema, allergic rhinitis and bronchial asthma are on the increase in all industrialized countries because of integrity of defense systems or disruption of immune response. Number of studies shows a significant reduction in respiratory infection of children and management of atopic disease by the administration of probiotic cultures (Hatakk et al., 2001; Murch, 2001; Kirjavainen et al., 2003). The protective action of probiotics to allergic inflammation and food allergies may be due to enhancement of gut specific IgA response, restoring normal intestinal permeability, improvement of intestinal immunological barrier function, alleviation of inflammatory cytokines characteristic of local and systemic allergic inflammation (Isolauri et al., 1992; Majamma and Isolauri, 1997; Isolauri et al., 2000; Isolauri, 2004).

2.6. Probiotics in functional foods

Functional foods are foods that claim to promote human health. The term functional food was first proposed in Japan two decades ago and legally approved them in terms of foods for specified health use (FOSHU). A large number of scientific evidences indicate that ingestion of LAB cultures exert health benefits not only in the gastrointestinal tract but also in the respiratory and urogenital tracts (Cadieux et al., 2008; Reid, 2008). Lactic acid bacteria (LAB) occupies a central role in fermentation processes and has long and safe history of application in the production of fermented foods and beverages (Caplice and Fitzgerald, 1999). They cause rapid acidification of the raw material through the production of organic acids, mainly lactic acid. LAB are able to produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, useful enzymes and nutraceuticals which represent the health promoting properties of LAB. These properties help in replacing chemical additives by natural compounds and at the same time providing the consumer with new, attractive food products with health benefits. Further they also enhance shelf life, improve texture and contribute to the pleasant sensory profile of the end product.

2.7. Probiotic: Dosage and Administration

As there are a variety of probiotic organisms with various delivery systems, there is no uniform dose recommendation. Studies to date have used oral doses of 1 to 10 billion colony forming units (cfu) per dose, with administration frequency ranging from twice daily to intermittent weekly schedules. Dose may also be dependent on the purpose of use that is treatment versus prophylaxis. With respect to site of action and strain used, it is suggested that each species function only at a particular site. Because of this industries are facing difficulties in establishing good measures of probiotic efficacy (Rolfe, 2000). Studies on lactose intolerance, diarrhea and colon cancer show that a daily dose of LAB is needed for any measurable effect (Rembacken et al., 1999).

The food and pharmaceutical industries have to be careful in assessing the efficacy of new species/ strain before incorporating them into the product. Many probiotic formulations are administered orally, either in capsule or powder form. These preparations result in a variable viability and are inadequate for therapeutic benefits. So the efficacy of the product is very essential and the optimal dose of probiotic for beneficial effect is to be considered before using them.

2.8. Probiotic: Market potential

Marketing functional foods was initiated in Japan in late 1980s. This concept is becoming increasingly popular with consumers heightened awareness of health linked with nutrition and diet. Today the food manufacturers are more enthusiastic in developing products with added ingredients to increase health. In this regard probiotic products are gaining popularity and acceptance throughout the world. These products are common in Japan and Europe (Lee et al., 1999; Sanders and Huis In't Veld, 1999; Stanton et al., 2001). The passage of Dietary supplement Health and Education Act (1994) has increased the scale of these products as dietary supplement. In 1997, these products accounted for 65% of European functional food market valued for US $ 889 million (Hilliam, 1998). Probiotic yogurt market in UK, France, Germany, Spain, Belgium, Netherlands, Denmark, Finland and Sweden is more than 250 million Kg (Hilliam, 1998). "Bikkle" a probiotic drink with *Bifidobacteria sp* was launched in 1993 (Osaka, Japan) which achieved a sale of 11 billion Yen in its first year (Young, 1996).

Interest in acceptance of functional foods is gaining importance in areas of developing new food processing, retailing and distribution technologies. Benefits of these functional foods are opening new opportunities to value added products for increasing profit. If a product can offer any measurable benefit to consumers in long term, there is a greater chance of re-buying the product (Childs, 1997). Hence, it is necessary that the

product should be of high quality that fits to the consumer's life style. As a reference, yogurt industry reported a 10.5% increase in sales from 1999-2000 and increase of 7.9% in 2001.

Probiotics products are becoming popular because of the desire by consumers to use natural methods for health maintenance rather than long term chemotherapeutic agents. The present challenge for nutrition and health professionals would be to guide consumers for their specific needs. They also should be aware of individual taste and food preference or nutrients of the consumers (Hoolihan, 2003).

2.9. Probiotic safety

The use of LAB in food products has a long safety record. The safety of probiotic bacteria has been reviewed by several authors (Mogensen et al., 2002; Saarela et al., 2002; Hammerman et al., 2006). The absence of pathogenicity and infectivity is the prerequisite of probiotic safety. There are a few published case reports of rare infections involving LAB, like liver abscess (Rautio et al., 1999), endocarditis (Husni et al., 1997), dental caries (Mackay et al., 1998), induction of inflammatory cytokines (Okitsu-Negishi et al., 1996) and septicemia (Barros et al., 2001). Therefore, safety assessment is an essential phase in the use of any culture.

Safety and efficacy assessment requires proper identification of probiotic culture to avoid any inclusion of pathogenic microorganism in the products. Pathogenicity, infectivity and virulence factors comprising of toxicity, metabolic activity and intrinsic properties of the microbes are the major factors that have to be addressed for the evaluation of safety of probiotic microorganisms (Salminen and Marteau, 1997; Ishibashi and Yamazaki, 2001). The culture should not bear any plasmid coding for antibiotic resistance (Franz et al., 2003; Cocconcelli et al., 2003).

2.10. Preservation of probiotic culture

Preservation of microorganism by desiccation has been the preferred method for long term storage. There are extensive culture collections that depend on these drying methods to preserve a huge diversity of cells. There are many applications in industries like food and pharmaceutical industries which utilize preserved aliquots of microorganism. These strains are subjected to different drying techniques to make cultures stable and transportable at ambient temperature. The different techniques employed are as follows:

Freeze drying: It is one of such technique used to preserve organisms for decades and is the preferred method for culture collections all over the world including American Type Culture Collection (ATCC) and National Collection of Type Culture (NCTC). Freeze dried materials allows easy and inexpensive shipping and handling. It is generally recommended to freeze dry concentrated cultures of $>10^7$ cells to ensure that there are sufficient cells remaining after the freeze drying process.

Foam formation: It is another technique which uses protective sugar matrics to transform biological suspensions into mechanically stable dry foams. The foam is then subjected to further drying at elevated temperatures to increase their stability (Bronshtein, 2004).

Spray drying: It is a drying technique that produces granulated powders from a slurry solution by atomizing the wet product at high velocity within the chamber (Corcoran et al., 2004).

Fluidized bed drying method: It is a method which uses an upward flow of heated air and mechanical shaking to create a fluidized effect in a solid product (Larena et al., 2003).

The process of dehydration can be detrimental to the viability of bacterial cell (Uzunova-Doneva and Donev, 2000). Hence protective agents are added prior to freeze drying to enhance the cell stability. Sugars like trehalose and sucrose have been reported to enhance the tolerance of numerous microorganisms by stabilizing membrane and proteins (Crowe et al., 1998; Gomez-Zavaglia et al., 2003; Streeter, 2003).

3. *Leuconostoc*: Characteristic and functional prospects

Leuconostoc spp are gram positive, catalase negative LAB with G+C DNA content less than 50%. They are involved in fermentation of food stuffs, production of gas in cheese, production of flavor compounds in dairy products and formation of dextran.

Leuconostoc spp are present in many natural ecological niches like green vegetation and roots from where they can easily propagate into various niches such as vegetables, silage (Ennahar et al., 2003) and fermented food products. They have been isolated from feces, vaginal samples and breast milk (Auge et al., 1987; Dal Bello et al., 2003). They have also been isolated from microflora of cattle (Brashears et al., 2003), fish (Ringo and Gatisoupe, 1998), insects (Ohkuma and Kudo, 1998; Reeson et al., 2003) and other environments such as treated bauxite residues (Hamdy and Williams, 2001). It is also one of the microbial components of kefir grain contributing to the production of ethanol and acetate (Robinson et al., 2002). They are commonly found in sugar processing liquors and fermented foods. They are desirable in foods because of their involvement in flavor development and preservation.

3.1. *Leuconostoc*: Taxonomy

Leuconostoc spp are non-motile, non-spore forming almost spherical cells, sometimes rather lenticular that resemble very short bacilli with rounded ends. Their size is approximately 0.5-0.7 × 0.7-1.2 μm. The cells are arranged in pairs or chains. They are facultative anaerobic bacteria with optimum growth at 20-30°C, but some species even grow at 10°C. The initial pH of the growth medium drops from 6.5 to 4.5 ± 0.1 during growth due to acid production. According to Bergey's manual one of the distinctive traits of *Leuconostoc spp* is their inability to hydrolyze Arginine. They are heterofermentative cultures producing CO_2 from glucose metabolism beside D-lactate and ethanol/ acetate (Table 6).

Nucleic acid hybridization, polymerase chain reaction, amplification and nucleic acid sequencing may be applied for *Leuconostoc sp* identification.

Cibik et al. (2000) have applied RAPD technique for identification of these strains which was confirmed using 16srDNA sequencing fragment amplification. DNA based methods are becoming widely useful for differentiation of *Leuconostoc spp* (Lee et al., 2000; Jang et al., 2003; Reeson et al., 2003). Separation of 16s ribosomal DNA by temporal temperature gradient gel electrophoresis (TTGE) is used for rapid identification of bacterial species present in dairy products including *Leuconostoc sp* (Ogier et al., 2002). Protein patterns or ribotyping is used for distinction between the sub-species of *Leuconostoc* (Villani et al., 1997; Perez and Hanson, 2002). *Leuconostoc* culture is also characterized based on the neutral volatile compounds produced in whey (Mauriello et al., 2001). Although molecular methods are useful for taxonomy and phylogeny of strains, phenotypic characterization also plays a predominant role (Table 6). Phenotypic features must be analyzed prior to molecular based techniques such as fermentation of sugar, citrate utilization, vancomycin resistance, CO_2 and dextran production (Cibik and Chartier, 2000). Table 7 represents the species included in the genus *Leuconostoc*.

Table 6 : Presumptive identification of *Leuconostoc* by phenotypical tests

General characters	Gram positive, cocci (ovoid-shaped), non-motile, non-spore forming, facultative anaerobic, catalase negative, production of gas from glucose, no arginine hydrolysis, production of D-lactate from glucose
Additional characters	Growth at 7% NaCl, no H_2S formation. Acid production from arabinose, arbutin, cellulose, cellobiose, fructose, galactose, lactose, maltose, mannitol, mannose, melibiose, raffinose, ribose, salicin, sucrose, trehalose and xylose

Table 7 : Species included in the genus *Leuconostoc*

***Leuconostoc* species**	**References**
L. mesenteroides *subsp cremoris* *subsp dextranicum* *subsp mesenteroides*	Garvie (1986)
L. lactis	Garvie (1986)
L. pseudomesenteroides	Farrow et al. (1989)
L. carnosuni	Shaw and Harding (1989)
L. gelideum	Shaw and Harding (1989)
L. fallax	Martinez-Murcia and Collins (1991)
L. citreum	Takahashi et al. (1992)
L. argentinum	Dicks et al. (1993)
L. gasicomitatum	Bjorkroth et al. (2000)
L. kimchi	Kim et al. (2000)
L. ficulneum	Antunes et al. (2002)
L. fructosuni	Antunes et al. (2002)
L. inhae	Kim et al. (2003)

3.2. *Leuconostoc*: growth and stability/ maintenance

Cultivation of *Leuconostoc sp* is carried out using enrichment broth and selective or non-selective media depending on a need to isolate either a particular genus from a mixture of microorganism or to maintain isolates (Bjorkroth and Holzapfel, 2003). The most commonly used media are API, Briggs, MRS, La and BHIYE. Inhibitory factors such as potassium sorbate (MRSS pH 5.7), thallous acetate (MRST pH 6.5), sodium azide (MSE), antibiotics such as vancomycin or tetracycline may be used alone or in combination as a selective ingredient for specific media.

MRS medium is the regular medium used for cultivation of pure cultures of *Leuconostoc sp*. Media for physiological studies of sugar fermentation, gas production, dextran formation, citrate degradation and others have been developed for their identification. Foucaud et al. (1997) have

developed a chemically defined medium that fulfills the nutritional requirement for rapid growth.

Leuconostoc sp grow at 30°C like other mesophilic *Lactococcus* starters but are favored by low temperature. Most strains grow well at 10°C (Hamasaki et al., 2003). Some are also thermoduric as they resist pasteurization (Martley and Crow, 1993). They are able to survive for a long time in unfavorable environment like surface of wooden gerl, glazed sandstone, iron or plastic moulds used in traditional cheese making (Devoyod and Poullain, 1988). They form slime or glycocalix in presence of saccharose and trace minerals resulting in a biofilm that protects cells from detrimental effect (Kim et al., 2000). Resistance to heat in *L. mesenteroides* is due to over expression of stress proteins that are homologous to chaperone proteins (Salotra et al., 1995; Derre et al., 1999).

Leuconostoc spp may be maintained for short duration in MRS stabs at 4°C (1-2 weeks). Cultures grown in litmus milk (supplemented with 5% yeast extract and 5% glucose) or in MRS broth (containing 1% lactose and 10% glycerol) can be stored for 6 months at -20°C. Lyophilization or freeze drying, the culture shows viability even on long storage for several months (Bellengier et al., 1997; Bjorkroth and Holzapfel, 2003).

3.3. *Leuconostoc*: Metabolism

Leuconostoc spp like other LAB do not contain a tricarboxylic acid cycle or a cytochrome system and so cannot derive energy from oxidative phosphorylation. Instead they obtain energy through substrate level phosphorylation during fermentation of sugars to lactic acid, CO_2 and ethanol/acetate (Cogan and Jordon, 1991). They take up carbohydrates by permeases enzymes which enter into phosphoketolase pathway leading to CO_2 and pyruvate. Pyruvate further gets converted into D-lactate in presence of D-lactate dehydrogenase enzyme. In presence of acetaldehyde in the medium, acetate kinase directs the flux for the formation of acetate and ATP. Additionally phosphotransacetylase produces acetyl CoA that is used for

biosynthesis of NADH with production of ethanol by alcohol dehydrogenase (Bourel et al., 2001).

Leuconostoc spp ferment fructose that enters pentose-phosphate pathway to be converted into mannitol which causes oxidation of NADH to NAD^+ in presence of mannitol dehydrogenase enzyme. Pentoses are converted to xylulose-5-phosphate that are further catabolized into glyceradehyde-3-phosphate and acetyl-phosphate (Cogan and Jordan, 1994). Galactose and mannose are used via the Leloir pathway. Lactose is cleaved to galactose and glucose by a β-galactosidase enzyme, which are further used via Leloir and pentose-phosphate pathway.

Organic acids like citrate and malate are metabolized by *Leuconostoc spp* that is important in aroma formation and gas production in fermented dairy products (McSweeney and Sausa, 2000; Konings, 2002). The ability of *Leuconostoc spp* to utilize amino acids independently or as mixture varied with strains and species and it shows the potential of the culture for flavor generation (Crowe et al., 1998; Tavaria et al., 2002; Liu et al., 2003).

3.4. *Leuconostoc*: Application

Leuconostoc spp plays a very important role in the technology of dairy products, in particular through production of gas and aroma compounds (Table 8). It is also applied for inhibition of undesirable bacteria in functional foods (Vedamuthu, 1994). They are involved in early and late blowing of some cheeses (Devoyod and Poullain., 1988; Narvhus et al., 1992). *Leuconostoc spp* utilizes diacetyl, acetate and ethanol contributing to aroma formation. They are also able to reduce acetaldehyde content under refrigerated condition thus avoiding 'green' flavor formation in butter and fermented milk. Some strains have shown to inhibit off-flavor production in cheddar cheese (Martley and Crow., 1993). They are used in many functional foods as probiotic culture with an intension of contributing health benefit to the consumer. Their presence in dahi is known to reduce diarrhea in children (Agarwal and Bhasin, 2002).

Table 8: Fermented foods that involve *Leuconostoc*

Products	Food stuff	Raw material	Country
Dairy	Butter and cream Cheeses Fermented milk (amasi, maziwa, lala, laban, filmjolk, kefir, pindidam, smetanka etc)	Milk Milk Milk	International International Europe, Africa, Asia
Meat	Sausages Salami	Meat Meat	Europe, Southeast Asia Europe
Fish	Sauce foods (belacham, chinchaluk, pekasam, sam-fak)	Fish, schrimp	Southeast Asia
Cereals	Beverages (beer, boza, bushera, idli, dadih, jangsu, ogi, pozol, sobia etc) Dough and starchy accompaniments (bread, flour, mawe, puto, trahanas) Sauce foods (tsauco)	Maize, corn, rice, millet Maize, rice, sorghum, tef Rice, soybeans	International Europe, Africa, Southeast Asia Southeast Asia
Vegetables	Sauerkraut Pickles, kimchi, sayur-asin Dough and starchy accompaniments (agbelima, flour, fufu, sapal etc) Cocoa Coffee Juices	Cabbage Olives, beetroot, cabbage, carrot, cucumber, sweet pepper Cassava, taro	International International Africa New Guinea, S. America, C. Africa S. America, C. Africa S. America, International
Fruits	Tempoyak, kocho	Durian fruit, Enseti ventricosa	Southeast Asia, Africa

Source: Hemme and Foucaud-Scheunemann, (2004)

4. Lactose intolerance

Inability of human beings to digest lactose is referred to as "Lactose intolerance" and has been attributed to insufficient amounts of lactase in the small intestine to hydrolyze lactose consumed in the diet (Littman and Hammond, 1965; Bayless and Rosenweig, 1966; Sarkar, 2006). Lactase deficient persons show symptoms like nausea, cramps, gas and diarrhea (Sieber et al., 1997). Temporary lactase deficiencies may also result from the damage of the intestinal lining. The disease is alarming and is of great concern to the society.

4.1. Significance of β-galactosidase/ lactase enzyme

Lactose, a disaccharide composed of glucose and galactose is the major solid component of milk. It is hydrolyzed into its components under the influence of enzyme lactase/β-galactosidase, a membrane bound enzyme present in the brush border of the small intestinal epithelial cell prior to its absorption by humans (Miller and Brand, 1980; Hourigan, 1984). In case the lactose ingested exceeds the hydrolytic capacity of the available intestinal lactase, the undigested portion of lactose is transported to the large intestine, where it increases the osmolarity of the intestinal fluids. Undigested lactose undergoes bacterial fermentation in the colon resulting in organic acid, CO_2 and H_2, which causes exclusion of large amount of water into intestine. This is primarily responsible for various symptoms such as bloating, flatulence, abdominal cramps, diarrhoea and loss of appetite (Hourigan, 1984; Hofi, 1990).

4.2. Lactose intolerance, maldigestion/ Malabsorption

Lactose maldigestion occurs due to either gastrointestinal disease or physiological decline in the intestinal lactase activity. Semenza and Auricchio (1995) registered reduction in lactase activity due to the digestion of lactase-

phlonizin hydrolase molecule by pancreatic proteases at the brush border membrane. Swaminathan (1998) has described three main reasons for deficiency of lactase enzyme.

1) Congenital lactase efficiency: caused due to the absence of lactase enzyme in intestinal mucosa.
2) Lactase deficiency in premature infants: occurs in premature infants due to decrease in lactase activity. Initially, infants cannot utilize lactose efficiently, however they are able to tolerate and digest milk after one month due to increase in lactase activity.
3) Acquired lactase deficiency: adults and older children cannot tolerate large amounts of milk due to their non-habitual consumption of milk resulting in low lactase in the intestinal mucosa.

4.3. Occurrence of lactose intolerance

According to Swagerty et al. (2002) about 70-90% of adults in most parts of the world are known to be lactose intolerant (Table 9). The prevalence of lactose maldigestion is 15-20% in Austria, 55% in Balkans, 70-90% in Africa, 80% in Central Asia, 90-100% in Eastern Asia, 30% in Northern India, 70% in Southern India and 65-75% in South America (de Vrese et al., 2001).

Table 9 : Incidence of lactose intolerance in different population group around the world

Group		Lactose intolerance (%)
USA	White	6-21
	Black	70-75
	Indian	67
Africa	Uganda	72
	S. Africa	90
	Nigeria	58-99
Europe	Greek crypriots	88
	Switzerland	17
	Finland	17
	Denmark	6
	Czechoslovakia	18
	Poland	29
	Germany	15
	Greece	38
	Turkey	15
Asia	Chinese	100
	Korean	100
	Japan	100
	Malaysia	100
	Philippines	97-100
	Thailand	81
	Australia	0-8

Source: Fernandes et al., 1987 and Sanul, 1990.

4.4. Lactose intolerance: Diagnosis

Lactose intolerance is either self diagnosed by the patient or diagnosed by a physician using subjective evaluation tools (i.e., description of symptoms, elimination of diets) rather than objective testing methods (i.e., Breath hydrogen test) (Lovelace and Barr, 2005). If symptoms are chronic, a physician should be consulted and an objective test should be conducted. Direct and indirect methods are available for diagnosis of lactase deficiency (Table 10). It is possible to directly assay the lactase activity in the small bowel by taking an intestinal biopsy. Indirect methods for diagnosis include

lactose tolerance test, stool acidity and breath hydrogen test. When lactose or any other dietary sugars is not completely absorbed, the unabsorbed portion is fermented by colonic bacteria forming hydrogen, which is exhaled in breath (Saavedra and Perman, 1989). According to test protocol, breath samples are taken every 30 min for 3 h and analyzed by GC. An increase in breath hydrogen of >10-20 ppm indicates positive for lactose maldigestion (Montes and Perman, 1990). A small percentage of people do not have colonic flora that ferment lactose, which can lead to false negative results. This situation may be the result of antibiotic use before the test. Smoking prior to the test may also lead to false positive result. The breath hydrogen test is also useful in diagnosing bacterial overgrowth that can cause secondary lactose intolerance.

Now that the genetic variant responsible for lactase non-persistence has been identified, researchers are evaluating whether genetic testing could replace other traditional diagnostic methods (Hogenauer et al., 2005; Ridefelt and Hakansson, 2005). Genotyping for the DNA variant associated with adult hypolactasia demonstrates an excellent correlation between both hydrogen breath test and rise in blood glucose following lactose challenge (Buning et al., 2005). Genetic testing may soon complement other indirect methods for identifying individuals at risk for both lactose malabsorption and osteoporosis (Obermayer-Pietsch et al., 2004).

Table 10: Diagnostic tests for lactose intolerance

Test	Result
	Rise in breath hydrogen >20 ppm
Stool pH	Acid pH (<6.0)
Small bowel biopsy	Disaccharide assay (<13 IU/g of mucosal protein)
Lactose absorption (fecal reducing substances)	Minimal to significant

Source: Rusynyk and Still (2001)

4.5. Approach towards treating lactose intolerance

The common therapeutic approach would be to exclude milk and dairy products with lactose from the diet. However this strategy may have nutritional disadvantages like reduced intake of calcium, phosphorus and vitamins (Di Stefano et al., 2002). Solid lactase preparation in the form of capsules and tablets are commercially available as an alternative enzyme replacement therapy (Di palma et al., 1989). However comparative studies have shown that these preparations are more expensive and significantly less effective probably due to enzyme gastric inactivation (Suarez et al., 1995). So the use of culture, which is resistant to gastrointestinal conditions, is the best method for lactose hydrolysis.

LAB contains *β*-galactosidase as an intracellular enzyme which is protected during passage through the harsh environment of stomach and is able to reach the small intestine. Shah and Lankaputhra (1997) have shown that rupturing of cell membrane of starter cultures of yogurt reduced viable counts but increased the release of intracellular *β*-galactosidase. Microorganisms residing in the large intestine were tolerant to lactose through modifications of their metabolic activity (Hertzler and Savaiano, 1996). In general, improved digestion results from lactase activity of bacteria or stimulation of host's mucosal lactase activity (Kolars et al., 1984; Gibson and Fuller, 2000).

4.6. *β*-galactosidase enzyme/ Lactase

Lactase is a trival name of the enzyme *β*-D-galactosidase (Gekas and Lopez-Leiva, 1985). *β*-galactosidase was among the first hydrolase enzyme to be discovered. The molecule breaks down the sugar lactose which is composed of two rings bound together by Oxygen Bridge. This bridge is broken when enzyme *β*-galactosidase binds to lactose and water molecule reacts with oxygen atom in the bridge (Hung and Lee, 2002). *β*-galactosidase not only catalyses the hydrolysis of *β*-galactosidic linkage, but also catalyses transglycosylation reactions. It is speculated that *β*-galactosidase is a spurious

enzymatic activity (Premi et al., 1972). In some cases they are able to catalyze the opposite direction of hydrolysis ie., glycosylation (Karasova, 2002). They are widely distributed in all organisms (Table 11). Possible sources of enzyme are plants, animals, organs, bacteria, yeast, fungi and moulds (Gekas and Leiva, 1985). The traditional source of *β*-galactosidase are of microbial origin mainly yeasts and molds (Mahoney, 1997).

Table 11: List of organisms that produce lactase

Category	Source	Product name
Yeats	*Candida pseudotropicalis* *Kluyveromyces fragilis* *Kluyveromyces lactis*	Neural lactase Hydrolact Maxilacet
Animal organs	*Intestine* *Brain and skin*	
Bacteria	*Bacillus megaterium* *Escherichia coli* *Lactobacillus acidophilus* *Lactobacillus bulgaricus* *Lactobacillus belatericus* *Lactobacillus crispatus* *Lactobacillus helveticus* *Lactobacillus pentosus* *Streptococcus lactis* *Streptococcus thermophilus* *Thermos aquaticus*	Acidophilus (Wakunga probiotics)
Fungi	*Aspergillus flavus* *Aspergillus faetidus* *Aspergillus niger* *Aspergillus oryzae* *Aspergillus phoenicis* *Curvularia inaqualis* *Micrococcus meibei* *Micrococcus purillus* *Neurospora crassa*	Valio lactase
Plants	Almonds Apricot Coffee berries Kefir grains Peach	

Source: Godfrey and West, 1996

In the present work *Leuconostoc mesenteroides*, a lactic acid bacterial strain has been isolated, characterized and adapted for its probiotic functional properties to be used in food formulation.

Objectives

1) Isolation and characterization of *Leuconostoc* from milk products
2) Properties of the isolated bacterium in relation to functional significance
3) Colonization of LAB in relation to homoserine lactone

Aim & Scope of Present investigation

AIM OF THE PRESENT INVESTIGATION

During the past several years, the focus of nutritional sciences has shifted from deficiency disease prevention to optimizing health and prevention of chronic diseases. Accordingly, the research has encompassed the health effects of bioactive food components. In this regard, probiotic therapy is being used increasingly in humans and veterinary medicine due to their apparent high index of safety and public perception about natural or alternative therapies (Gherty 1995; Sander 1998; FAO Report 2002).

Probiotics are a category of 'nutraceuticals' i.e., viable cultures added to food with the intention of maintaining or improving the nutritional health of consumers. Lactic acid bacteria (LAB) are in the focus of extensive research because of their probiotic nature.

Today, probiotic market promises the disease prevention and better health for all as a natural alternative therapy. In the present scenario, with increase desire of consumer for natural food products as source of providing nutrition and other desirable benefits, research work towards the selection of strain with functional properties has become very important.

Considering all these aspects, the aim of the present work is to isolate an indigenous food grade lactic acid bacterial culture from different milk and milk products to be used as a potential probiotic culture. The selected strains will be adapted to survive under gastrointestinal conditions. The culture will be analyzed for its potential probiotic functional properties like antimicrobial, antibiotic resistance, antioxidative activity, anticholesterolemic activity and adherence ability. Volatile compounds of therapeutic importance produced during growth will be studied.

The study will be focused on the ability of the culture to hydrolyze the non-reducing disaccharide lactose into simple sugar for easy absorption. Strain will be improved for enhanced β-galactosidase activity which is responsible for lactose hydrolysis. Work will be carried out to preserve the culture on shelf storage by different drying techniques.

The potent isolated culture will be used as a starter culture for the preparation of probiotic fermented milk beverage. Different adjuvants will be supplemented to enhance the nutritional properties of the fermented milk beverage. Further the aim will be to preserve the fermented milk beverage from spoilage bacterial flora by interfering with the quorum sensing signal molecules using furanones. Safety and beneficial effect of the culture will be analyzed *in-vivo* using albino rats. Ability of the culture to hydrolyze lactose and reduce lactose intolerance problem will be analyzed in lactose intolerance induced rats.

SCOPE OF THE PRESENT INVESTIGATION

With the rise in the consumer's awareness of individual health, nutrition and well being, the interest and demand for value added foods and beverages has expanded. In this regard, the present isolate having potential probiotic functional properties promises to be a source of natural alternative cure. The β-galactosidase activity of the culture will show its positive impact in reducing lactose intolerance problem which is prevalent all over the world. The fermented beverage prepared by the probiotic *Leuconostoc mesenteroides* (M7-PLsr-$1_{(W)}$) will serve as a nutritive source for all ages.

Chapter 1

Isolation & Screening of Lactic Acid Bacteria from Milk & Milk Products

CHAPTER - 1

ISOLATION AND SCREENING OF LACTIC ACID BACTERIA FROM MILK AND MILK PRODUCTS

ABSTRACT

In the present work bacterial cultures were isolated from milk and milk products and screened for lactic acid bacteria with characteristics of being tolerant to gastrointestinal conditions. Totally forty five bacterial strains were isolated and screened for gram positive, catalase negative, non-motile, non-spore forming and vancomycin sensitive strains. The selected strains were adapted to grow at pH 2.0 and 4.0% bile salt mix concentration. The three adapted strains were subjected to simulated gastrointestinal condition. The strains that survived under such environment were identified through biochemical and molecular methods. The identified culture *Leuconostoc mesenteroides* (Lsr-$1_{(W)}$) and *Lactobacillus plantarum* (Lsr-$12_{(cu)}$) were then screened for resistance to digestive enzymes pepsin and trypsin. *Leuconostoc mesenteroides* (Lsr-$1_{(W)}$) that confirmed resistance to these enzymes was coded as **PLsr-$1_{(W)}$** and used for further studies.

1.1. Introduction

Interest in microorganisms as a component of biological diversity has been renewed in recent years. Probiotics include lactic acid bacteria (LAB). Mankind has exploited these bacteria for many years for the production of fermented foods because of their ability to produce desirable changes in taste, flavor and texture. The demand for the probiotic foods is increasing all over the world reflecting the awareness among the public between diet and health. Numerous scientific papers and review articles have been published on health benefits of these fermented products (Ostlie, 2005; Hughes and Hoover, 1995).

Probiotics have health promoting effects including inhibition of pathogens, antimutagenic, anticarcinogenic activity, prevention of diarrhea, stimulation of immune response and reduction of serum cholesterol (Tannock, 1999). The development of new applications such as live vaccines and probiotic foods reinforces the need for these characteristics.

Considering these, study was carried out to isolate a potential LAB culture from milk and milk products with the ultimate aim of using the culture in food formulations. In this study, isolated cultures were adapted for gastrointestinal condition of low pH and high bile salt mix concentration. The cultures that could survive such stress conditions were characterized and identified through bio-chemical assays and molecular techniques. These cultures were studied for the tolerance to digestive enzymes. The best culture showing all these properties was selected for further studies.

1.2. Materials

Samples: Milk, curd, butter, cheddar cheese, buttermilk and whey.

Chemicals: All chemicals used in this study were of analytical reagent grade purchased from HiMedia Pvt Ltd., India unless otherwise mentioned.

1) *De Mann Rogosa Sharpe media*: Table (1.1) represents the media composition. All the ingredients are accurately weighed and dissolved in double distilled water. Sterilization was carried out at 121^0C at 15 lb pressure for 20 min (For preparation of solid media bacteriological agar was added (2%) before sterilization).
2) Saline: 0.8% NaCl.
3) Hydrogen peroxide (30%).
4) Vancomycin antibiotic (30 μg).
5) Buffers: Glycine-HCl buffer (0.2 M; pH 2.0-3.0), Citrate buffer (0.1 M; pH 4.0-5.0), Sodium-phosphate buffer (0.2 M; pH 6.0-8.0) and Glycine-NaOH buffer (0.2 M; pH 9.0).
6) Bile salt mix: Bile salt mix contains 0.3% each of sodium salts of glycocholic acid, glycodeoxycholic acid, taurocholic acid and taurodeoxycholic acid.
7) Digestive enzyme: Pepsin and trypsin (Sigma-Aldrich Company).
8) Chemicals for DNA isolation: TAE buffer (40 mM Tris acetate, 1 mM EDTA; pH 8.0), PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 M KH_2PO_4), proteinase K, SDS, agarose, ethidium bromide and Sodium acetate (Sigma-Aldrich Company, India).
9) PCR chemicals: Primer, Taq polymerase, Taq polymerase buffer, $MgCl_2$ and dNTPs (Sigma-Aldrich Company, India).
10) Lysis buffer (100 mM EDTA, 34 mM SDS)

Equipment : Microscope (Olympus BX 40, Japan), pH meter (Genei, Control Dynamics, India), Centrifuge (Labline, India), DNA isolation unit (Genei, Bangalore), PCR machine (Primers Company, MWG Biotech, Germany), Gel documention (Bio-Rad Laboratories, USA).

Table 1.1 : Composition of MRS media (deMan Rogosa Sharpe Media)

Ingredients	Quantity (g/L)
Peptone	10.0
Beef Extract	10.0
Yeast Extract	05.0
Dextrose	20.0
Polysorbate	0.10
Tri Ammonium Citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Di potassium hydrogen phosphate	2.00

pH 6.5

*All the ingredients were appropriately weighed and dissolved in distilled water (1L). Sterilization was done at 121°C for 15 min at 15lb pressure. For solid media, bacteriological agar (2%) was added to media, homogenized and then sterilized as described above.

1.3. Methods

1.3.1. Isolation of lactic acid bacteria (LAB)

Five samples each of milk, curd, butter, cheddar cheese, buttermilk and whey were collected from different areas in Mysore, India. Each sample (1 g) was suspended in saline (9 ml), serially diluted and plated on MRS media. Plates were then incubated at 37°C for 24 h. The colonies that exhibited distinct morphology in color, shape and size were selected, purified by repeated streaking and subsequently sub-cultured in MRS broth. The purified cultures were preserved in MRSA stabs overlaid with liquid paraffin and stored at 4°C until use.

1.3.2. Preliminary selection and characterization of isolated cultures

The selected bacterial cultures were grown in MRS broth and observed under the microscope for their morphological structure. The cultures were subjected to gram staining (Beveridge, 2001). Catalase test was performed by suspending the culture in H_2O_2 and production of effervescence was checked (Smibert and Krieg, 1981). Motility of the cultures was tested using cavity

slide by hanging drop method under the microscope (Priest et al., 1988). Vancomycin susceptibility was tested by BSAC standardized disc sensitivity testing method using 30µg vancomycin disc. Sensitivity or resistance was determined by the growth of the culture around the antibiotic disc.

1.3.3. Screening the isolated cultures for acid and alkaline tolerance

The gastric pH varies from pH 2.0-3.5 and intestinal pH from 7.5-9.0. Therefore to check the growth of LAB under these conditions the selected bacterial strains were inoculated (1% v/v) into MRS broth at different pH (2.0, 3.0, 4.0, 7.5 and 9.0) and incubated at 37^0C for 24 h. Initial and final cell count of treated cultures were determined by plating on MRSA media. Bacterial cultures that exhibited survival under such conditions were selected for further screening for bile resistance.

1.3.4. Screening the cultures for bile salt tolerance

Bacteria that survived the acidic condition of stomach have to face further challenge of bile that is released into upper small intestine after ingestion of fatty meals (Hong et al., 2005). As the bile salt concentration varies in different regions of human intestine from 1.5 to 4.0% (Chou and Weimer, 1999; Berrada et al., 1991) the selected cultures were further screened for their growth under the conditions. Experimentally, MRS broth was prepared by supplementing different concentrations of filter sterilized bile salt mix (0.5 to 4.0% w/v) separately in different conical flasks. The selected cultures were inoculated (1% v/v) to these flasks and incubated at 37°C for 24 h. Cultures that grew in the presence of bile salt mix were selected for further studies.

1.3.5. Adaptation of culture to pH 2.0 and 4% bile salt concentration

The selected cultures were adapted for acidic pH by repeated sub-culturing for 3-4 generations in MRS media prepared in a descending gradient from pH 4.3 to pH 2.0 using 1N HCl. Similarly, for adaptation to bile salts,

the cultures were grown in MRS broth supplemented with bile salt mix in ascending succession gradually from 0.5 to 4.0% and sub-culturing 3-4 times in the medium at each concentration.

1.3.6. Survival under simulated intestinal tolerance

An *in-vitro* method was designed that resembled the intestinal conditions of human to check the tolerance of the culture that were adapted to grow at low pH (2.0) and high bile salt mix (4%). MRS broth was adjusted to that of jejunum condition (pH 7.5 and 4% bile salt) and inoculated with the selected cultures (one culture/flask) at a concentration of 2×10^8 cfu/ml. After an incubation period of 4 h at 37°C, cells were collected by centrifugation and then transferred to another flask with MRS broth at pH 8.0 and 2% bile salt mix (condition of small intestine) and incubated for 12 h at 37°C. Again after the incubation period the cell pellet was collected and transferred to another flask of MRS broth at pH 9.0 and 1.5% bile salt mix (condition as of large intestine) and incubated for 24 h at 37°C. At each simulated condition culture viability was determined by plating the appropriate dilution on MRSA media. After an incubation period of 24 h, colonies grown were counted and expressed as colony forming units per ml (cfu/ml).

1.3.7. Identification and characterization of selected cultures

The adapted cultures that were able to survive under the simulated intestinal conditions were identified by bio-chemical assays and molecular techniques.

1.3.7.1. Phenotypic characterization of selected culture

Physiological and biochemical characterization of the selected cultures were tested according to Bergey's manual of Systematic Bacteriology (Kreig, 1984). Growth at different temperatures (10°, 37° and 45°C) and pH (4.2, 8.5 and 9.6) were evaluated by incubating the selected cultures at the respective temperature and pH. Similarly salt tolerance was determined by growing the

culture in MRS broth supplemented with different concentration of NaCl (2.5, 6.5 and 18.0%).

The carbohydrate fermentation ability of the selected strains was tested in basal media (MRS broth devoid of beef extract and dextrose) with bromocresol purple as an indicator. The filter sterilized sugar was added to the basal media at a final concentration of 2g/100 ml. The selected strains were inoculated (50 µl) into the basal medium and incubated at 37°C for 24 h. Growth and color change in the medium were observed after 24 h of incubation (Sneath et al., 1986; Rashid et al., 2007). Deamination of arginine was tested in Thornley's semi solid arginine medium (Terzaghi and Sandine, 1975).

1.3.7.2. Molecular characterization of selected cultures

1.3.7.2.1. Extraction of genomic DNA

Bacterial genomic DNA was extracted according to the method of Perez et al (2002). The exponentially grown culture (1.4 ml) was centrifuged to collect the cell pellet. Cell pellet was washed twice with PBS buffer and suspended in 700 µl of lysis buffer followed by addition of proteinase K (35 µl; 20mg/ml). The mixture was then incubated at 50°C for 80 min. The aqueous phase was then extracted with an equal volume of phenol followed by phenol: chloroform: iso-amyl alcohol (25:24:1) and finally with chloroform: iso-amyl alcohol (24:1). The aqueous supernatant was combined with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol. The pellet was washed with 70% ethanol and dissolved in 50 µl 1X TAE buffer.

1.3.7.2.2. Amplification of 16srRNA by polymerase chain reaction and identification by sequence analysis

PCR based typing method was used for identification of the strain using the universal primer M13 (5' CCC AGT CAC GAC GTT 3'). PCR was performed in a total volume of 30µl containing 1 ng of template DNA, 0.8 µM of primer, 0.5 U Taq polymerase, 1X Taq polymerase buffer, 2 mM $MgCl_2$ and 0.2 mM of dNTPs. Amplification reaction was performed with the

following temperature profile: 10 min at 94°C, 25 cycles of 1 min at 94°C, 2 min at 61°C and 5 min at 72°C. The amplified product (15 µl) was loaded into agarose gel (1.5%) in TAE buffer and the bands were observed after staining with ethidium bromide. Gels were then photographed under gel documentation.

For identification of *Leuconostoc* culture, the specific primer was designed with a sequence of F 5' ATT GGG ACT GAG ACA CGG 3' and R 5' TGA TGA CCT GAC GTC GTC C 3'. Amplification of the 16srRNA sequence was performed with the following thermal cycle: initial denaturation at 94°C for 2 min, followed by 40 cycles of 1 min denaturation at 94°C, annealing at 32°C for 2 min and extension for 7 min at 72°C and a final extension for 7 min at 72°C. PCR product was then sequenced and through BLAST search of multiple sequence alignment programme, the cultures were identified and a phylogram was drawn with the nearest cultures.

1.3.8. Determination of tolerance to digestive enzymes

1.3.8.1. Preparation of simulated gastric and small intestinal juices

Simulated gastric and small intestinal juice was prepared fresh as per the procedure of Huang and Adams (2004). Simulated gastric juice was prepared by suspending pepsin (3 g/L) in sterile saline (0.5% w/v) which was previously adjusted to pH 2.0 using buffer. Simulated small intestinal juice was prepared by suspending trypsin (1 g/L) in sterile saline (0.5% w/v) with supplementation of 4% bile salt mix. The pH was adjusted to 7.5 with sterile 0.1 M NaOH.

1.3.8.2. Determination of GI transit tolerance

The cell biomass of the selected cultures was collected by centrifugation at 8000 rpm for 10 min at 4°C. The cell biomass collected was washed thrice in phosphate buffer (pH 7.0) and suspended in saline (1 ml). An aliquot (0.1 ml) of this suspension was mixed with simulated gastric (10 ml) and intestinal juice (10 ml) separately and incubated at 37°C for 3 h.

Transit tolerance of the cultures were determined by counting the viable cells after serial dilution and plating.

1.3.9. Bacterial growth

The growth pattern of the selected culture isolate was studied in MRS broth. The culture was inoculated into MRS broth at a concentration of 1.8×10^5 cfu/ml and incubated at 37°C. At each time interval, an aliquot of sample was taken, serially diluted and appropriate dilution was plated on MRSA plates. Plates were then incubated at 37°C for 24 h. The colonies grown were counted and expressed as colony forming units per ml (cfu/ml).

1.4. Results and Discussion

1.4.1. Isolation of lactic acid bacteria

A screening procedure was performed to select a potential probiotic culture from milk and milk products. The aim of the present study was to select a possible lactic acid bacterial culture to be used as probiotic that is tolerant to gastrointestinal tract conditions.

Isolation and identification of lactic acid bacteria has been carried out from different sources like goat's milk, katyk, cheese (Tserovska et al., 2002) and fermented milk (Beukes et al., 2001; Savadogo et al., 2004). In the present work milk and milk products were selected as a source for isolation of LAB. Totally 45 bacterial strains were isolated and purified. The isolates were selected randomly based on their differences in colony morphology, color, texture and margin (Fig. 1.1). The isolates were either cocci (30 isolates) or bacilli (16 isolates) of different sizes.

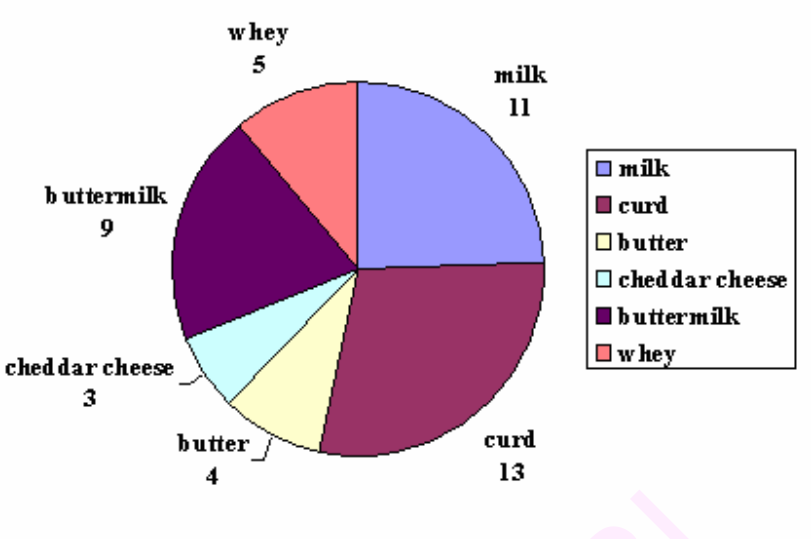


Fig 1.1: Distribution of isolated cultures from milk and milk products.
Values are the number of isolated cultures.

1.4.2. Preliminary selection and characterization of isolated cultures

Out of 45 strains isolated two strains did not grow on further propagation. From the remaining 43 strains, 10 were gram negative and 9 were catalase positive and were not considered to be presumptive LAB. Motility test confirmed that 5 isolates were motile and other 4 strains showed sporulation. The remaining 15 cultures were tested for Vancomycin sensitivity. It was observed that 3 isolates were Vancomycin resistant. All these were discarded.

Out of 12 selected strains, 3 strains were rod shaped. They did not produce gas on glucose fermentation and were not able to ferment pentose sugars (arabinose, ribose and xylose). Therefore, they were characterized as obligate homofermentative bacilli. One isolate with rod-shaped morphology did not produce gas from glucose fermentation but could ferment pentose sugars and was characterized as facultative heterofermentative rod/ bacilli. Four strains which were cocci produced gas from glucose and L-lactate indicating that they belong to heterofermentative cocci. The other 4 strains with cocci morphology did not produce gas from glucose and L-lactate. Therefore they were characterized as homofermentative cocci (Table 1.2).

Table 1.2: Preliminary characterization of isolated cultures

Culture code	Source of isolation	Morphology	Gram stain	Catalase	Gas from glucose	Arabinose	Ribose	Xylose
Lsr-$1_{(W)}$	Whey	Cocci	+	-	-	-	+	+
Lsr-$2_{(W)}$	Whey	Rod	+	-	-	-	-	-
Lsr-$3_{(B)}$	Butter	Cocci	+	-	-	+	+	+
Lsr-$4_{(B)}$	Butter	Cocci	+	-	+	-	-	-
Lsr-$5_{(BM)}$	Buttermilk	Rod	+	-	-	-	-	-
Lsr-$6_{(BM)}$	Buttermilk	Cocci	+	-	+	+	-	-
Lsr-$7_{(CC)}$	Cheddar cheese	Rod	+	-	-	-	-	-
Lsr-$8_{(CC)}$	Cheddar cheese	Cocci	+	-	-	-	+	+
Lsr-$9_{(M)}$	Milk	Cocci	+	-	+	-	-	-
Lsr-$10_{(M)}$	Milk	Rod	+	-	-	-	-	-
Lsr-$11_{(CU)}$	Curd	Cocci	+	-	+	-	+	+
Lsr-$12_{(CU)}$	Curd	Rod	+	-	-	+	+	+

*(+) = positive reaction; (-) = negative reaction

1.4.3. Screening for gastrointestinal tolerance

Survival under gastrointestinal environmental condition is one of the most important characteristic features for a culture to be used as a probiotic. As the pH is acidic in stomach and alkaline in the intestinal region (Pennacchi et al., 2004; Klingberg et al., 2006), the selected cultures were screened for their ability to grow at both acidic and alkaline pH.

pH of secreted HCl in stomach is 0.9, but presence of food raises the pH value to 2.0-3.0 (Erkkila and Petaja, 2000). Hence in the present study the isolated cultures were screened for their ability to grow at varying pH from 2.0 to 9.0 by inoculating the cultures at a rate of 5×10^5 cfu/ml. The cultures show variable degree of tolerance to different pH. Zarate et al. (2000) have reported four dairy *Propionibacterium* to survive at pH 4.0 but did not grow substantially at pH 2.0. Similarly in the present work, at pH 4.0 all the tested bacterial strains survived but at pH 3.0, four strains lost the viability.

Earlier reports have shown large number of LAB like *L. sakei, L. plantarum, L. pentosus, P. acidilactici* and *P. pentosaceus* to be tolerant to acidic condition (Pennacchia et al., 2004; Klingberg et al., 2006). Prasad et al. (1998) during screening a large number of LAB (200 isolates), selected four resistant strains which were able to survive at pH 3.0. In the present study 6 cultures were selected that were able to survive at pH 3.0. The culture Lsr-$1_{(W)}$ was able to survive at pH 3.0 with maximum survival rate (20%). The other cultures Lsr-$3_{(B)}$, Lsr-$8_{(Cc)}$, Lsr-$10_{(M)}$, Lsr-$11_{(Cu)}$ and Lsr-$12_{(Cu)}$ showed a survival rate of 10, 1, 4, 3 and 8% respectively. These six cultures which were able to grow at acidic pH 3.0 were selected for bile salt tolerance.

Bile plays a fundamental role in specific (Marteau et al., 1997) and non-specific (Kalambaheti et al., 1994) defense mechanism of the gut and the magnitude of its inhibitory effect is determined primarily by the bile salt concentration (Charteris et al., 2000). Since the cell membrane of microorganism is composed of lipids and fatty acids the bile salts are critical for their survival.

With this in mind, the present work was carried out to screen the isolated cultures for bile salt tolerance. The selected cultures that were able to tolerate pH 3.0 were grown in MRS broth supplemented with varying concentration of bile salt mix (0.5–4.0%). It was observed that there was a delay in the growth on supplementation of bile. Chateau et al. (1994) have also reported an extreme variability of resistance to bile salts in *Lactobacillus sp* and all the tested strains showed a delayed growth as compared to a reference culture without bile salts. Similar results have been noticed by Gilliland et al. (1984), Gupta et al. (1996) and Mustapha et al. (1997).

Gomes-Zavaglia et al. (1998) have shown that 10 *Bifidobacterium* strains out of 41 tested were resistant to 0.5% bile. In the present work all the six cultures selected were able to grow at 0.5% bile salt mix. The cell viability was found to decrease with increase in bile salt mix concentration. The culture Lsr-$1_{(W)}$ that was isolated from whey exhibited maximum survival (12%) followed by Lsr-$10_{(M)}$, Lsr-$12_{(Cu)}$ and Lsr-$3_{(B)}$. Further these four cultures were adapted to tolerate the gastrointestinal condition of pH 2.0 and 4% bile salt concentration.

1.4.4. Adaptation of the culture to GIT condition

Adaptation to low pH and high bile is a valuable tool for increasing bacterial survival in the harsh conditions of GIT. Earlier reports have suggested that inhibition of microbial growth due to stress condition can be overcome by progressive adaptation to increasing concentration of these compounds (Chung et al., 1999; Margolles et al., 2003). In the present work, the selected culture isolates were grown in MRS broth through sequential sub-culturing by increasing the concentration of bile salt and decreasing the pH (Table 1.3). The results determined that the cultures Lsr-$1_{(W)}$, Lsr-$10_{(Cu)}$ and Lsr-$12_{(Cu)}$ were better ($P<0.05$) adapted to such stress conditions.

Table 1.3 : Adaptation of isolated cultures to low pH and high bile salt mix concentration

Treatment		Optical density (600 mn) after 24 h of incubation			
		Lsr-3$_{(B)}$	**Lsr-1$_{(W)}$**	**Lsr-10$_{(M)}$**	**Lsr-12$_{(Cu)}$**
pH	4.3	0.613 ± 0.03	0.613 ± 0.03	0.600 ± 0.10	0.612 ± 0.01
	3.8	0.086 ± 0.03	0.201 ±0.02	0.194 ± 0.02	0.163 ± 0.10
	3.2	0.059 ± 0.12	0.086 ± 0.01	0.039 ± 0.01	0.068 ± 0.03
	2.4	0.032 ± 0.01	0.072 ± 0.01	0.044 ± 0.01	0.060 ± 0.02
	2.0	0.024 ± 0.10	0.064 ± 0.02	0.034 ± 0.01	0.038 ± 0.20
Bile salt mix concentration (%)	0.5	1.090 ± 0.10	1.290 ± 0.10	1.112 ± 0.10	1.190 ± 0.02
	1.0	0.383 ± 0.15	0.475 ± 0.15	0.381 ± 0.10	0.388 ± 0.01
	2.0	0.152 ± 0.01	0.257 ± 0.01	0.177 ± 0.05	0.183 ± 0.01
	2.5	0.148 ± 0.12	0.170 ± 0.12	0.150 ± 0.25	0.146 ± 0.01
	3.0	0.138 ± 0.10	0.159 ± 0.05	0.138 ± 0.03	0.139 ± 0.01
	4.0	0.092 ± 0.05	0.133 ± 0.12	0.093 ± 0.02	0.096 ± 0.01

*Results are average of three experiments (Mean ± SD). Initial optical density of the culture broth for pH and bile salt adaptation were 0.024 ± 0.01 and 0.091 ± 0.01 respectively.

1.4.5. Survival under simulated intestinal conditions

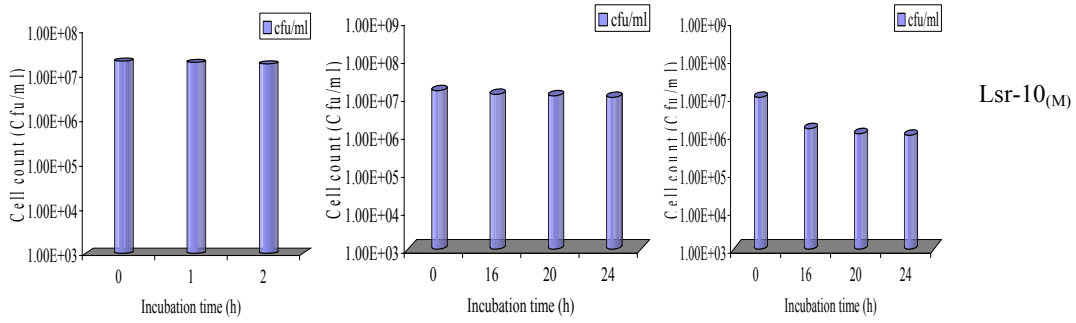
Bile salt concentration in human intestine is known to vary in different region of small intestine (jejunum-4% bile, pH 7.5; ileum-2% bile, pH 8.0; large intestine-1.5% bile, pH 9.0) with varying residence time passage at each compartment of intestinal tract (Berrada et al., 1991; Chou and Weimer, 1999). Accordingly, stock solutions were prepared with different pH and bile salt concentration and added to the growth media. At every stage total cell count i.e., survival rate was measured (Fig. 1.2).

Earlier researchers have described various *in-vitro* approaches to measure the efficacy of probiotic culture. There have been some studies in which green fluorescent protein is expressed by target strain and thus allowing the probiotic to be tracked (Collins and Gibson, 1999). A popular approach for determining bacterial fermentability was to use agar, however such method was not wholly reliable, they do not recover the full gut diversity and the technique was laborious and susceptible to operator subjectivity (Gibson and Fuller, 2000).

Another simplest *in-vitro* method was the use of fermenters of static batch culture (Wang and Gibson, 1993). Continuous culture system was also used by Gibson and Wang (1994), to simulate the intestinal conditions. MacFarlane et al. (1998) have validated a model consisting of 3 vessels aligned in series, the first vessel is set to resemble proximal colon, the second the transverse colon and the third the distil colon.

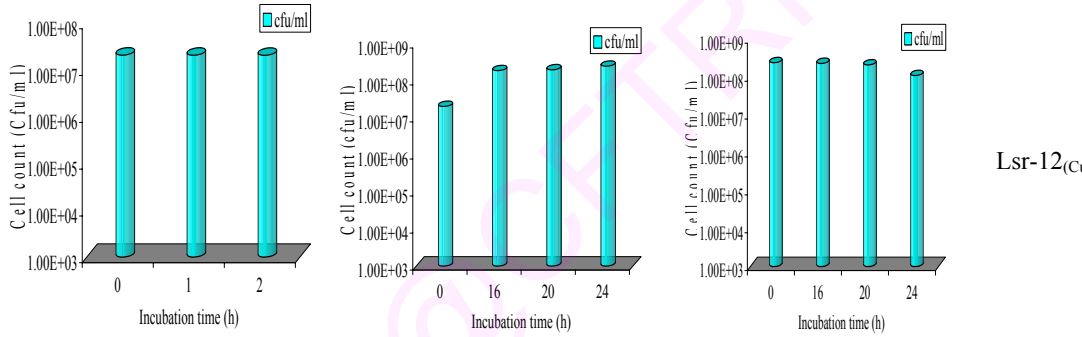
In the present work, experiment was designed in different conical flasks that resemble the human intestinal condition. Figure 1.2 represents the effect of simulated intestinal transit on the viability of selected cultures. In jejunum condition (pH 7.5 and 4% bile), Lsr-$1_{(W)}$ showed an increase in the cell count from 2.08×10^7 to 2.20×10^7 cfu/ml whereas Lsr-$10_{(M)}$ and Lsr-$12_{(Cu)}$ decreased to 1.80×10^7 and 2.05×10^7 cfu/ml from an initial cell count of 2.08 x 10^7 cfu/ml each. Further under ileal conditions (pH 8.0 and 2% bile salt mix) an increase in cell count was observed in Lsr-$1_{(W)}$ (2.2×10^7 to 4.0×10^8 cfu/ml) and Lsr-$12_{(Cu)}$ (2.05×10^7 to 2.5×10^8 cfu/ml) whereas reduction (1.5×10^7 to 1.0×10^7 cfu/ml) was observed in Lsr-$10_{(M)}$. Under induced conditions of large intestine (pH 9.0, 1.5% bile salt mix) a slight reduction in the viability was observed in Lsr-$1_{(W)}$ (4×10^8 to 2.46×10^8 cfu/ml) followed by Lsr-$12_{(Cu)}$ (2.5×10^8 to 1.1×10^8 cfu/ml) whereas significant reduction was observed in Lsr-$10_{(M)}$ (1×10^7 to 1.0×10^6 cfu/ml). It can be confirmed that Lsr-$10_{(M)}$, isolated from milk was sensitive to stress conditions of GIT whereas the cultures Lsr-$12_{(Cu)}$ and Lsr-$1_{(W)}$ were tolerant and were able to survive the intestinal environment to exert their beneficial effect.

Error!



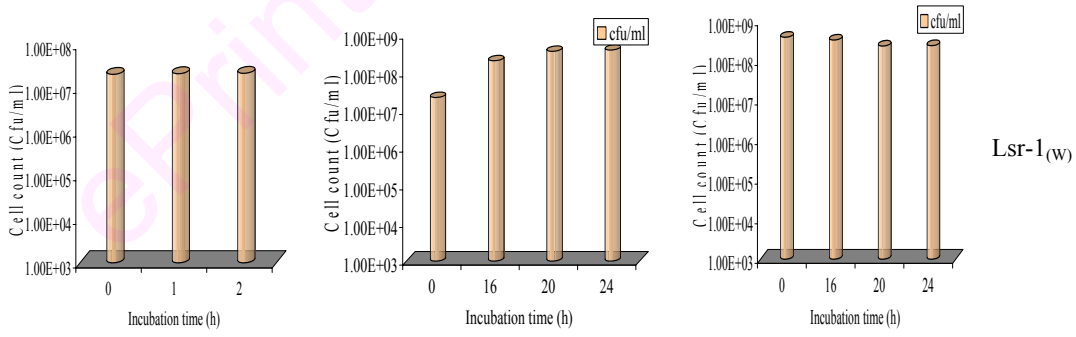
Lsr-10$_{(M)}$

Bile salt (4%); pH 7.5 Bile salt (2%); pH 8.0 Bile salt (1.5%); pH 9.0



Lsr-12$_{(Cu)}$

Bile salt (4%); pH 7.5 Bile salt (2%); pH 8.0 Bile salt (1.5%); pH 9.0



Lsr-1$_{(W)}$

Bile salt (4%); pH 7.5 Bile salt (2%); pH 8.0 Bile salt (1.5%); pH 9.0

Fig 1.2: Survival of isolated cultures under simulated intestinal conditions.

Values are mean ± SD.

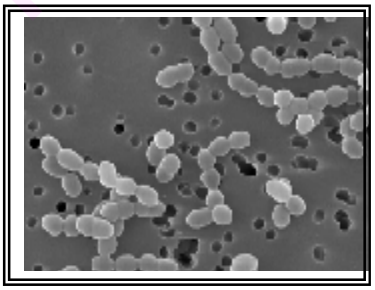
1.4.6. Characterization and identification of selected cultures

Phenotypic characters have been used for a long time to characterize bacterial cultures. As it is difficult to identify species or sub-species, new methods of biochemical characterization and molecular techniques are presently used for reliable and consistent identification of the culture. Table 1.4 represents the biochemical and physiological characteristics of Lsr-$1_{(W)}$ and Lsr-$12_{(Cu)}$ isolates. By comparing the results with Bergey's manual of systematic bacteriology (Krieg, 1984), the cultures were identified as *Leuconostoc* sp and *Lactobacillus* sp respectively (Fig. 1.3).

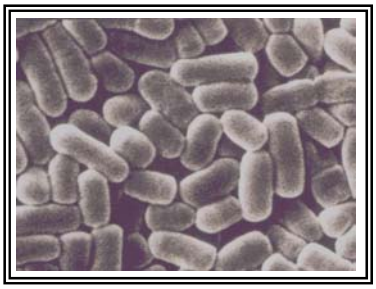
Table 1.4: Physiological and biochemical identification of culture isolates

Characters		Response	
		Lsr-$1_{(W)}$	**Lsr-$12_{(Cu)}$**
Morphology		Cocci	Rod
Gram staining		+	+
Catalase		-	-
Growth temperature (^oC)	10	+	+
	37	+	+
	45	+	-
Growth in NaCl (%)	2.5	+	+
	6.5	+	+
	18	-	-
Arginine hydrolysis		-	-
Casein hydrolysis		+	ND
Starch hydrolysis		-	-
Citrate utilization		-	ND
Gas production		-	-
Sugar utilization	Arabinose	-	+
	Cellobiose	+	+
	Fructose	+	+
	Galactose	D/+	+
	Glucose	+	+
	Lactose	+	+
	Maltose	+	+
	Mannitol	+	+
	Mannose	D	+
	Melibiose	-	+
	Raffinose	-	+
	Rhamnose	+	-
	Ribose	ND	+
	Sorbitol	+	+
	Sucrose	+	+
	Trehalose	+	+
	Xylose	D	D

'+'- Positive; '-'-negative, D-delayed; ND-not determined
*Characterized according to Bergey's manual of systematic bacteriology



SEM of Lsr-$1_{(W)}$



SEM of Lsr-$12_{(Cu)}$

Fig 1.3: SEM photograph of selected cultures

Further 16srDNA fragment of the total genomic DNA from selected cultures were amplified and sequenced for identification. Amplification using M13 primer produced a PCR product of approximately 1528 bp from Lsr-$12_{(Cu)}$ whereas no amplification was observed in Lsr-$1_{(W)}$. With *Leuconostoc sp* specific primer an amplified PCR product of approximately 854 bp was found in Lsr-$1_{(W)}$ (Fig. 1.4). PCR product obtained was sequenced and aligned along with other known sequence of *Leuconostoc* sp and *Lactobacillus* sp (NCBI) using cluster W18 program (Thompson et al., 1994). It was confirmed that the selected cultures were *Leuconostoc mesenteroides* (Lsr-$1_{(W)}$) and *Lactobacillus plantarum* (Lsr-$12_{(Cu)}$) (Fig. 1.5 and 1.6).

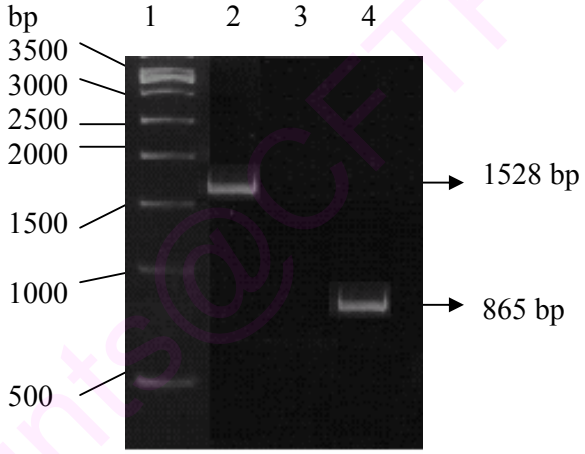


Fig 1.4: PCR product obtained by amplification of 16srDNA.
Lane 1: DNA marker, Lane 2: Lsr-$12_{(Cu)}$ amplified with M13 primer, Lane 3: no amplification found in Lsr-$1_{(W)}$ with M13 primer, Lane 4: Lsr-$1_{(W)}$ amplified with specific primer.

Primer : F 5' ATT GGG ACT GAG ACA CGG 3'

R 5' TGA TGA CCT GAC GTC GTC C 3'

ALIGNED SEQUENCE DATA: (865 bp)

GGTGCTTGCACCTTTCAAGTGAGTGGCGAACGGGTGAGTAACACGTGGACAACCTGCCT
CAAGGCTGGGGATAACATTTGGAAACAGATGCTAATACCGAATAAAACTTAGTGTCGCA
TGACACAAAGTTAAAAGGCGCTTCGGCGTCACCTAGAGATGGATCCGCGGTGCATTAGT
TAGTTGGTGGGGTAAAGGCCTACCAAGACAATGATGCATAGCCGAGTTGAGAGACTGAT
CGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCTGCAGTAGGGAAT
CTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGCTTTCGG
GTCGTAAAGCACTGTTGTATGGGAAGAACAGCTAGAATAGGAAATGATTTTAGTTTGAC
GGTACCATACCAGAAAGGGACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGT
CCCGAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTTTATTAAGTCTG
ATGTGAAAGCCCGGAGCTCAACTCCGGAATGGCATTGGAAACTGGTTAACTTGAGTGCA
GTAGAGGTAAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACA
CCAGTGGCGAAGGCGGCTTACTGGACTGCAACTGACGTTGAGGCTCGAAAGTGTGGGTA
GCAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAACACTAGGTGTTAG
GAGGTTCCGCCTCTTATGCCGAGCTAAGCATTAGTGTTCCCCTGGGGAGTCNACCCCAG
GTTGAACTCAAGGATTGANGGACCNCCAGCGGGGACATGTG

Fig 1.5(a): 16srDNA sequence data of Lsr-1$_{(W)}$: *Leuconostoc mesenteroides*

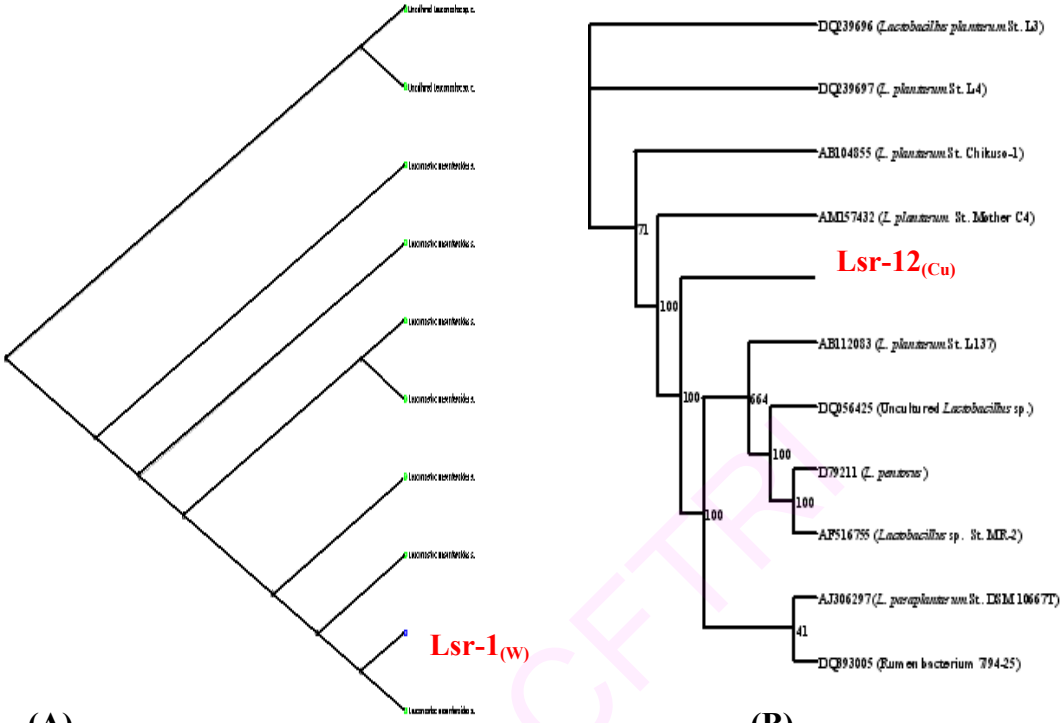
Primer: F 5' CCC AGT CAC GAC GTT 3'

ALIGNED SEQUENCE DATA: (1528 bp)

AGAGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAA
CGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTG
GTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGC
TAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATC
ACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCA
ATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCA
AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAG
CAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAAC
ATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGT
AAAGCGAGCGCAAGCGGTTTTTTAAGTCTGATGTGAACGCCTTCGGCTCAACCGAAGAA
GTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAG
CGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGT
AACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCC
ATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTA
ACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGAAGCATGTGGTTTAATTTGAAGCTACGCGAAGAACCTT
CCCAGGTCTTGACATCCTATGCAAATCTAAGAGATTAGACGTTTCCGTCGGGGACATGG
ATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCTCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGC
CGGTGACAAACCGGATGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG
GGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTA
ATCTTTTAAAGCCATTTTCAGTTTGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGA
ATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGGGTAACCTTTTAGGA
ACCAGCCGCCTAAGGTGGGACAGATGATTAGGGTGAA GTCGTAACAAGGTAAC

Fig 1.5(b): 16srDNA sequence data of Lsr-12$_{(Cu)}$: *Lactobacillus plantarum*

Error!



(A) **(B)**

Fig 1.6 : Phylogenetic tree of (A) Lsr-1$_{(W)}$: *Leuconostoc mesenteroides* (B) Lsr-12$_{(Cu)}$: *Lactobacillus plantarum*

Earlier investigators have used various techniques for the identification of bacterial species. DNA based method was used for differentiation of *Leuconostoc* (Lee et al., 2000; Schonhuber et al., 2001; Matte-Tailliez et al., 2001; Jang et al., 2003; Reeson et al., 2003). Separation of 16srDNA by temporal temperature gradient gel electrophoresis (TTGE) and protein pattern or ribotyping also permits identification of bacterial species (Villani et al., 1997; Ogier et al., 2002). Cibik et al. (2000) have estimated molecular diversity of 221 dairy strains by RAPD and strains were classified as *Leuconostoc mesenteroides* or *citreum* using 16srDNA sequence and 16srDNA fragment amplification.

Barrangou et al. (2002) have isolated 6 strains from fermented Sauerkraut and identified them by biochemical finger printing, endonuclease digestion of 16s-23s intergenic transcribed spacer region and sequencing of various regions V1 and V2 of the 16srRNA gene as *Leuconostoc fallax* strain.

Other methods have also been proposed like characterization through neutral compounds produced in whey (Mauriello et al., 2001) and cellular fatty acid profile (Rementzis and Samelis, 1996). Some discrepancy is seen in each of these results and hence molecular method using 16srRNA analysis is followed for the identification of isolated cultures. In the present study primarily presumptive identification was done by biochemical assay and sugar fermentation pattern according to Bergey's manual of systematic bacteriology. Further the cultures were identified by 16sRNA sequencing as *Leuconostoc mesenteroides* and *Lactobacillus plantarum*.

Benkerroun et al. (2003) have shown only 1% of *Leuconostoc sp* to be present out of the total isolates from camel's milk. Gomes-Zavaglia et al. (1998) have isolated 25 *Bifidobacterium* strains from infant feces and identified them by sugar fermentation pattern and whole cell protein analysis. Probiotic properties were checked in the isolated strain and were found that not all desirable characteristics were present in a single strain.

Leuconostoc mesenteroides has been considered to be a predominant species during first week of fermentation (Fleming et al., 1995). Several reports confirm the presence of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* as dominant microflora of fermented product (Beukes et al., 2001; Gadaga et al., 2001). Obodai and Dodd (2006) have characterized LAB from Nyarmie, traditional Ghanaian fermented milk and have found *Leuconostoc mesenteroides*, *Streptococcus thermophilus, Lactobacillus delbruekii, Lactobacillus helveticus* and *Lactococcus lactis* to be the dominant microflora. Similarly out of 45 LAB isolates from Kenyan traditional fermented camel milk 24% were found to be *Leuconostoc mesenteroides* and 16% *Lactobacillus plantarum* (Lore et al., 2005).

In the present study, the selected cultures Lsr-$1_{(W)}$ and Lsr-$12_{(Cu)}$ were identified as *Leuconostoc mesenteroides* and *Lactobacillus plantarum* respectively.

1.4.7. Determination of tolerance to digestive enzymes

The probiotic microorganisms must have the ability to survive and persist in the GIT. The low pH of the stomach and the antimicrobial action of pepsin are known to provide an effective barrier against the entry of bacteria into the intestine (Holzapfel et al., 1998). Another barrier to probiotic bacteria is the adverse condition of intestine that includes bile salts and pancreatin (Le Vay, 1988). With this in mind, the present culture isolates were tested for their tolerance to digestive enzymes (pepsin and trypsin) under simulated gastrointestinal conditions.

The effect of digestive enzymes on the viability of selected LAB cultures ie., *L. mesenteroides* and *L. plantarum* is presented in Fig. 1.7. According to the data obtained the isolated culture Lsr-$1_{(W)}$ was found to be more tolerant to digestive enzymes than Lsr-$12_{(Cu)}$ and can be considered as intrinsically tolerant. After 3 h of incubation there was a reduction in cell count of Lsr-$1_{(W)}$ by 3 logs (3.49×10^{10} to 3.59×10^{7} cfu/ml) whereas Lsr-$12_{(Cu)}$ exhibited reduction by 6 logs in presence of pepsin (3.49×10^{10} to 8.19×10^{4} cfu/ml). With trypsin Lsr-$1_{(W)}$ exhibited 8.22×10^{7} cfu/ml of viable cells after 3 h of incubation whereas Lsr-$12_{(Cu)}$ showed only 5.20×10^{5} cfu/ml under the same condition.

Charteris et al. (1998) have studied the transit tolerance of potential probiotic *Lactobacillus sp* and *Bifidobacterium sp* by exposing them to simulated gastric juice (pH 2.0; 0.3% pepsin) and intestinal juice (pH 8.0; 1g/L pancreatin). They found that only *Lactobacillus fermentum* was intrinsically resistant with 30% survival. In the present study the culture *L. mesenteroides*-Lsr-$1_{(W)}$ showed higher capacity to tolerate the gastrointestinal conditions.

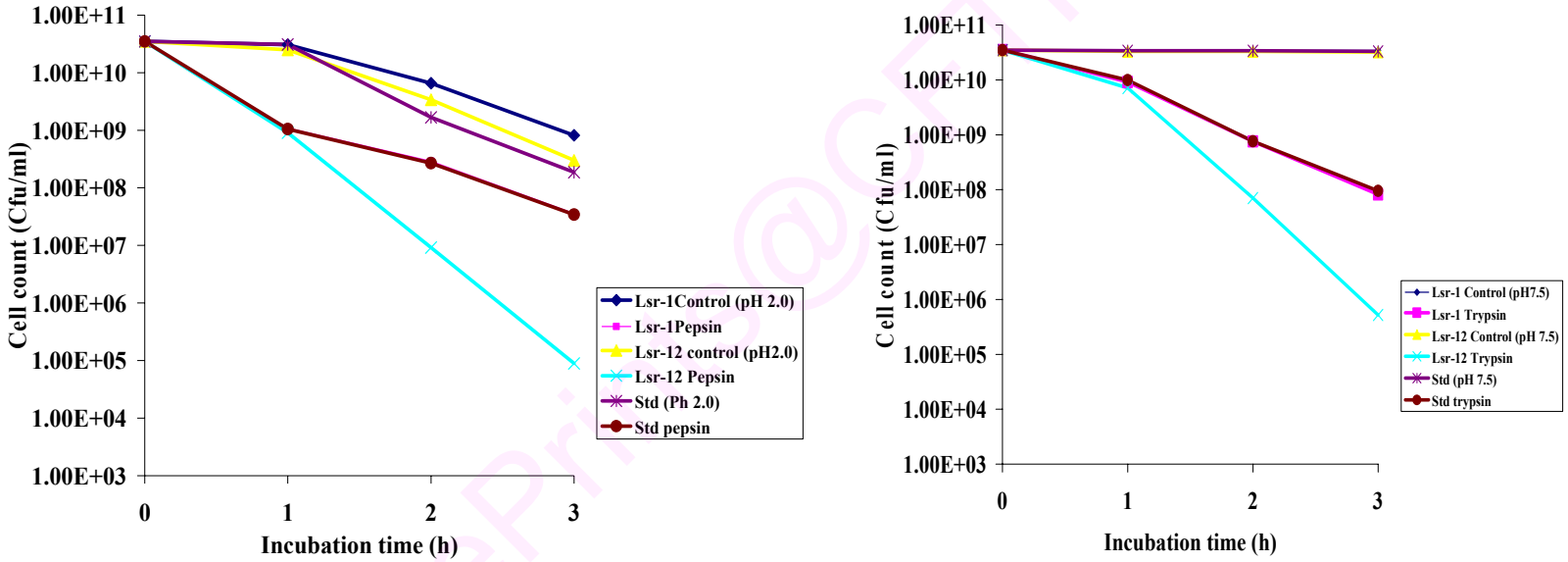


Fig 1.7 : Tolerance of the selected cultures to digestive enzymes. (A) tolerance to Pepsin (B) tolerance to trypsin

1.4.8. Bacterial growth

The growth pattern of the culture was studied in MRS broth (pH 6.5) incubated at 37°C. A typical growth curve of Lsr-$1_{(W)}$ is shown in the fig 1.8. An exponential growth was initiated after 5 h of lag phase and continued till 32 h. After 32 h the culture entered the stationary stage. Cell decline was observed after 64 h of incubation.

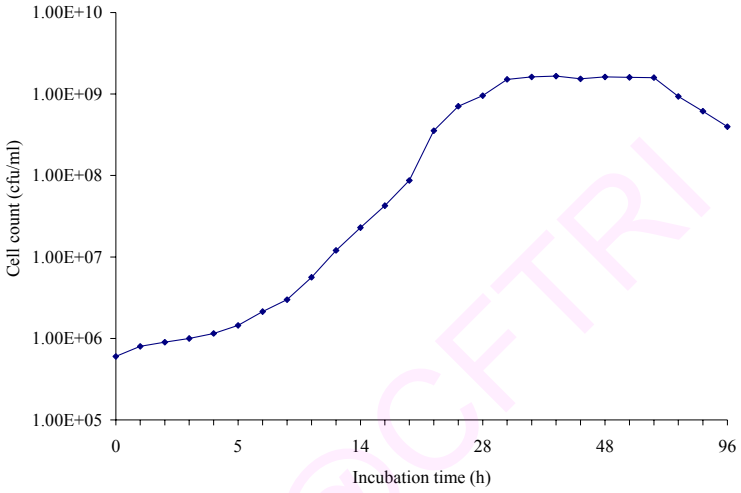


Fig 1.8 : Growth curve of Lsr-$1_{(W)}$ in MRS broth at 37^0C

1.5. Conclusion

The probiotic lactic acid bacterial culture (Lsr-$1_{(W)}$) isolated from whey has the ability to resist low pH and high bile of GIT condition. This was identified as *Leuconostoc mesenteroide*s according to biochemical assays and 16srDNA sequence analysis. The culture was able to resist digestive enzymes and has a long exponential growth phase from 5 to 32 h. **This probiotic culture was coded as PLsr-$1_{(W)}$ and was used for further studies.**

Chapter 2

Probiotic Functional Properties of Culture Isolate

CHAPTER – 2

PROBIOTIC FUNCTIONAL PROPERTIES OF CULTURE ISOLATE

ABSTRACT

The probiotic culture *Leuconostoc mesenteroides* (PLsr-$1_{(W)}$) was evaluated for its potential functional properties. The culture exerts antimicrobial activity against seven food borne pathogens and is resistant to three common antibiotics tested. The inhibitory activity of intracellular cell free extract of culture to ascorbate autooxidation, ferrous ion chelating ability and scavenging activity of oxygen radical represents the antioxidative property of the culture isolate. Cell hydrophobicity with different hydrocarbon confirms the adhesion ability of the culture. The SDS-PAGE analysis of the surface protein showed a prominent protein band at 42 KD which is the S-layer protein responsible for the adhesion of the culture. The culture has cholesterol lowering ability, β-galactosidase activity and produces volatile compounds of therapeutic value which shows the importance of probiotic culture (PLsr-$1_{(W)}$) as compared to native isolate Lsr-$1_{(W)}$.

2.1. Introduction

In the quest of discovering how food can enhance health or prevent chronic diseases, researchers have stumbled on to another range of components in foods beside nutrition. The idea of being healthy by consuming bacteria with beneficial gastrointestinal effects was promoted by the notable immunologist Metchnikoff around a century ago. These bacteria are characterized as "probiotics" that favorably maintain or improve the intestinal microflora, inhibit growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Haudault et al., 1997; Tannock, 1999).

Industrialization of food bio-transformation increased the economical importance of lactic acid bacteria as they play a crucial role in the development of organoleptic and hygienic quality of fermented products. Therefore the reliability of starter strains in terms of their quality, functional properties and growth performance has become essential (van de Guchte et al., 2002). With the growing threat of food borne pathogens, discovery of new food processes and the consumers demand for natural preventive measures has made these microorganisms as potential biopreservatives for foods. Substantial research on these microorganisms has been focused on such application in treatment of diseases (Hoover, 1993; Ennahar et al., 1999).

A number of requirements have been identified for strains to be effective probiotic microorganism with functional and technological aspects. The required characteristics include stress adaptation towards gastric acid, physiological concentration of bile and adherence to intestinal epithelial cells (Dunne et al., 2001; Schillinger et al., 2005; Khalil et al., 2007). These characters are frequently suggested for the evaluation of the probiotic potential of bacterial strain.

In this regard the probiotic culture *L. mesenteroides* (PLsr-$1_{(W)}$) (Chapter 1) was studied for the changes in cellular fatty acids and protein profile when exposed to these stress conditions. The culture PLsr-$1_{(W)}$ was also studied for antimicrobial activity, susceptibility to common antibiotics, antioxidant activity, cholesterol lowering ability, β-galactosidase activity, adherence ability and the release of volatile compounds.

2.2 Materials

- Standard culture *Leuconostoc mesenteroides* (B640) and dairy starter culture *Streptococcus thermophilus* were procured from MTCC culture collection centre, Chandigard, Hariyana.
- Bacterial strains and media: The probiotic strain (PLsr-$1_{(W)}$) of *Leuconostoc mesenteroides* preserved in MRSA-stabs was propagated twice in MRS broth at 37°C before use. Native culture isolate (Lsr-$1_{(W)}$), *Leuconostoc mesenteroides* (B640) and *Streptococcus thermophilus* were used for comparison.

Chemicals:

1) Brain heart infusion agar (BHI; HiMedia Pvt Ltd, India).
2) Antibiotic disc (Octadisc, HiMedia Pvt Ltd, Mumbai, India).
3) Phosphate-Urea-Magnesium sulphate buffer (pH 7.1; 16.9g K_2HPO_4, 7.3g KH_2PO_4, 1.8g Urea, 0.2g $MgSO_4.7H_2O$, 1000 ml Distilled water).
4) Lysis buffer (0.06 M Tris HCl, pH 6.8, 10% glycerol, 2 % SDS, 5% β-mercaptoethanol), sample buffer (0.12 M Tris HCl, 0.1% SDS, 5% β-mercaptoethanol, 25% glycerol and 0.01% bromophenol blue).
5) *Chemicals:* NaOH, HCl, H_2SO_4, ascorbic acid, $FeSO_4$, trichloroacetic acid, O-phenanthroline, 0-phthalaldehyde, ONPG, KOH and hexadecane were of analytical grade purchased from HiMedia Pvt Ltd, India. Protein marker was purchased from Genei, Bangalore, India. Cholesterol, acrylamide, bisacrylamide and DPPH (1,1-Diphenyl-2-picryl hydrazyl) were procured from Sigma-Aldrich company, India. Hexane, methanol, chloroform, dichlormethane, octane, xylene, toluene and ethanol were of HPLC grade (SRL Company, India).

Equipments: Spectrophotometer (UV-1601 A, Shimadzu Corporation, Japan), pH meter (Genei, Bangalore, India), Vortex, Centrifuge (Labline Company, India), Microscope (Olumpus, Japan), Electrophoretic unit (Genei, Bangalore), Gel documentation system (Bio-Rad Laboratories, USA), Gas Chromatograph and Mass Spectrometer (Shimadzu Corporation, Japan) with SE-30 and ELITE-1 column.

2.3 Methods.

2.3.1. Antimicrobial activity

***2.3.1.1. Indicator bacterial strains*:** The antagonistic activity of PLsr-$1_{(W)}$ was assessed against the following target bacteria: *E. coli, Staphylococcus aureus, Salmonella typhi, Salmonella paratyphi, Shigella dysenteriea, Pseudomonas aeroginosa, Listeria monocytogenes, Yersinia enterocolitica* and *Vibrio cholerae* (obtained from JSS Hospital, Mysore). These pathogenic cultures were grown in BHI broth at 30°C for 18 h.

***2.3.1.2. Preparation of cell free supernatant*:** PLsr-$1_{(W)}$ was grown in MRS broth for 24 h at 37°C. After incubation period culture broth was centrifuged at 8,000 rpm for 10 min to collect the cell free supernatant**.**

***2.3.1.3. Antimicrobial assay*:** The culture was tested for antimicrobial activity by agar well diffusion assay (Schillinger and Lucke 1989). Plates were prepared with MRS media supplemented with 0.2% glucose and 1.5% agar. These plates were overlaid with 7 ml of soft MRS agar (0.8% agar) preinoculated with pathogenic strain (0.5% v/v). Wells of 4 mm in diameter were made in these agar plates and the culture supernatant of PLsr-$1_{(W)}$ (30 µl) was placed into each well. Plates were then incubated for 24 h at 30°C and subsequently examined for the zone of inhibition.

The inhibitory activity of heat treated and neutralized supernatant of PLsr-$1_{(W)}$ was assessed against facultative anaerobes *Shigella dysenteriae, Salmonella typhi and Staphylococcus aureus*. Thermal stability of the antibacterial compound was determined by heating the cell free supernatant at 100°C for 10 min and tested for antimicrobial activity after cooling. In order to find out that the antimicrobial activity is due to proteinaceous compound and not due to low pH (acidic pH) was determined by neutralizing the culture supernatant using NaOH (0.1 N). This neutralized cell free supernatant was then tested for antimicrobial activity against food borne pathogens as described earlier. All experiments were performed in triplicate.

2.3.2. Antibiotic susceptibility test

The susceptibility to antibiotics which are commonly used by animals and humans (Food and Drug administration, 2001) was tested by disc diffusion method (Brashears and Durre, 1999). The culture PLsr-$1_{(W)}$ (1%) in MRS soft agar (0.8% agar) was overlaid on MRS agar plate. An antibiotic disc (Octadisc) was placed on it to allow the diffusion of antibiotics into the medium and then incubated at 37°C for 24 h. The inhibition zone around each antibiotic was measured to check the susceptibility of the culture. The minimal inhibitory concentration (MIC, expressed in µg/ml) was determined by using antibiotics at variable concentrations. The MIC was defined as the smallest amount of antibiotic needed to inhibit the growth of bacteria after incubation for 24 h.

2.3.3. Antioxidative activity

***2.3.3.1. Preparation of intracellular cell free extract*:** The exponentially (16 h) grown culture (PLsr-$1_{(W)}$) was harvested by centrifugation at 8000 rpm for 15 min. Cell biomass obtained was resuspended in deionized water after washing. The cell suspension was then homogenized for 5 min at 4°C. Cell debris was removed by centrifugation at 10,000 rpm for 10 min and the resulting supernatant was used as the intracellular cell free extract.

2.3.3.2. Ascorbate autooxidation assay: The antioxidant activity of the culture was determined by ascorbate autooxidation assay (Mishra and Korachich, 1984). Intracellular cell free extract (0.1 ml) was added to the mixture of ascorbate stock solution (0.1 ml; 5 mM) and 9.8 ml of phosphate buffer (0.2 M; pH 7.0). The mixture was then quickly measured for absorbance at 265 nm using spectrophotometer.

Percentage of inhibition of ascorbate autooxidation was calculated by using the following formula:

$$\% \text{ Inhibition} = 1 - \left(\frac{A_{265\ (\text{Sample})}}{A_{265\ (\text{blank})}} \times 100 \right)$$

***2.3.3.3. Metal ion chelating assay*:** The chelating ability of intracellular cell free extract of the culture for ferrous ion was determined by ion chelating assay. The method developed by Yamauchi et al. (1984) and modified by Lin and Yen (1999) was used for assaying. The intracellular cell free extract (0.5 ml) was mixed with 0.1 ml of ascorbate (1g/dl), followed by 0.1 ml of $FeSO_4$ (0.4 g/dl) and 1 ml of NaOH (0.2 M). The mixture was incubated at 37°C in a water bath for 20 min and then 0.2 ml of trichloroacetic acid (10%) was added. Mixture was then centrifuged at 5000 rpm for 10 min to obtain the supernatant. O-phenanthroline (0.5 ml; 1 g/L) was added to the supernatant and incubated for 10 min. Absorbance of the mixture was then measured at 510 nm.

2.3.3.4. DPPH assay (1,1-Diphenyl-2-picryl hydrazyl): The scavenging of DPPH by *L.mesenteroides* (PLsr-$1_{(W)}$) was analyzed by the method of Brand Williams et al. (1995) which was modified by Pyo et al. (2005). Intracellular cell free extract (0.02 ml) and 1.0 ml of freshly prepared DPPH solution (0.1 mM in methanol) were mixed and the absorbance was measured spectrophotometrically at 517 nm after 30 min of incubation. The scavenging activity was calculated by the following equation. Blank sample contained deionized water.

$$\text{Scavenging activity (\%)} = Aa - \left(\frac{(Ab - Ac)}{Aa} \times 100 \right)$$

Where, Aa – absorbance of DPPH solution without sample

Ab – absorbance of mixture containing sample and DPPH

Ac – absorbance of blank solution without DPPH

2.3.4. Anticholesterolemic activity

An exponentially (16 h) grown culture *L. mesenteroides* (PLsr-$1_{(W)}$) was inoculated (1%) into MRS broth (10 ml) supplemented with water soluble cholesterol (100 mg/L) and incubated anaerobically for 24 h at 37°C. After the incubation period, the cell biomass was collected by centrifugation

at 8,000 rpm for 15 min at 4°C and suspended in distilled water (10 ml). The cholesterol content was then measured by O-phthalaldehyde method as described by Rudel and Morris (1973) and modified by Gilliland et al. (1985).

Sample extract (0.5ml) was placed in a clean test tube and mixed with 3 ml of ethanol (95%). This was followed by the addition of 2 ml of potassium hydroxide (50%). The mixture was thoroughly vortexed and heated on a water bath for 10 min at 60°C. After cooling, hexane (5 ml) was added into the test tube and mixed thoroughly. This was allowed to stand at room temperature for 10 min and then 3 ml of distilled water was added and vortexed. The hexane (2.5 ml) layer was carefully transferred into a clean test tube and evaporated at 60°C. To this dry tube, 4 ml of O-phthalaldehyde reagent (0.5 mg per ml of glacial acetic acid) was added and allowed to stand at room temperature for 10 min followed by slow addition of concentrated H_2SO_4 (2 ml). The contents of the tube were mixed thoroughly. After 10 min of incubation the absorbance was read at 550 nm against a reagent blank. The absorbance was compared with the standard curve (Fig. 2.1) to determine the concentration of the cholesterol. Results are expressed as µg of cholesterol per ml.

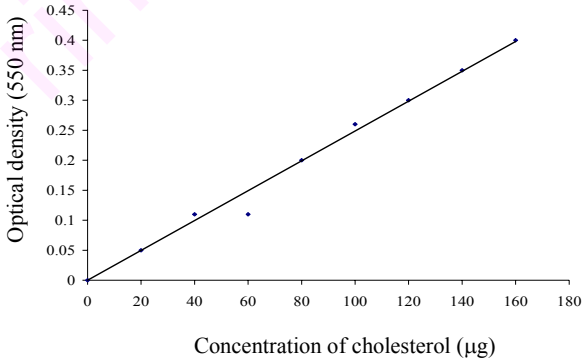


Fig 2.1 : Standard graph of cholesterol estimation

Note: Standard curve was prepared by following the same procedure but in place of sample extract different concentrations of cholesterol (20-140 µg) were used. The absorbance values were plotted against µg of cholesterol. Values are average of three experiments (n=3).

2.3.5. β-galactosidase activity

The culture PLsr-$1_{(W)}$ was tested for β-galactosidase activity by qualitative assay using ONPG discs. The culture grown in MRS broth was harvested by centrifugation at 5,000 rpm for 15 min. Cell biomass was washed twice in phosphate buffer (pH 7.0) and resuspended in the same buffer. The cell suspension (1 ml) was permeabilized with 50 µl of toluene:acetone (1:9) mixture. ONPG disc was immersed in this suspension and incubated for 30 min at 37°C. After the incubation time, the development of yellow color was recorded as a positive reaction for β-galactosidase activity. Quantitatively the enzyme activity was estimated by using ONPG as substrate according to the method of Bhowmik and Marth (1989). Specific activity (µM mg^{-1}) was then expressed as the amount of O-nitrophenol (ONP) released per mg of protein.

2.3.5.1. Influence of carbon source on enzymatic activity: PLsr-$1_{(W)}$ was inoculated at a rate of 1% (v/v) in MRS basal broth supplemented with glucose, galactose, lactose, sucrose and fructose at a concentration of 2% (w/v) in different test tubes and incubated at 37°C for 24 h. After the incubation period, cell biomass was collected by centrifugation and assayed for enzyme activity.

2.3.6. Adherence ability

2.3.6.1. Cell hydrophobicity assay (Microbial adhesion to hydrocarbons)

The *in-vitro* method by Rosenberg et al. (1980) was used to assess the bacterial adhesion to hydrocarbons (toluene, xylene, octane and hexadecane). The culture *L. mesenteroides* (PLsr-$1_{(W)}$) was grown at 37°C for 16 h in MRS broth containing cystine hydrochloride. Cell biomass was collected by centrifugation at 5000 rpm for 10 min, washed twice with phosphate-urea-magnesium sulphate buffer (pH 7.1) and resuspended in the same. The optical density of the suspension was adjusted to 0.8-0.9 at 610 nm using a spectrophotometer. To this bacterial suspension (2.4 ml) hydrocarbons were added (0.4 ml) separately and mixed well for 2 min followed by incubation at

37°C for 1 h. The aqueous phase was carefully removed and the absorbance was measured at 610 nm.

The fraction of adherent cells was calculated as percent decrease in absorbance of aqueous phase as compared to that of original cell suspension. Cell surface hydrophobicity or the percent adhesion was calculated by the following formula

$$\% \text{ adhesion} = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100$$

2.3.6.2. Adhesion of PLsr-1$_{(W)}$ to rat intestinal epithelium

The *in-vitro* adhesion to intestinal epithelium layer was performed by the method of Mayra-Makinen et al. (1983) with modifications. Ileal sample was collected from male Albino Wistar rats. The tissue was held in PBS at 4°C for 30 min to loosen surface mucus, and then washed three times with buffer (pH 7.0). The adhesion test was performed by incubating tissue sample (1 cm^2) in bacterial suspension (10^8 cfu/ml in buffer) at 37°C for 30 min. Treated tissue sample was fixed in 10% formalin, dehydrated by increasing concentrations of ethanol, and embedded in paraffin. Serial sections (5 μm) were cut, mounted on standard microscope slides and stained for identification of Gram-positive and Gram-negative bacteria. Slides were examined and photographed using a light microscope.

2.3.6.3. Analysis of cell surface protein of PLsr-1$_{(W)}$

(a) Extraction of S-layer protein

The exponentially grown (16 h) culture PLsr-1$_{(W)}$ was centrifuged at 8,000 rpm for 10 min at 4°C. The cell biomass was washed twice in ice-cold water and resuspended in 10 ml of Lithium chloride (5 M). This was incubated at 37°C for 1 h. The supernatant was then collected by centrifugation (10,000 rpm for 15 min) at 4°C and dialyzed against water at 4°C. The dialyzed sample was freeze dried and resuspended in 1 ml of Tris buffer (pH 7.5).

Note: *Dialyzing bag preparation*: Cutoff the dialyzing tube to required length and boil it in water for 10 min containing Na_2CO_3 (2%) and 1mM EDTA. After cooling, wash with distilled water and again boil for 10 min in distilled water. On cooling rinse with distilled water thrice and store in water at 4°C until use.

(b) SDS-PAGE analysis of S-layer protein

Electrophoresis was performed as described by Laemmli (1970). The polyacrylamide gel was composed of a 4% stacking gel and a 12.5% separating gel.

The S-layer protein was diluted in an equal volume of the tracking buffer (0.12 M Tris HCl; pH 6.8 and 0.01% bromophenol blue) and then applied to the gel. Electrophoresis was done at 50 mA through the stacking gel and 70 mA through separating gel until the tracking dye migrates to the bottom of the gel. Further the gel was stained in a mixture of 1% (wt/v) coomassie blue, 40% (v/v) methanol and 20% (v/v) acetic acid. After overnight staining it was decolorized under continuous shaking in a 25% (v/v) methanol solution containing 10% (v/v) acetic acid. The stained protein patterns were then scanned with a gel documentation system. Molecular masses of the protein extracts were estimated using the linear relationship between the marker and the protein band.

2.3.7. Analysis of volatile compounds

2.3.7.1. Extraction of volatile compounds

PLsr-$1_{(W)}$ was grown in MRS broth at 37°C. Formation of volatile compounds were analyzed at different growth stages. An aliquot (5 ml) of culture broth was drawn at regular interval of time and the cell biomass was collected by centrifugation (5,000 rpm for 20 min). The cell biomass was suspended in dichloromethane (10 ml) and mechanically homogenized. Extraction procedure was repeated thrice and the solvent layer was collected, dried over anhydrous sodium sulphate and concentrated to 0.5 ml under a stream of nitrogen.

2.3.7.2. Gas chromatographic analysis and GCMS conditions

GC analysis was carried out with a Packed series 15A gas chromatograph, equipped with a flame ionization detector. A (3%) SE-30 column (3m X 3mm id) with mesh size 80/100 was used. O_2 flow of 300 ml/min and H_2 flow of 30 ml/min was employed. The operating conditions were as follows: the temperature was programmed from 100°C to 250°C (100°C with 6 min hold, 100-150°C; 4°C/min, 150-220°C; 8°C/min). The injector and detector temperature were set at 250°C. The nitrogen carrier velocity was 30 ml/min.

Mass spectra were obtained with Turbomass gold mass spectrometer (Perkin Elmer International, Switzerland) coupled with gas chromatograph equipped with turbomass version-4 software. Sample injection was done in the split mode (40:1) in an ELITE-1 column, 30 m X 0.25 mm id and 0.25 μm film thickness coated with 100% poly dimethoxy siloxane. Pure Helium was used as the GC carrier gas at a flow rate of 1 ml/min. The GC injector temperature was set at 250°C, oven temperature at 100°C. The detector was adjusted to 250°C. The mass spectra were determined at 70eV with emission current 100 μA and ion source was held at 150°C. Acquisition and processing of mass spectra were carried out by means of a computer and the compounds were identified with the aid of mass spectral data bases.

2.3.8. Changes in cellular fatty acids and proteins of PLsr-$1_{(W)}$

2.3.8.1. Extraction of cellular membrane fatty acids

Cell biomass of the culture PLsr-$1_{(W)}$ and native isolate Lsr$1_{(W)}$ collected separately by centrifugation at 8000 rpm for 10 min. The cellular fatty acids were then extracted by the method of Bligh and Dyer (1959). The cell suspension (1 ml) in distilled water was mixed with methanol:chloroform (2:1) mixture (3.7 ml), vortexed thoroughly and incubated for 2 h at 28°C. The mixture was then centrifuged at 5000 rpm for 15 min at 4°C to collect the supernatant. Extraction was repeated with 4.75 ml of methanol:chloroform:water

mixture (2:1:0.8) and the supernatant extract was collected by centrifugation. To the pooled extract water (2.5 ml) and chloroform (2.5 ml) were added and vortexed thoroughly. This was kept at room temperature for phase separation. The chloroform layer was quantitatively taken into a clean dry test tube and evaporated under nitrogen flow. The dried tubes were stored at -20°C.

Analysis of fatty acids by GC and GCMS method

The dried tubes containing the fatty acids were dissolved in hexane (2 ml). To this methanolic KOH (2 N; 0.1 ml) was added and mixed thoroughly. The upper hexane layer was taken in a clean test tube and evaporated to dryness under N_2 flow. The dried residues were then dissolved in 0.1 ml of hexane and analyzed by GC with the following operating conditions: injection temperature 220°C and detector temperature 250°C. The temperature was programmed from 100°C to 220°C (100°C held for 5 min, 100-220°C; 10°C/min, at 220°C held for 10 min). Carrier gas N_2 was used at a flow rate of 1.5 ml per min. Injection volume was 1µl. The identification of peaks was based on the comparison of fragmentation pattern of GCMS chromatogram with GCMS library data base and then GCMS volumes by Noever et al. (1998).

2.3.8.2. Extraction of cellular proteins

Cell biomass of the culture PLsr-$1_{(W)}$ and Lsr-$1_{(W)}$ was collected separately by centrifugation at 8000 rpm for 10 min, washed in Tris HCl buffer (pH 7.0) and resuspended in the same. Cells were then solubilized by boiling for 5 min in disruption buffer and analyzed on SDS PAGE as described in section 2.3.6.3.

2.4. Results and Discussion

2.4.1. Antimicrobial activity

One of the most frequent health claims for probiotics is the reduction and prevention of infectious diseases in the gastrointestinal tract (GIT). Enteric pathogens infect the host in different atmospheric conditions of GIT causing diarrhoel diseases. Many scientists all over the world have reported

the occurrence of food borne pathogens contaminating processed foods and vegetables thus causing health hazards (Vescovo et al., 1996; Kannappan and Manja, 2004; Wilderdyke et al., 2004). Innovative approaches have been tried as an alternative to antibiotics in treating these diseases which include usage of live biotherapeutic agents such as bacterial isolates (Daly and Davis, 1998; Soomro et al., 2002; Oyetayo et al., 2003). The antimicrobial effect of LAB has been used to extend the shelf life of many foods and in treating food borne diseases (Savadogo et al., 2004).

The effect of probiotic strains depends on their ability to persist and compete with pathogens in GIT. Antimicrobial activities of LAB have been widely investigated in the past few years (Daeschel, 1989; Piard and Desmazeaud, 1991; Hechard et al., 1992). The inhibitory activity of LAB against spoilage bacteria and food borne pathogens is mainly based on acid production, competition for nutrients and space, formation of hydrogen peroxide, carbon dioxide and other products of catabolism and bacteriocins (Lucke and Earnshaw, 1990). Broad-spectrum inhibition is generally attributed to organic acid production and / or to H_2O_2. Narrow inhibitory spectrum includes synthesis of bacteriocins (Tagg et al., 1976; Klaenhammer, 1988).

In the present experiment, agar well diffusion assay was used to determine the antimicrobial activity of probiotic *L. mesenteroides* (PLsr-$1_{(W)}$) against nine food borne pathogens. The antimicrobial activity of PLsr-$1_{(W)}$ was found to be better than the native isolate Lsr-$1_{(W)}$ (Table 2.1). The culture PLsr-$1_{(W)}$ inhibited the growth of seven food borne pathogens with the inhibition zone of 11-16 mm whereas Lsr-$1_{(W)}$ inhibited with the inhibition zone of 8-12 mm. The culture PLsr-$1_{(W)}$ exhibited maximum inhibition to *V. cholerae* (16 mm) as compared to Lsr-$1_{(W)}$ (12 mm), dairy starter culture *S. thermophilus* (10 mm) and Standard *L. mesenteroides* (B640) (11 mm). Other pathogens like *S. typhi, P. aeroginosa* and *S. aureus* (Kannappan and Manja, 2004; Vescovo et al., 1996; Wilderdyke et al., 2004) were inhibited by PLsr-$1_{(W)}$ with an inhibition zone of 13, 14 and 11 mm diameter

respectively. PLsr-$1_{(W)}$ had inhibitory activity of 14 mm diameter against *S. dysenteriae* which is known to infect colonic mucosa (Dupont, 2005; Pegues et al., 2005) whereas Lsr-$1_{(W)}$, B640 and dairy starter *S. thermophilus* had 10, 8 and 7 mm zone of inhibition respectively. *E. coli* causing urinary tract infections (Franz and Horl, 1999) was inhibited by PLsr-$1_{(W)}$ with 13 mm inhibition zone.

Tadesse et al (2005) have reported antimicrobial activity in *Leuconostoc sp* against *Salmonella, S. flexneri, S. aureus* and *E. coli* with an inhibition zone of 16.28, 16.38, 16.16 and 15.34 mm diameter respectively, whereas PLsr-$1_{(W)}$ showed 13, 14, 11 and 13 mm zone of inhibition against *S. typhi, S. dysenteriae, S. aureus* and *E. coli* respectively. Additionally PLsr-$1_{(W)}$ inhibits other pathogenic cultures like *Y. enterocolitica, P. aeroginosa* and *V. cholerae*.

Table 2.1: Antimicrobial activity of *L. mesenteroides* (PLsr-$1_{(W)}$ and Lsr-$1_{(W)}$) in comparison with standard cultures

Pathogenic strains	Inhibition zone (mm in diameter)			
			Standard cultures	
	PLsr-$1_{(W)}$	Lsr-$1_{(W)}$	*Leuconostoc mesenteroides* (B640)	*Streptococcus thermophilus*
Escherichia coli	13 ± 0.4	13 ± 0.1	07 ± 0.1	10 ± 0.2
Salmonella typhi	13 ± 0.2	10 ± 0.1	10 ± 0.1	10 ± 0.4
Salmonella paratyphi	-	-	-	07 ± 0.4
Shigella dysenteriae	14 ± 0.4	10 ± 0.2	08 ± 0.4	07 ± 0.4
Pseudomonas aeroginosa	14 ± 0.4	11 ± 0.1	12 ± 0.3	07 ± 0.3
Vibrio cholerae	16 ± 0.1	12 ± 0.3	11 ± 0.2	10 ± 0.2
Yersinia enterocolitica	12 ± 0.3	10 ± 0.1	07 ± 0.1	-
Staphylococcus aureus	11 ± 0.1	11 ± 0.1	08 ± 0.1	10 ± 0.1
Listeria monocytogenes	-	-	10 ± 0.1	-

*Values are Mean ± SD (n=3). (-) indicate no inhibitory activity.

To determine the effect of heat/ higher temperature, cell free culture supernatant was treated at 100°C for 10 min and then tested for antimicrobial activity against *S. dysenteriae, S. typhi* and *S. aureus*. These are important food borne pathogens which are known to cause disease outbreaks all over the world. From the result it was observed that the heat treated cell free supernatant shows activity less (≤ 9 mm) as compared to non-thermal treated sample (≥ 11 mm) (Table 2.2; Fig. 2.2). This shows that the antimicrobial or bacteriocin like compound present in the cell free supernatant is heat sensitive.

To determine that acidity is not the only criteria responsible for antimicrobial activity of PLsr-$1_{(W)}$, the cell free supernatant was adjusted to pH 6.0 using 0.1 N NaOH and assayed for antimicrobial activity. The results indicate that the culture supernatant adjusted to pH 6.0 showed very slight reduction ($P>0.01$) in the activity. Inhibitory activity against *S. dysenteriae, S. typhi* and *S. aureus* was reduced by 14.29, 15.39 and 9.09% respectively (Fig. 2.2).

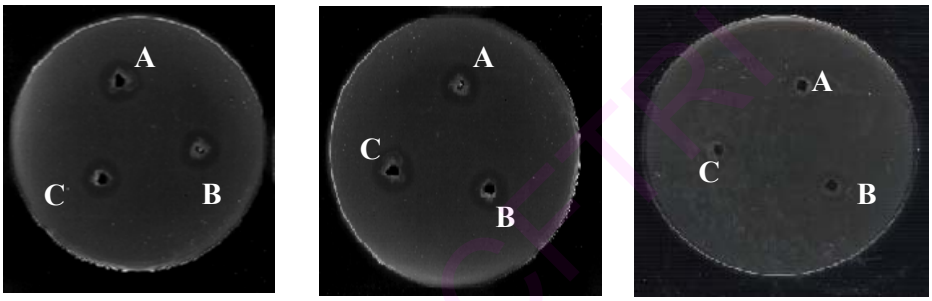
Draksler et al. (2004) have shown *Lactobacillus* DDL48 having antimicrobial activity against *Salmonella typhi* with an inhibition zone of 8–10 mm diameter. The effect was observed with a non-adjusted pH supernatant. However, the supernatant adjusted to pH 6.0 was unable to inhibit the growth of the pathogen. In the present study, PLsr-$1_{(W)}$ inhibits *S. typhi* with an inhibition zone of 13 mm diameter. The culture supernatant when adjusted to pH 6.0 had inhibitory activity against *S. typhi* with an inhibition zone of 11 mm diameter.

The results indicate that PLsr-$1_{(W)}$ can be used as a starter culture for traditional fermented foods to improve its safety. It could be helpful in treatment of diseases like typhoid, shigellosis, lung infection, cholera, enterocolitis and food poisoning.

Table 2.2 : Antimicrobial activity of heat killed and neutralized culture supernatant of PLsr-$1_{(W)}$

Indicator strain	Inhibition zone (mm) in diameter		
	Normal	Heat treated (100^oC)	Neutralized
Shigella dysenteriae	14.0 ± 0.4	9.0 ± 0.1	12.0 ± 0.2
Salmonella typhi	13.0 ± 0.2	8.0 ± 0.1	11.0 ± 0.2
Staphylococcus aureus	11.0 ± 0.1	9.0 ± 0.2	10.0 ± 0.4

*values are Mean ± SD (n=3)



Shigella dysenteriae *Salmonella typhi* *Staphylococcus aureus*

Fig 2.2 : Antimicrobial activity of *L. mesenteroides* (PLsr-$1_{(W)}$).

(A) Culture supernatant (B) Supernatant heated for 30 min (C) Supernatant treated with NaOH to neutralize

2.4.2. Antibiotic susceptibility test

LAB are common microorganisms in food products and also constitute the natural intestinal microflora of humans and animals (Tannock, 1995). Antibiotic therapy can significantly affect this microbial balance in the intestine by reducing 0the viability of indigenous microflora. This results in the stimulation of opportunistic microorganism and pathogens to establish in the intestinal region thus causing diarrhea or other intestinal disorders. Restoring the microbial balance and reestablishing the normal intestinal microflora is considered necessary in order to prevent the number of intestinal pathogens. In the present work susceptibility of probiotic culture *L. mesenteroides* (PLsr-$1_{(W)}$) was assessed against common antibiotics to

determine the capability of the culture to maintain the microbial balance in the intestine during antibiotic treatment.

The antibiotic susceptibility of PLsr-$1_{(W)}$ is given in table 2.3. The growth inhibition of PLsr-$1_{(W)}$ was observed with chloramphenicol (25 µg), clindamycin (2 µg), erythromycin (15 µg), vancomycin (30 µg), ampicillin (10 µg), co-trimoxozole (25 µg), oflaxacin (1 µg) and penicillin (10 µg) with inhibition zone of ≥ 16 mm. On the other hand, no growth inhibition was observed with cephalothin (30 µg), gentamycin (10 µg) and oxacillin (1 µg) which shows that the culture has intrinsic resistance to these antibiotics (Fig. 2.3). The native isolate Lsr-$1_{(W)}$ was resistant to only 2 antibiotics (gentamycin and oxacillin). The standard culture B640 also was found resistant to cephalothin, gentamycin and oxacillin.

Zhou et al. (2005) have detected that the *Lactobacillus* and *Bifidobacterium* strains were susceptible to β-Lactam antibiotics (penicillin, ampicillin, and cephalothin) also Gram-positive spectrum antibiotics (erythromycin and novobiocin) and broad-spectrum antibiotics (chloramphenicol, rifampin, spectinomycin and tetracycline). Some of the LAB starter/ probiotic strains belonging to genera *Lactobacillus, Pediococcus, Leuconostoc* and *Streptococcus* are known to be sensitive to clinically relevant antibiotics such as penicillin, ampicillin, tetracycline, erythromycin and chloramphenicol (Hummel et al., 2007).

Bacteria are known to develop numerous mechanisms to counteract the action of antibiotics such as inactivation of antibiotics by enzymes, active efflux pumps that exclude the molecule outside the cell and /or modification of the sub cellular target, where the antibiotics binds (Walsh, 2003).

Bezares et al. (2006) have assessed the antibiotic susceptibility of LAB strains isolated from wine and have reported 4 µg gentamycin as the minimal inhibitory concentrations to inhibited *Leuconostoc sp* whereas the present culture PLsr-$1_{(W)}$ was found resistant even at higher concentration of

gentamycin (10µg) (Table 2.4). PLsr-$1_{(W)}$ is also resistant to Oxacillin (1 µg) and Cephalothin (30 µg) and hence can maintain the normal intestinal microflora in patients under these antibiotic treatment.

Table 2.3 : Antibiotic susceptibility of PLsr-$1_{(W)}$

Antibiotic	Concentration (µg/disc)	Inhibition zone (mm)		
		PLsr-$1_{(W)}$	Lsr-$1_{(W)}$	Std- *L. mesenteroides* (B640)
Cephalothin	30	R	11 ± 0.01	R
Chloramphenicol	25	32 ± 0.01	30 ± 0.01	30 ± 0.01
Clindamycin	2	31 ± 0.02	30 ± 0.01	32 ± 0.01
Erythromycin	15	25 ± 0.02	25 ± 0.02	22 ± 0.10
Gentamycin	10	R	R	R
Oxacillin	1	R	R	R
Vancomycin	30	16 ± 0.03	17 ± 0.02	17 ± 0.01
Ampicillin	10	25 ± 0.05	23 ± 0.03	23 ± 0.02
Co-trimoxozole	25	32 ± 0.01	30 ± 0.02	30 ± 0.03
Ofloxacin	1	30 ± 0.01	30 ± 0.01	28 ± 0.01
Penicillin G	10	32 ± 0.01	32 ± 0.02	32 ± 0.10

*values are Mean ± SD (n=2). R = resistant

Fig 2.3 : Antibiotic susceptibility test of PLsr-$1_{(W)}$

Growth inhibition can be observed around the antibiotic disc indicating the sensitivity of the culture isolate

Table 2.4 : Minimum inhibitory concentration of antibiotics tested against probiotic *L. mesenteroides* (PLsr-1$_{(W)}$)

Antibiotics	Concentration (µg)	Inhibition zone (mm)
Cephalothin	10	R
	25	R
	30	R
	40	10 ± 0.20
	50	12 ± 0.13
Gentamycin	2.5	R
	5	R
	10	R
	20	08 ± 0.22
	30	10 ± 0.40
Oxacillin	0.25	R
	0.5	R
	0.75	R
	1.0	R
	1.25	08 ± 0.11

*values are Mean ± SD (n=2), R = resistant

2.4.3. Antioxidant activity

Oxidation is essential to living organisms for the production of energy to biological processes. It is well established that free radicals are normal part of metabolism that are continuously produced *in-vivo* but cause oxidative damage (Halliwell and Cherico, 1993). According to the theory proposed by Farmer et al. (1942) free radicals, reactive oxygen species and other toxic compounds produced by oxidation process can damage biological molecules. These free radicals are very unstable and react rapidly with other groups/ substances in body leading to cell or tissue injury. Cancer, emphysema, cirrhosis, artherosclerosis, arthritis, cardiovascular disease, cognitive decline, diabetes and mascular degeneration have been correlated with oxidative damage (Halliwell and Gutteridge, 1984; Tapiero et al., 2004; Astley et al., 2004; Tyssandier et al., 2002; Dwyer et al., 2001).

Our body has its own defense mechanism against reactive oxygen radical based on antioxidant enzyme and low molecular mass non-enzymatic antioxidant compounds. These defense systems are not effective enough to

totally prevent the damage. Therefore, food supplements containing antioxidants may be useful to human body in reducing oxidative damage (Zommara et al., 1996; Oxman et al., 2000; Terahara et al., 2001; Kullisaar et al., 2003).

Various synthetic and natural antioxidants have been reported. However Lin and Yen (1999) have predicted doubts about the safety and long term effects on health of synthetic antioxidants, hence antioxidants from natural sources are likely to be desirable. In this regard, antioxidative property of LAB has been exploited to be used as a natural source of antioxidants. There are various reports on LAB that are able to degrade the superoxide anion and hydrogen peroxide (Alander et al., 1997; Miller and Britigan, 1997; Kullisaar et al., 2002). Considering this, the present isolate *L. mesenteroides* (PLsr-$1_{(W)}$) was studied for its antioxidative property.

Earlier authors have described various methods to measure the antioxidative property and the results are expressed in a variety of ways. Inhibition of lipid peroxidation (Wanasundrara et al., 1994; Bertelsen et al., 1995), ascorbate autooxidation (Rashid et al., 1993) and norepinephrine oxidation (Mishra and Korachich, 1984) are used. In the present study, antioxidative activity was measured by method of ascorbate autooxidation, metal chelating and DPPH scavenging activity.

2.4.3.1. Inhibition of ascorbate autooxidation

The inhibition of ascorbate autooxidation by intracellular cell free supernatant of PLsr-$1_{(W)}$ increases with incubation time and reaches maximum (5.7%) after 6 min, whereas the native isolate Lsr-$1_{(W)}$ had 4.9% inhibition after 6 min (Table 2.5). Some of the earlier investigators have found the ability of LAB cultures to inhibit ascorbate autooxidation (Esaki et al., 1994; Berghofer et al., 1998; Sheih et al., 2000; Chung et al., 2002). Wang et al. (2006) have shown that fermentation of soymilk with lactic acid bacteria and *Bifidobacteria* significantly increases the inhibition rate of

ascorbate autooxidation. The inhibition of ascorbate autooxidation observed in soymilk has been attributed to the action of isoflavones and tocopherols found in soybean (Murakami et al., 1984; Persky and Van Horn, 1995). Chien (2004) has demonstrated that the activity is due to liberation of aglycone genistein and diadzein during fermentation. However, they have observed a significantly higher rate of inhibition when *L. acidophilus* (14.43–15.03%) was used for fermentation of soyamilk than by *S. thermophilus* (14.01–14.04%). But there is no mention of *Leuconostoc* culture and the activity in broth media.

Table 2.5 : Inhibition of ascorbate autooxidation by intracellular cell free extract of *L. mesenteroides* (PLsr-1$_{(W)}$ and Lsr-1$_{(W)}$)

Incubation time (min)	Inhibition (%)	
	PLsr-1$_{(W)}$	Lsr-1$_{(W)}$
0	4.6 ± 2.1	3.8 ± 1.1
2	5.4 ± 1.1	4.1 ± 0.6
4	5.5 ± 2.0	4.5 ± 0.1
6	5.7 ± 1.1	4.9 ± 1.3
8	5.6 ± 1.1	4.7 ± 1.1
10	5.4 ± 1.2	4.6 ± 1.1

*values are Mean ± SD

2.4.3.2. Metal chelating assay

According to Halliwell et al. (1995) metal ions like iron and copper are highly reactive and can initiate lipid peroxidation and start a chain reaction by decomposition of hydroperoxidase to form peroxyl and alkoxyl radicals. It has been proposed that iron may contribute to the risk of heart disease, cancer and arthritis (Halliwell and Gutteridge, 1984). In the present work the chelating ability of PLsr-1$_{(W)}$ towards iron ions was investigated (Table 2.6). The culture shows a good ($P<0.05$) iron chelating ability (53.4 ± 0.11 ppm). The activity observed in the present culture is higher than reported by Lee et al. (1992) who have found *L. casei* KCTC 3260 (10.6 ppm) to exhibited the

higher metal ion chelating ability followed by *L. rhamnosus* GG (8.14 ppm) and *L. casei* KCTC 3109 (1.1 ppm) for Fe2+ ions. Earlier it has been reported that *Streptococcus thermophilus* had highest metal ion chelating ability (42.8 ppm) for iron among the 19 LAB strains tested for antioxidative activity (Yamauchi et al., 1984; Lin and Yen, 1999).

Table 2.6 : Ferrous ion chelating ability of intracellular cell free extract of PLsr-$1_{(W)}$ and Lsr-$1_{(W)}$

Sample	Concentration of chelated metal ion (ppm)
PLsr-$1_{(W)}$	53.4 ± 0.11
Lsr-$1_{(W)}$	44.2 ± 0.23

*values are mean ± SD (n=3)

2.4.3.3. DPPH scavenging ability

DPPH is a stable free radical which is used in the present assay to determine the free radical scavenging ability of PLsr-$1_{(W)}$. Table (2.7) represents the DPPH scavenging activity of PLsr-$1_{(W)}$ is compared with native isolate Lsr-$1_{(W)}$ and the dairy starter culture *S. thermophilus*. The data determines that PLsr-$1_{(W)}$ has comparatively high scavenging activity (20.5%) than Lsr-$1_{(W)}$ and *S.thermophilus* and hence may act as a potential natural antioxidant supplement.

Table 2.7 : DPPH scavenging activity of intracellular cell free extract

Culture	Cell count (cfu/ml)	Dry weight (g)	Scavenging activity (%)	Scavenging activity/ g dry weight
PLsr-$1_{(W)}$	1×10^9	0.030	20.5	6.83 ± 0.11
Lsr-$1_{(W)}$	1×10^9	0.030	19.0	6.33 ± 0.06
Streptococcus thermophilus	1×10^9	0.042	19.1	4.55 ± 0.21

*values are Mean ± SD (n=3)

2.4.4. Anticholesterol activity

Cardiovascular disease is the most important cause of death in westernized countries and it is strongly associated with hypercholesterolemia (Lee et al., 1992). The disease has been accounted for 16.7 million or 29.2% of total global death in 2003. In India it is 11% and in China it is 53.4%. Therefore to decrease the cholesterol concentration is a very important.

The cholesterol concentration can be regulated by the biosynthesis of cholesterol from saturated fat, removal of cholesterol from the circulation, absorption of dietary cholesterol and excretion of cholesterol via bile and feces (Lee, 1997; Hay et al., 1999; Lim et al., 2004).

Some natural microorganisms in the intestine are known to be beneficial in terms of lowering serum cholesterol (Fernandes, 1987; Fukushima et al., 1999; Mann and Spoerry et al., 1974). LAB in particular *Lactobacillus* and *Bifidobacteria* are known to have the ability to metabolize cholesterol (Gilliland and Walker, 1990; De Smet, 1995; Canzi et al., 2000; Park et al., 2002).

The present study examines the cholesterol assimilation ability of culture PLsr-$1_{(W)}$. According to the data obtained, the culture was found to assimilate 37.23% cholesterol from the media which was higher as compared to Lsr-$1_{(W)}$ (35.2%) and B640 (32.74%) (Table 2.8).

Table 2.8 : Cholesterol assimilation ability of PLsr-$1_{(W)}$

Culture	Cholesterol assimilation (%)
PLsr-$1_{(W)}$	37.23 ± 0.10
Lsr-$1_{(W)}$	35.20 ± 0.11
Std-*L. mesenteroides* B640	32.74 ± 0.09
Dairy starter *S. thermophilus*	40.11 ± 0.13

*values are Mean ± SD (n=3). Initial cholesterol concentration in broth was 75.2µg/ml.

2.4.5. β-galactosidase activity

The probiotic culture *L. mesenteroides* (PLsr-$1_{(W)}$) shows a positive reaction to β-galactosidase with development of yellow color in the reaction mixture with ONPG disc (Fig. 2.4).

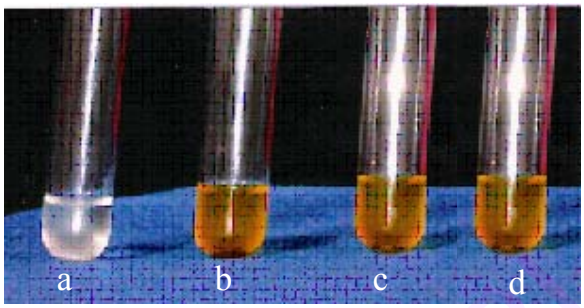


Fig. 2.4 : Qualitative assay for β-galactosidase activity using ONPG disc
(a) Control (b) PLsr-$1_{(W)}$ (c) Lsr-$1_{(W)}$ (d) Dairy starter *S. thermophilus*. Reaction of cell extract with ONPG develops yellow color in reaction mixture in presence of β-galactosidase to release O-nitro phenol.

Influence of carbon source on enzymatic activity

The effect of carbohydrate in growth medium of PLsr-$1_{(W)}$ was determined for β-galactosidase activity (Fig. 2.5). Activity was found to be maximum with lactose (9.135 μM mg^{-1}) followed by glucose (5.92 μM mg^{-1}), sucrose (5.18 μM mg^{-1}) and fructose (4.9 μM mg^{-1}) showing that lactose is the most effective inducer of β-galactosidase in the culture.

Several investigators have described the carbon source regulation of β-galactosidase biosynthesis in various microorganisms (Fantes and Roberts, 1973; Montero et al., 1989; Fiedurek and Szezodrak, 1994; Nikolaev and Vinetski, 1998; De Vries et al., 1999; Nagy et al., 2001; Fekete et al., 2002). Selection of appropriate carbon source is one of the most critical stages in the development of an efficient and economic production of enzyme.

Amount and the type of carbon source are known to affect the expression of β-galactosidase (Inchaurrondo et al., 1998). Biosynthesis of β-galactosidase varies widely in different organisms. Lactose is considered to be the best carbon source to induce maximum β-galactosidase by *Rhizomucor sp* (Shaikh et al., 1997), *K. fragiles* (Fiedurek and Szczodrak,

1994) and *B. longum* CCRC 15708 (Hsu et al., 2005) whereas in the case of *L. crispatus*, highest β-galactosidase activity is observed in the presence of galactose (Kim and Rajagopal, 2000). In *B. subtilis* maximum activity is obtained with starch as carbon source (Konsoul and Kyriakides, 2007). In the present study highest enzyme activity was observed in presence of lactose.

Synthesis of β-galactosidase is governed by transcription rate of lac genes constituting lac operon and is enhanced or suppressed in presence of inducer or repressor (Wallenfels et al., 1972). Hichey (1986) has observed a significant decline in β-galactosidase of *L. bulgaricus* upon addition of small amount of glucose probably due to partial repression of lac operon. resulting Similar results have been observed in the present study where the culture shows less β-galactosidase activity (5.92 μM mg^{-1}) in presence of glucose as compared to lactose (9.13 μM mg^{-1}). In another study Smart et al. (1993) evaluated 21 strains of *Lactobacillus* out of which 19 strains had low β-galactosidase activity in presence of glucose. In case of *B. longum* β-galactosidase was highest with lactose and lowest in presence of glucose.

It has been reported that lactose is hydrolyzed by many LAB via phosphoenol pyruvate dependent phosphotransferase system (McKay et al., 1970; Nader de Macias et al., 1983; Michaela et al., 1992), which may be the probable pathway in *Leuconostoc sp* for lactose hydrolysis.

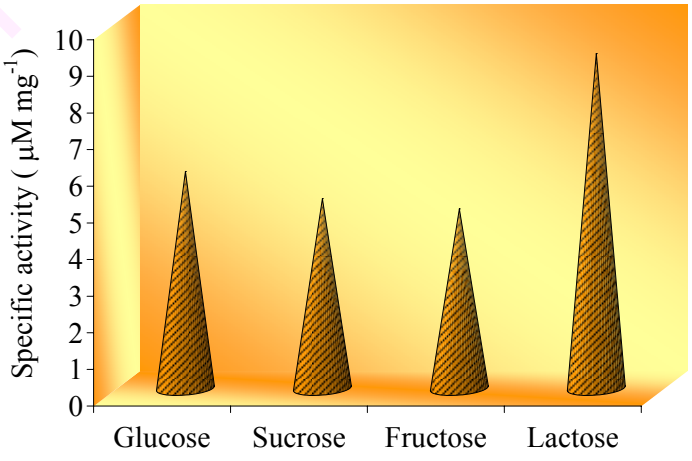


Fig 2.5 : Effect of carbon source on β-galactosidase activity of PLsr-$1_{(W)}$

2.4.6. Adherence ability

Adherence is one of the most important selection criteria for probiotic bacteria (Shah, 2001). Adhesion of bacteria to gastrointestinal epithelium is considered to be a prerequisite for exclusion of enteropathogenic bacteria (Bernet et al., 1993; Mack et al., 1999) or immunomodulation of the host (Perez et al., 1998; Isolauri et al., 1999; Blum et al., 2002).

Adherence of bacterial cells is related to cell surface characteristics (Bibiloni et al., 2001; Canzi et al., 2005). Therefore the identification and characterization of bacterial cell wall properties is important to understand their role in adhesion to hydrocarbons, autoaggregation and relation to co-aggregation mechanism and also the relevance to future probiotic food development from natural strains (van-Loosdrecht et al., 1987; Zammaretti and Ubbink, 2003). Adhesion to the colon surface occurs through the association of bacteria with secreted mucus gel or by adherence to underlying epithelium (Fontaine et al., 1994). High cell surface hydrophobicity favors the colonization of mucosal surfaces and plays a role in adhesion of bacteria to epithelial cells and extracellular membrane proteins (Zareba et al., 1997).

As bacterial adhesion to epithelial cells is been considered as one of the selection criteria for probiotic strains, the present probiotic *L. mesenteroides* (PLsr-$1_{(W)}$) was studied for its adherence activity. Hydrophobic interactions with different solvents are evaluated *in-vitro* and the S-layer responsible for adhesion is extracted with LiCl and analyzed by SDS-PAGE.

2.4.6.1. Cell surface hydrophobicity

For probiotics to exert maximum effect on the host, it should have the ability to adhere and colonize the intestine apart from being resistant to GI condition (Clark et al., 1993). In the present study PLsr-$1_{(W)}$ was studied for its adhesion ability using different hydrocarbons which is an important parameter for bacterial adhesion and colonization in the GI tract (Rosenberg et al., 1980). Bacterial adhesion to hydrocarbons (BATH test) has been extensively used for measuring cell surface hydrophobicity in LAB (Kos et al., 2003; Vinderola et al., 2004; Canzi et al., 2005).

Earlier reports have correlated the adhesion ability and hydrophobicity as a measure of microbial adhesion (Wadstrom et al., 1987; Del Re et al., 2000). Hydrophobicity is one of the physico-chemical properties that facilitate the first contact between the microorganisms and the host cells. This non-specific initial interaction is weak and reversible and precedes the subsequent adhesion process mediated by more specific mechanisms involving cell surface proteins and lipoteichoic acid (Granato et al., 1999; Rojas et al., 2002; Roos and Jonsson., 2002). A number of reports have described the composition, structure and forces of interaction related to bacterial adhesion (Pelletier et al., 1997; Tuomola et al., 2000; Collado et al., 2005).

The present study was carried out to determine adhesion ability of the culture by using toluene, xylene, octane and hexadecane (Table 2.9). The culture exhibited maximum ($p<0.05$) adhesion when grown in MRS medium as compared to Rogosa media. Similar results were found by Ram and Chander (2003) who speculated that the MRS media which contain yeast extract synthesize certain enzymes. These enzymes are responsible for the selective intake of nutrients and synthesize cell-surface components that play a vital role in hydrophobic interaction. Rogosa media contains $FeSO_4$ that might probably inhibit hydrophobic components on cell surface. With different hydrocarbons, maximum adhesion of PLsr-$1_{(W)}$ was with toluene (50.81%) which was higher than Lsr-$1_{(W)}$ (46.22%), B640 (47.11%) and dairy starter culture *S. thermophilus* (49.13%). Ram and Chander (2003) have studied the influence of different growth media and observed that maximum percent (80-89%) adhesion of *B. bifidum* was with MILS media followed by MRS-lactose broth (68-79%). Draksler et al. (2004) have demonstrated the adhesion ability of *Lactobacillus* strains to three solvents namely hexadecane, xylene and toluene. The highest hydrophobic percentages was found in *Lactobacilli* strains DDL19 (60, 57 and 63%) and DDL48 (47, 68 and 69%). In the present study highest hydrophobic adhesion is found with toluene (50.81%) followed by xylene (41.69%), octane (34.78%) and hexadecane (24.47%).

Table 2.9 : Cell hydrophobicity to different hydrocarbons

Medium	Hydro-carbon	Adhesion (%)			
		PLsr-1$_{(W)}$	**Lsr-1$_{(W)}$**	**B640**	***S. thermophilus***
MRS	Toluene	50.81 ± 0.12	46.22 ± 0.11	47.11 ± 0.11	49.13 ± 0.12
	Xylene	41.69 ± 0.15	40.11 ± 0.11	40.23 ± 0.06	42.36 ± 0.15
	Octane	34.78 ± 0.15	33.60 ± 0.09	02.56 ± 0.12	32.12 ± 0.16
	Hexadecane	24.47 ± 0.16	24.00 ± 0.12	22.15 ± 0.15	20.15 ± 0.11
Rogosa	Toluene	20.15 ± 0.15	19.11 ± 0.15	19.15 ± 0.12	19.61 ± 0.09
	Xylene	23.77 ± 0.12	23.00 ± 0.11	23.11 ± 0.15	20.15 ± 0.05
	Octane	18.33 ±0.30	18.00 ± 0.13	18.22 ± 0.15	17.33 ± 0.15
	Hexadecane	17.21 ± 0.12	15.12 ± 0.11	16.54 ± 0.30	19.21 ± 0.16

*values are mean ± SD (n=3). Lsr-1$_{(W)}$ is the native isolate, B640 is standard *Leuconostoc mesenteroides, S. thermophilus* is the dairy starter

2.4.6.2. Adhesion of PLsr-1$_{(W)}$ to intestinal epithelium

The adhesiveness of PLsr-1$_{(W)}$ to the intestinal tissue was investigated by incubating the culture with intestinal tissue at 37°C for 30 min. Microscopic examinations showed that this species strongly adhered to ileal epithelial cells. Figure 2.6 indicates the adherence of PLsr-1$_{(W)}$ to intestinal epithelial cell.

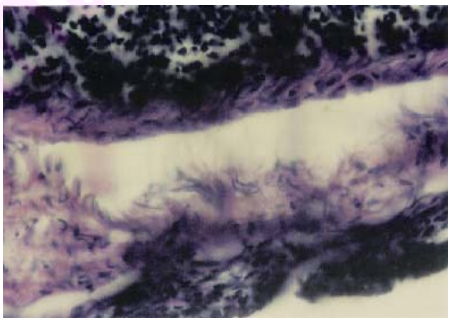


Fig 2.6 : Adhesion of *L. mesenteroides* (PLsr-1$_{(W)}$) to the intestinal epithelium of the rat. Magnification (40 X)

2.4.6.3. Analysis of S-layer protein

Crystalline surface layers (S-layer) are common features among eubacteria and archaeobacteria (Sleytr and Messne, 1988; Messner and Sleytr, 1992; Boot et al., 1993). They are composed of identical subunits consisting of a single protein species which may be glycosylated and are known to

completely cover the cell surface (Koval, 1988). It helps in cell protection, adhesion and surface recognition (Gruber and Sleytr, 1991; Frece et al., 2005). S-layers are not covalently attached to cell surface and can be extracted either as sheets or as individual subunits in presence of dissociating agents such as LiCl, EDTA or chaotropic denaturants such as guanidine hydrochloride (Navarre and Schneewind, 1999).

Different authors have used various methods for the extraction of S-layer protein. Barker and Thorne (1970) have used negative staining and electrophoresis for the detection of S-layer. Kawata et al. (1974) and Masuda (1992) have extracted S-layer from *L. fermenti* and *L. brevis* by freeze etching techniques. Enzymatic hydrolysis of peptidoglycans and extraction with guanidine hydrochloride, urea and SDS has been used in *L. fermenti* and *L. brevis* (Lortal et al., 1992). In the present work, the S-layer protein was efficiently extracted by treatment of LiCl (5M).

S-layer protein is known to constitute upto 10% of the total protein of the cell (Pouwels et al., 1998; Sara and Sleytr, 2000). Further, it has been proposed that S-layer acts as a potential mediator in autoaggregation and adhesion (Schneitz et al., 1993; Green and Klaenhammer, 1994). Mukai and Arihara (1994) have found that glycoproteins in the S-layer bind to lactins on the intestinal epithelial cells. Earlier studies have also shown that presence of S-layer is an important criterion for bacterial adhesion to intestinal epithelial cells and extracellular matrix (Hynonen et al., 2002; Smit et al., 2002).

Frece et al. (2005) and Kos et al. (2003) have revealed the presence of S-layer protein in *L. acidophilus* with a molecular mass of 45 KD. Chen et al. (2007) revealed the presence of potential S-layer proteins in *L. crispatus* ZJ001 with a molecular mass of about 42 KD. In the present work, the surface protein extracted from the culture PLsr-$1_{(W)}$ and analyzed by SDS-PAGE showed a prominent protein band at 42 KD (Fig. 2.7).

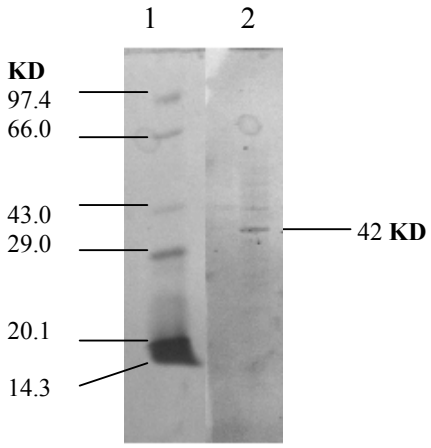


Fig 2.7 : SDS PAGE analysis of S-layer protein of PLsr-1$_{(W)}$

Lane 1: Marker protein, Lane 2: surface protein extract of PLsr-1$_{(W)}$

2.4.7. Analysis of volatile compounds

Flavor in LAB play a major role in dairy industries. LAB produces lactic acid as major end product of metabolism. Diacetyl, acetoin, 2-3-butanediol, acetate, ethanol, formate, CO_2 and many others are also produced in different ratios according to species or strain (Boumerdassi et al., 1997; Tzanetaki and Mastrojiannaki, 2006). These substances are important in flavor perception and texture of many fermented foods.

Flavor development in dairy fermentation results from a series of biochemical processes in which starter cultures provide enzymes and thus contribute to sensory perception of dairy products (Smit et al., 2005). LAB added as starter culture transform lactic acid, citrate, lactate, proteins and fats into volatile compounds that together with amino acids and other products play a critical role in the development of flavors (Martley and Crow, 1993).

Flavor molecules can be produced during growth by biosynthesis (*de novo* synthesis) and bioconversion (precursor biotransformation) (Mauriello et al., 2001). Biochemical routes leading to flavor may include proteolysis and peptidolysis. Enzymatic activity of hydroxyacid dehydrogenase, decarboxylase, esterase, acyl transferase, alcohol, aldehyde

and keto acid dehydrogenases also contribute to flavor development (Smit et al., 2005).

Earlier Alonso and Fraga (2001) have analyzed the volatile flavor compounds in yogurt by headspace gas chromatography-mass spectrometry (GCMS), wherein important compounds identified were acetone and acetic acid. Fermented products formed from starter mixture of *Lactococcus lactis subsp lactis* and *Lactococcus lactis subsp cremoris* are known to produce volatile compounds like acetaldehyde, acetone, 2-butanone, diacetyl and ethanol (Richelieu et al., 1997). Agrawal et al. (2000) have studied the flavor profile of idli batter prepared from *Pediococcus pentosaceus* and *Candida versitalis* as starter cultures, wherein diols and acids were found upto 8 days of storage.

According to the literature volatile compounds produced from LAB are found to be therapeutically important (Murti et al., 1993; Gallardo-Escamilla et al., 2005). In the present work the volatile compounds of therapeutic importance produced by the PLsr-$1_{(W)}$ are identified at different stages of its growth.

Table 2.10 represents the volatile compounds extracted at different time intervals during growth. The compounds were identified by comparing the fragmentation pattern with the mass spectra of respective standards (Fig. 2.8).

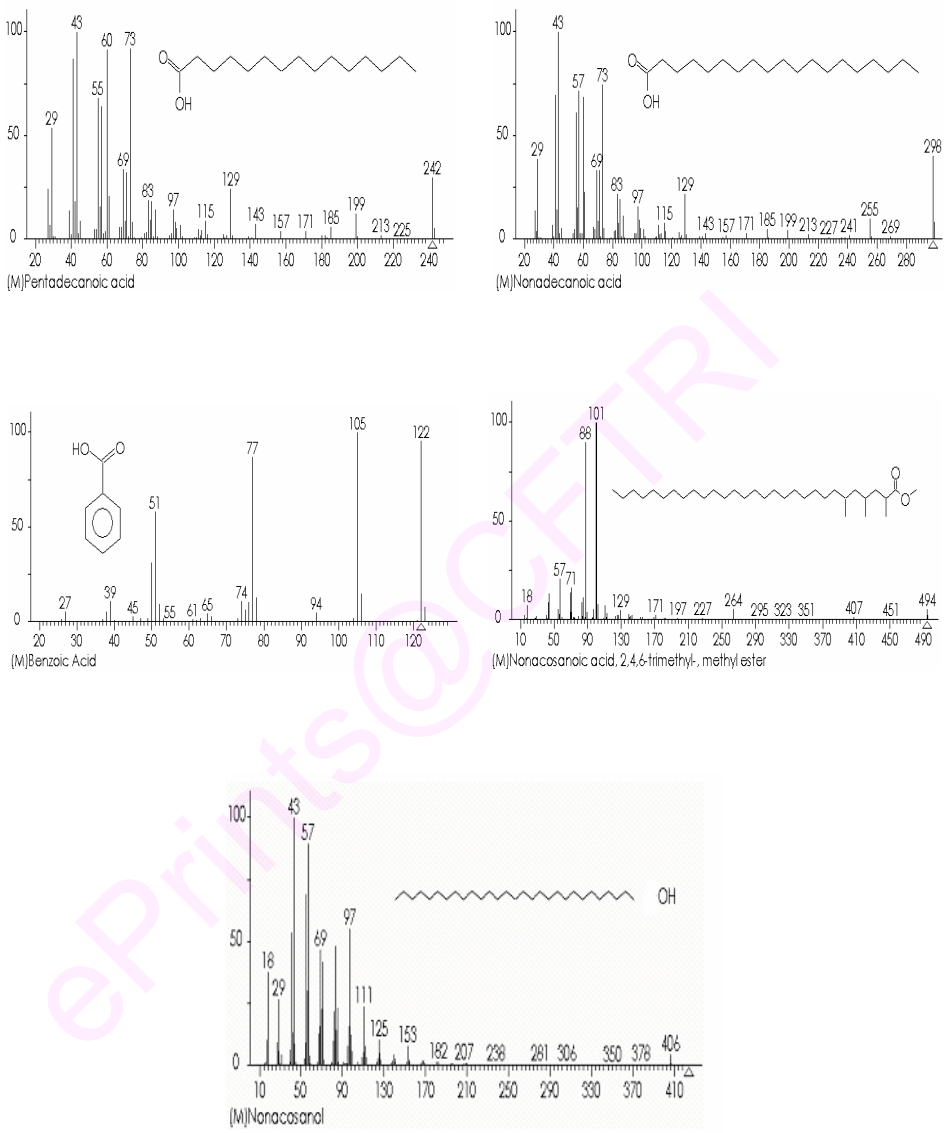


Fig 2.8 : Mass spectra of isolated volatile compounds

Table 2.10: Yield of volatile compounds at different time intervals

Compounds Yield (mg %)	Incubation time (h)							Mol. Wt.	Fragmentation pattern	Uses
	0	**8**	**16**	**24**	**32**	**40**	**48**			
Benzoic acid	-	-	0.281	0.488	0.697	0.799	1.072	278	105,122,77,51,50	Antirheumatic and antiseptic property
Nonacosane	27.90	35.73	44.44	56.95	68.81	55.93	26.76	408	57,43,71,85,99	Antibacterial activity
Nonacosanol	8.574	8.568	8.628	8.816	10.42	11.55	12.63	424	43,57,97,83,69	Constituent of policosanol-reduces LDL and HDL cholesterol
Nonadecanoic acid	0.538	0.858	0.907	0.739	0.435	0.341	0.239	298	43,73,57,29,69	Anti-inflamatory action
Pentadecanoic acid	0.356	0.602	-	-	0.602	1.115	1.426	242	43,60,73,41,55	Used as marker for intake of milk. Constituent of manaca's plant which is used for arthritis and rheumatism and has anti-inflamatory action

The major compounds produced were identified as pentadecanoic acid, nonadecanoic acid, benzoic acid, nonacosane and nonacosanol. Pentadecanoic acid, which is used to cure arthritis and also as an anti inflammatory agent was initially 0.356% which increased to 1.426% after 48h of incubation. Nonadecanoic acid which is used for anti-inflammation was produced initially at a concentration of 0.538% and reached maximum at 8 h of growing phase (0.858%) then gradually declined to 0.239% after 48 h. Benzoic acid which is used as antirheumatic and antiseptic agent was produced 0.281% at 16 h of growth which increased upto 1.072% after 48 h. Nonacosane which has antibacterial property reached a maximum 68.81% after 32 h and declined on further incubation.

In the present work the pentadecanoic acid which was increasing even after 48 h of incubation was also reported by Hannes et al. (2000) where it is formed at the end of alcoholic fermentation by *Saccharomyces bayanus* and *Saccharomyces cerevisiae*. Hirschler et al. (1998) has shown the formation of pentadecanoic acid by carboxylation at C_3 of ketone and the removal of two terminal carbon atoms at C_1 and C_2 position of hexadecane. Kato et al. (2001) have also shown the accumulation of pentadecanoate in *Bacillus thermoleovorans*.

Nonacosanol which is a constituent of policosanol was increasing even after 48 h of bacterial growth. Policosanol is known to be effective in lowering LDL and cholesterol (Pons et al., 1994; Ortensi et al., 1997; Fernandez et al., 2001).

Benzoic acid which is a natural antibiotic is known to be produced by *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (Shahani et al., 1976). In the present work PLsr-$1_{(W)}$ produces benzoic acid after 16 h of incubation. The culture shows the importance for its use in food preservation. According to Masja et al. (1998) benzoic acid is formed by aminotransferase reaction of keto acids. Thierry and Maillard (2002) has shown that when amino acids are used as a substrate it is catabolised by several reactions like deamination, decarboxylation, oxidative deamination and transamination leading to keto acids that enters the pathway of fatty acid synthesis.

All these volatile compounds produced by PLsr-$1_{(W)}$ in the present study are shown to have therapeutic value for various ailments like anti-inflammation, antirheumatic, antiseptic, antibacterial and antitumor activity.

Today, flavor industry has come a long way in terms of making nutraceutical products more acceptable to the consumers, wherein this potent probiotic culture may play an important role. Food formulations with addition of such culture or byproduct or metabolite of the culture may have an immense health effect on humans and can prove to be a good functional food.

2.4.8. Changes in the cellular fatty acids and proteins in probiotic PLsr-$1_{(W)}$

Bacteria are known to develop a set of mechanism leading to their protection against GIT conditions. A combination of constitutive and inducible methods like removal of protons (H^+), alkalization of external environment, changes in the composition of cell envelope, production of general shock proteins, expression of transcriptional regulators and responses to changes in cell density all can contribute to their survival (Christensen et al., 1999; Nomura et al., 1999; Sanders et al., 1999; Cotter and Hill, 2003).

LAB have also evolved specific mechanism to resist the toxic effect of bile thus enabling them to live in intestinal environment (Gunn, 2000). The mechanism may be the release of bile salt hydrolases (BSH) that deconjugate bile acid or by hydrolyzing amino acid glycine or taurine from steroid core thus altering the properties of bile (Adamowicz et al., 1991; De Smet et al., 1995).

Cellular membrane fatty acids and cellular proteins are the primary target of any stress. It is established that organisms regulate membrane composition in response to environmental condition to maintain optical membrane fluidity for normal cellular function (Swan and Watson, 1997). In the present study cellular fatty acid and proteins of probiotic PLsr-$1_{(W)}$ is compared with native isolate Lsr-$1_{(W)}$ to determine the adaptive nature of PLsr-$1_{(W)}$.

Cellular fatty acids profile

Table 2.11 represents the cellular fatty acids composition of the probiotic strain PLsr-$1_{(W)}$ and the native isolate Lsr-$1_{(W)}$. An increase of unsaturated fatty acids like palmitoleic acid (9.08%), oleic acid (5.84%), linoeic acid (5.35%) and linolenic acid (2.8%) was found in PLsr-$1_{(W)}$ as compared to Lsr-$1_{(W)}$. Ma and Masquis (1997) have also reported an increase in unsaturated fatty acids which is known to reduce the permeability of protons in acid adapted cells.

Table 2.11: Cellular fatty acids composition of PLsr-$1_{(W)}$ and Lsr-$1_{(W)}$

Fatty acid concentration (%)		PLsr-$1_{(W)}$	Lsr-$1_{(W)}$
Butyric acid	$C_{4:0}$	1.027	0.194
Caprylic acid	$C_{6:0}$	0.844	0.020
Capric acid	$C_{8:0}$	1.284	1.089
Lauric acid	$C_{10:0}$	1.138	0.154
Myristic acid	$C_{12:0}$	0.778	0.177
Palmitic acid	$C_{14:0}$	28.31	53.01
Palmitoleic acid	$C_{16:0}$	9.084	0.088
Stearic acid	$C_{18:0}$	19.65	39.66
Oleic acid	$C_{18:1}$	5.845	2.103
Linoeic acid	$C_{18:2}$	5.351	0.342
Linolenic acid	$C_{18:3}$	2.809	0.283
Arachidic acid	$C_{20:0}$	2.068	0.719

*ND: not detected

Lipids in microbial cells play a very important role in cell physiology (Ratledge and Wilkinson, 1989; Russell and Fukanaga, 1990). The changes in lipid compositions enable microorganisms to maintain membrane function in stress environments. Van-Schaik et al. (1999) have suggested that the increase in production of straight chain fatty acids C16:0 and decrease in C18:0 levels is associated with acid adaptation. Similarly Guerzoni et al. (2001) has reported an increase of vernolic acid (upto 37%) an epoxide of linoleic acid when exposed to low pH. Cellular fatty acids are one of the factor that protect

cells from acid shock (Brown et al., 1997). The present work the probiotic culture PLsr-$1_{(W)}$ shows increase in unsaturated fatty acids which shows the adaptation to GIT conditions.

Cellular protein analysis of PLsr-$1_{(W)}$

Figure 2.9 shows the SDS PAGE analysis of cell extract of probiotic PLsr-$1_{(W)}$ and native isolate Lsr-$1_{(W)}$. Molecular mass of the protein was determined by comparing the prominent protein band with that of protein marker. A prominent protein band was observed at 20.1 and 43 KD in the protein extract of PLsr-$1_{(W)}$ as compared to Lsr-$1_{(W)}$.

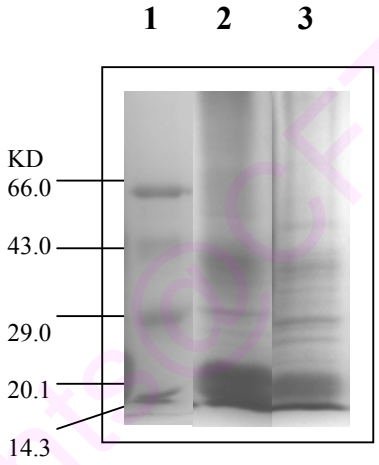


Fig 2.9 : SDS-PAGE showing the protein pattern of *L. mesenteroides* (PLsr-$1_{(W)}$ and Lsr-$1_{(W)}$).
Lane 1: Molecular weight Markers
Lane 2: Cell extract of PLsr-$1_{(W)}$
Lane 3: Cell extract of Lsr-$1_{(W)}$

Protein synthesis is known to confer tolerance to a stressful environment. Proteomic study is hence one of the best way to investigate changes in the genome expression profile when cells are subjected to environmental stress (Leverrier et al., 2004). Increased synthesis of 42 KD proteins was observed in acid adapted mutant of *L. oenos*, which suggests that this protein could be a specific protein of acid stress involved in the mechanism of acid tolerance of *L. oenos* (Drici-Cachon et al., 1996). A study by Leverrier et al. (2004) shows the presence of nine proteins (14.1, 18.6,

21.5, 26.9, 41.9, 49.6 and 56.2 KD) as a result of pH drop in the media. Savijoki et al. (2005) have observed an upregulated induction of proteins like HtrA (67 KD), Dna K (66 KD) and GroEL (57 KD) in *B. longum* on bile salt treatment. In *E. coli* (aEPEC) bile treatment induced LdaG (25 KD) protein (Torres et al., 2007). These results strongly suggest the involvement of these proteins in cell adaptation to environmental changes.

In the present culture increase in 20.1 and 43 KD protein has been observed PLsr-$1_{(W)}$ as compared to Lsr-$1_{(W)}$ which suggests the culture PLsr-$1_{(W)}$ adaptation to stress conditions GIT.

Conclusion

This study focused on the functional properties of *Leuconostoc mesenteroides* PLsr-$1_{(W)}$. The culture has antimicrobial activity against seven food borne pathogens, which determines the capability of the culture to be used as a preservative or in pharmacological industries against large number of diseases. The antibiotic susceptibility test confirmed the culture to be resistant to 3 antibiotics which enables the culture to maintain the balanced intestinal microflora under antibiotic therapy. The antioxidative activity of the culture demonstrates the scavenging activity against oxygen free radicals. Therefore, it can be used as an antioxidant source in food and pharmaceutical preparation. Further the adhesion assays indicate the ability of the culture to adhere to the intestinal epithelial cells which imparts beneficial health effect to the consumer. The culture has cholesterol lowering ability, β-galactosidase activity and produces volatile compounds of therapeutic value.

Chapter 3

Leuconostoc as a Source for β-Galactosidase Enzyme

CHAPTER – 3

LEUCONOSTOC AS A SOURCE FOR β-GALACTOSIDASE ENZYME

ABSTRACT

The focus of the present chapter was to evaluate the ability of the culture (PLsr-$1_{(W)}$) to hydrolyze lactose. β-galactosidase responsible for the breakdown of non-reducing disaccharide lactose was studied in the culture isolate. The strain was improved by UV irradiation and chemical mutagenesis for enhanced enzyme activity. The UV-mutant (coded as **M7-PLsr-$1_{(W)}$**) which had 2 folds higher activity than parent strain (PLsr-$1_{(W)}$) was subjected to different permeabilization methods to optimize maximum release of the enzyme. Further RSM studies were undertaken for optimization of chemical and physical parameters. The enzyme produced under optimum conditions of pH 7.5 with 1.25% lactose was further precipitated by ammonium sulphate. The results indicate a 25 fold increase in the enzyme activity after ammonium sulphate precipitation as compared to the crude extract.

3.1. Introduction

Probiotics beneficially affect the health of the host by providing enzymatic activities that improve the utilization of nutrients within the intestine (Rowland, 1992). In this sense, these probiotic cultures are included in dairy products to improve lactose absorption (Montes et al., 1995; Jiang et al., 1996; Mustapha et al., 1997).

Lactose is a non-reducing disaccharide, hydrolyzed into glucose and galactose in presence of lactase/ *β*-galactosidase enzyme. During infancy, all humans and mammals possess high levels of enzyme lactase in their small intestine which enables digestion of lactose. After weaning stage, a large part (≈ 75%) of world population undergoes a genetically determined decline in lactase activity which can lead to maldigestion of lactose causing abdominal discomfort (Sahi, 2001).

The development of lactose hydrolyzed products with *β*-galactosidase is one of the possible approaches to diminish the lactose maldigestion problem. By hydrolyzing lactose with *β*-galactosidase, the problem associated with whey disposal, lactose crystallization in frozen concentrated deserts and lactose intolerance problems can be alleviated (Mahoney, 1998; Kim and Rajagopal, 2000; Hsu et al., 2007). Although pharmaceutical preparation of *β*-galactosidase have been developed for treating lactose intolerance (Moskovitz et al., 1987; Sanders et al., 1992) they are found to be less effective (Solomons et al., 1985; Onwulata et al., 1989). They are either expensive, not available or are in insufficient quantity for industrial application (Albayrak and Yang, 2002). Therefore selection of microorganisms which is capable of producing high level of *β*-galactosidase is very important.

β-galactosidase has been characterized in yogurt cultures *L. delbrueckii* and *S. thermophilus* (Itoh et al., 1980; Greenberg and Mahoney, 1982). Gilliland and Kim (1984) suggested that microbial *β*-galactosidase which survives gastric digestion improves lactose digestion.

Jiang et al. (1996) have shown that ingestion of milk containing *B. longum* at a dose of 5×10^8 cfu/ml improved lactose digestion and caused a moderate reduction in total excretion of breath hydrogen. To alleviate lactose intolerance it is important to have a potent probiotic culture capable of producing large amounts of *β*-galactosidase enzyme.

For this approach, the probiotic culture *L. mesenteroides* (PLsr-$1_{(W)}$) was improved and optimized for maximum *β*-galactosidase production.

The study was undertaken as

1) Strain improvement using UV-radiation and chemical mutagenesis for enhanced *β*-galactosidase activity.
2) Optimization of permeabilization technique for maximum release of *β*-galactosidase.
3) Optimization of conditions for *β*-galactosidase using response surface methodology
4) Ammnium sulphate precipitation of *β*-galactosidase.

3.2. Materials

Chemicals and reagents

All chemicals used in this study were of analytical reagent grade.

O-nitrophenyl *β*-galactopyranoside (0.012 M), glucose, galactose, lactose, sucrose, fructose, sodium carbonate (2 ml; 0.6 M), Sodium thiosulphate, Bovine Serum Albumin (BSA), O-nitrophenol purchased from HiMedia Pvt Ltd, Mumbai, India..

Ethyl methyl sulphonate was purchased from SRL Chemicals, India.

Solvents: toluene, acetone, chloroform were used of HPLC grade purchased from Qualigens company, India.

Equipments:

1) Homogenizer, deep freezer, sonicator, lyophilizer, UV-lamp
2) Spectrophotometer, Centrifuge, Vortex ,Incubator (37, 40 and 60°C), GC and GCMS (as described in earlier chapters)

3.3. Methods

3.3.1. Bacterial growth and media

PLsr-$1_{(W)}$ was grown in MRS broth (1 L) supplemented with lactose (1.5%) as carbon source. Cells were harvested by centrifugation at 8000 rpm for 15 min at 4°C, washed twice with phosphate buffer (0.02 M; pH 7.0) and resuspended in the same.

3.3.2. Estimation of β-galactosidase activity

Extraction of cell free enzyme extract: β-galactosidase activity was determined according to the method of Bhowmik and Marth (1989). One ml of bacterial suspension in buffer was permeabilized with 50 µl of toluene: acetone mixture (1: 9 v/v), vortexed thoroughly and incubated at 37°C for 10 min. Suspension was then centrifuged at 10,000 rpm for 10 min at 4°C to collect the cell free enzyme extract. This enzyme extract was then assayed for β-galactosidase activity.

Estimation of enzyme activity: Cell free enzyme extract (1 ml) was taken in a clean test tube and treated with O-nitrophenyl β-galactopyranoside (1 ml; 0.012 M) at 37°C for 30 min. After the incubation period ice cold sodium carbonate (2 ml; 0.6 M) was added to stop the reaction and absorbance was recorded on a spectrophotometer at 420 nm. Specific activity (µM mg^{-1}) was expressed as the amount of ONP released per mg of protein (Fig. 3.1 and 3.2). Protein was estimated by Lowry's method (Lowry et al., 1951).

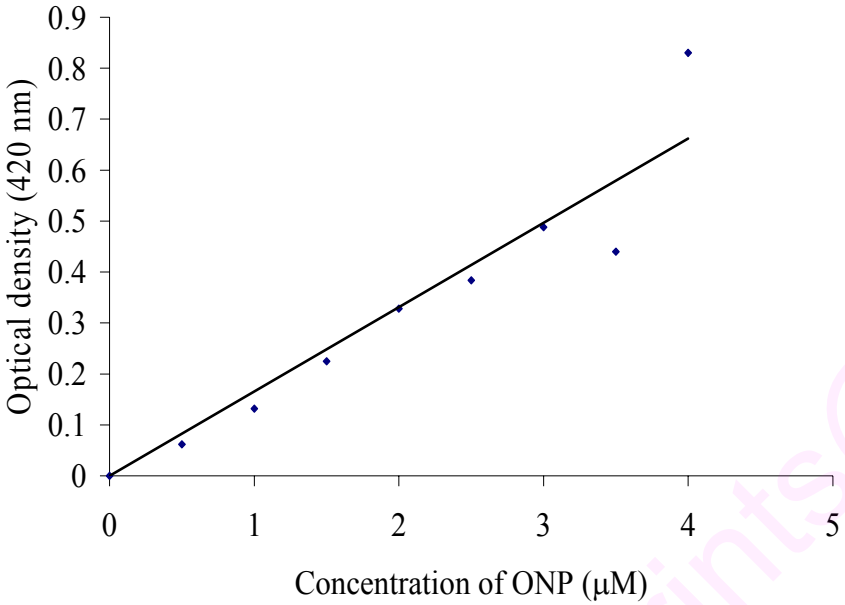


Fig 3.1: Standard graph for O-nitrophenol

Standard graph was prepared by using O-nitrophenol (ONP). Different concentration of ONP solution was prepared (0.5-4.0 µM) and absorbance was measured at 420 nm in a spectrophotometer. The values of absorbance obtained were then plotted against the concentration of ONP to obtain a standard graph

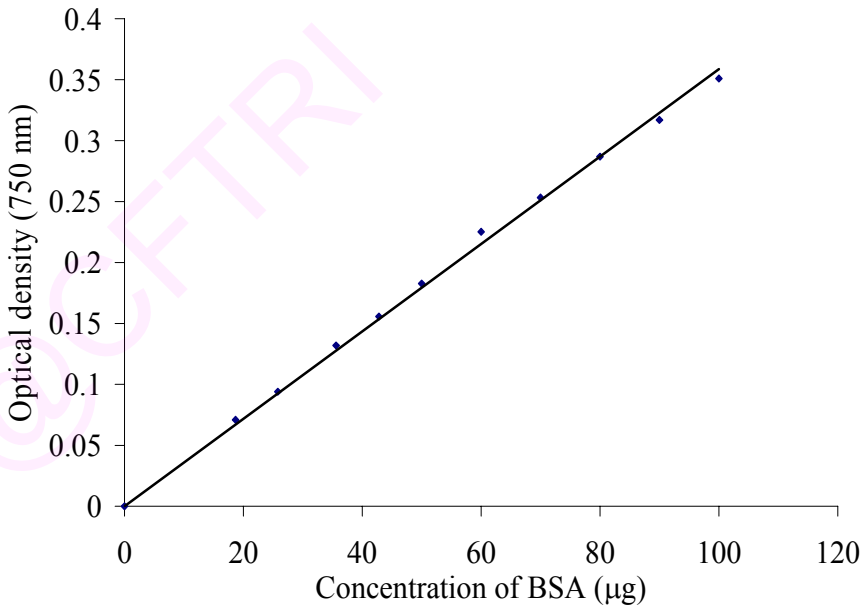


Fig 3.2: Standard graph for protein

Bovin serum albumin (BSA) was used as a standard protein. For preparation of standard curve, same procedure was followed. In place of sample, different concentration of BSA solution was used (10-100µg/ml). The absorbance values (750 nm) were plotted against the concentration of BSA (µg)

3.3.3. Strain improvement for enhanced β-galactosidase activity

Chemical mutation: The bacterial suspension (8 ml) was distributed into 4 equal portions. A stock of 1.17g/L of ethyl methyl sulphonate (EMS) was prepared and added to each portion at a final concentration of 23.4, 46.8 and 70.2 µg/ml respectively (one portion was kept as a control without EMS treatment). Test tubes were shaken well on a shaker at room temperature for 30 min. To this suspension 10% (w/v) of filter sterilized sodium thiosulphate was added and centrifuged at 10000 rpm for 10 min to remove the EMS residues. Further cell pellet was washed thrice and resuspended in phosphate buffer (pH 6.2). The cell suspension was serially diluted and appropriate dilutions were plated on MRSA plates and incubated at 37°C for 24 h. The colony grown was selected, purified and then checked for β-galactosidase activity as described earlier.

$$\text{Relative activity (\%)} = \frac{\text{Activity of mutant strain}}{\text{Activity of parent strain}} \times 100$$

UV mutation: The bacterial suspension (1 ml) was exposed to UV-radiation for different time intervals (5 sec, 30 sec, 1 min, 1.5 min and 2 min). These UV-treated suspensions were plated on MRSA plates, covered with a black cover and incubated at 4°C for 24 h. After incubation, the colonies were taken in saline (1 ml) serially diluted and plated on MRSA media. These plates were incubated at 37°C for 24 h. The developed colonies were selected, purified and assayed for β-galactosidase activity as described earlier. Relative activity was determined as above.

3.3.4. Permeabilization of cell for β-galactosidase enzyme

3.3.4.1. Screening of method for disruption of cell to release maximum β-galactosidase enzyme

(1) Repeated freezing and thawing: The bacterial cell suspension in buffer was frozen at -20°C. The frozen sample was removed from the freezer and

thawed with warm water. This process was repeated three times and then supernatant was assayed for enzyme activity.

(2) Lyophilization: Cell biomass was freeze dried in a lyophilizer programmed to operate for 10 min initial freezing later pressure reduces to 10^{-1} Torr for a period of 24 h. The lyophilized powder was then suspended in Z-buffer and assayed for enzyme activity.

(3) Sonication: Cell biomass suspended in buffer was sonicated under ice and assayed for enzyme.

(4) Homogenization: Cell suspension was subjected to homogenization to disrupt the cells and the enzyme released was estimated in the supernatant.

(5) Acetone powder preparation: Cell biomass collected after centrifugation was washed twice with phosphate buffer and pressed in between whatman No 1 filter paper to remove excess water. The biomass was taken and treated with HPLC grade chilled acetone. Immediately it was filtered and then pellet was dried until acetone is completely evaporated. This acetone powder was then suspended in buffer to estimate the enzyme activity.

3.3.4.2. Optimization of method for disruption to release maximum β-galactosidase enzyme

Sonication: Cell biomass suspended in buffer was sonicated for different time intervals (0.5-5 min) and assayed for enzyme activity.

Homogenization: Cell biomass suspended in buffer was homogenized for 1-2 min and assayed for enzyme activity.

Solvent permeabilization: Cell biomass suspended in buffer was treated with toluene:acetone and toluene: chloroform at varying ratio (1:3 to 1:9) and then assayed for enzyme activity.

3.3.5. Application of the response surface methodology for optimizing the culture condition for maximum β-galactosidase synthesis

Experimental design and analysis: Response surface methodology was used to estimate the effect of temperature, pH and lactose concentration during the growth of the culture for β-galactosidase activity. Central composite design,

face centered with three factors, two responses and five center points were used to evaluate the experimental data (Liong and Shah, 2005). Quadratic model used to describe the response variable was as follows:

$Y = b_0 + b_1X_1 + b_2X_1^2 + b_3X_2 + b_3X_2^2 + b_4X_3 + b_5X_3^2 + b_6X_1X_2 + b_7X_1X_3 + b_8X_2X_3$

Where Y = response (dependent variable)

X_1 = lactose levels

X_2 = temperature conditions

X_3 = pH values with b_0, b_1,……..b_8 as the regression co-efficients.

The above model was used to optimize the values of independent parameters for the response. The CCRD of three independent variables with five levels of each was chosen to design matrix.

3.3.6. Ammonium sulphate precipitation of β-galactosidase

After optimization of physical and chemical parameter for maximum enzyme release the enzyme extract in supernatant was collected after centrifugation at 10,000 rpm for 15 min at 4°C (All the steps were carried out at 4°C). This cell free enzyme extract was precipitated by ammonium sulphate at 30-60% (w/v) saturation according to the method of Bhowmik and Marth (1989). The enzyme precipitate was collected after centrifugation and was dissolved in Z-buffer. This was then dialyzed against Z- buffer for 24 h with several changes of liquid to remove the sulphate salts.

Analysis on SDS PAGE: Electrophoresis was carried out according to the method of Laemmli (1970) using 12% running gel and 4% stacking gel. Protein was stained with coomassie blue. On destaining the protein bands were observed and photograph using gel documentation.

3.3.7. Stability of the enzyme

(a) Effect of pH and temperature: Three buffer systems, citrate buffer (0.1 M; pH 4.0–6.0), sodium phosphate buffer (0.2 M; pH 7.0-8.0), and glycine-NaOH buffer (0.2 M; pH 9.0–10.0) were used to study the effect of pH on

enzyme activity. The enzyme was preincubated in different buffers at room temperature for 1 h and then checked for the activity.

Thermal stability was estimated by incubating the enzyme at different temperature (20-50°C). The residual activity was then measured as described earlier under standard assay conditions.

(b) Effect of substrate and enzyme concentration: Enzyme was treated with different concentration of ONPG (2-50 μM) and then assayed for the activity. To determine the effect of enzyme concentration, varying concentration of enzyme (25-500 mg protein) was incubated with ONPG (0.012 M) and assayed for enzyme activity.

(c) Effects of inhibitors and activators: Enzyme samples were incubated with different modulators like EDTA, $HgCl_2$, 1,10-phenothroline and ascorbic acid at varying concentrations (0.1-10.0 mM). Enzyme activity without modulators was used as control. The effect of metal ions was determined by incubating the enzyme with $CaCl_2$, $MgSO_4$, $MnSO_4$ and $ZnSO_4$ at a concentration of 0.1-10.0 mM each. The treated sample were then incubated with ONPG (0.012 M) and then assayed for enzyme activity as described earlier. Enzyme activity measured without added cations was used as control.

3.4. Results and discussion

3.4.1. Estimation of β-galactosidase activity

β-galactosidase enzyme has been found in numerous biological systems. Among these special attention has been paid to LAB because of GRAS status (Stiles and Holzapfel, 1997). In the present study, the production of β-galactosidase by a probiotic lactic acid bacteria *L. mesenteroides* (PLsr-$1_{(W)}$) was studied.

3.4.2. Strain improvement for enhanced β-galactosidase activity

Deficiency of lactase enzyme causes lactose intolerance problem, which is of great concern. Today, consumers are aware of many side effects caused by medicines and are looking forward for natural means to cure

diseases. Preparation of lactose treated dairy foods and improvement in enzyme production and hydrolysis reaction can lead to improved lactose digestion.

To alleviate the symptoms of lactose intolerance, it is important to select cultures capable of producing large amounts of *β*-galactosidase. Although gene over expression technique is established it is not presently applicable to most probiotic cultures because of the lack of knowledge and tools for genetic manipulation (Ibrahim and O'Sullivan, 2000). Method of recombinant DNA technology by mutagenesis offers powerful approach for manipulating strains for optimal *β*-galactosidase production and it is a good approach to obtain overproducing mutants. In the present work, the classical mutagenesis is used for increasing *β*-galactosidase production to improve its potential in treating lactose intolerance.

Chemical mutation

Chemical mutation of culture (PLSR-$1_{(W)}$) using ethyl methane sulfonate (EMS) shows a reduction in cell count with increase in the mutagen concentration. There was a decrease in 4 logs compared to control. A total of 30 mutants were isolated and purified on MRSA plate by repeated streaking. These were then analyzed for *β*-galactosidase activity by qualitative method using ONPG disc. The mutants with high *β*-galactosidase were tested quantitatively using ONPG as substrate by standard procedure as described earlier. The mutant strains showed *β*-galactosidase activities ranging from 6.5-13.1μM mg^{-1}. Five mutants showed high inducible level of *β*-galactosidase (>10 μM mg^{-1}). The best mutant strain coded as M3-PLsr-$1_{(W)}$ exhibited 1.75 folds higher level of *β*-galactosidase than the control (PLsr-$1_{(W)}$) (Table 3.1).

Different chemical mutagens are used for different purposes. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and EMS was used as chemical mutagen by Ibrahim and O'Sullivan (2000) to enhance

β-galactosidase synthesis in *Bifidobacterium sp, L. delbruckii* and *S. thermophilus*. Chemical mutagenesis with hydroxylamine or methoxylamine was performed on *E. coli* expression vector gene for enhanced production of *β*-galactosidase. Rasouli and Kulkarni (1994) subjected *Aspergillus niger* to mutagenesis with MNNG to scale up the production of *β*-galactosidase and found 28% increased enzyme activity. In the present study the *β*-galactosidase activity of EMS mutant strain (M3- PLsr-$1_{(W)}$) was increased by 74.31% activity which was higher than reported by Yu et al. (1986) wherein an increase of 40.9% *β*-galactosidase activity in *L. sporogenes* was found compared to parent strain with MNNG chemical.

UV-mutation

UV-irradiation of the culture shows a decrease in the survival rate as the duration of exposure increases (Table 3.2). There was a reduction in viable cells (1-3 logs). A maximum survival was observed when the cells were exposed for 5 sec (10×10^8). On 2 min exposure there was a significant reduction in the cell count of the culture (53×10^5). A total of 50 mutants were isolated and tested for *β*-galactosidase production using ONPG disc. The positive strains were then checked for enhanced *β*-galactosidase production using ONPG as a substrate. The *β*-galactosidase activity in mutant strains ranged from 7-15 μM mg^{-1}. Maximum enzyme activity (15.18 μM mg^{-1}) was observed when the culture was exposed to 1 min. Hence this strain that was **coded as M7-Plsr-$1_{(W)}$** was taken for all further studies. The mutant strain was checked every 15 days for its stability and *β*-galactosidase activity. According to the results enzyme activity did not vary even after 15 months of storage and was stable.

Table 3.1 : Strain improvement by chemical mutation using EMS

Treatment (μg/ml)	Culture code	Colony count (Cfu/ml)	No of colonies selected	Specific activity (μM mg^{-1})
Control	PLsr-$1_{(W)}$	48×10^8	NA	7.550 ± 0.11
23.4	M1- PLsr-$1_{(W)}$	40×10^4	7	10.23 ± 0.12
46.8	M2- PLsr-$1_{(W)}$	20×10^4	8	13.16 ± 0.06
70.2	M3- PLsr-$1_{(W)}$	10×10^4	15	8.225 ± 0.15

*Values are mean±SD. NA- not applicable

Table 3.2 : Strain improvement by UV-irradiation

Treatment (time)	Culture code	Colony count (Cfu/ml)	No of colonies selected	Specific activity (μM mg^{-1})
Control	M4- PLsr-$1_{(W)}$	14×10^8	NA	07.527 ± 0.23
5 sec	M5- PLsr-$1_{(W)}$	10×10^8	7	07.647 ± 0.24
30 sec	M6- PLsr-$1_{(W)}$	10×10^7	16	10.180 ± 0.19
1 min	M7- PLsr-$1_{(W)}$	10×10^7	10	15.182 ± 1.00
1.5 min	M8- PLsr-$1_{(W)}$	10×10^6	12	14.328 ± 0.10
2 min	M9- PLsr-$1_{(W)}$	53×10^5	5	13.877 ± 0.11

*Values are mean±SD. NA- not applicable

3.4.3. Permeabilization of cell for β-galactosidase

3.4.3.1. Screening of method for disruption of cell to release maximum β-galactosidase enzyme

As β-galactosidase is an intra cellular enzyme it is difficult to release the active enzyme from the cell. Different methods are therefore applied to increase cell permeability. Techniques of cell disruption like sonication, bead milling and high pressure homogenization have been used (Bury et al., 2001). Detergents such as digitonin and cetyltrimethylammonium bromide (CTAB) have been successfully employed in yeast cells (Joshi et al., 1989; Gowda et al., 1988; Bhat et al., 1993; Bachhawat et al., 1996). Pearmeabilization with oxgall and triton X-100 have also displayed higher levels of enzyme activity on *Streptococcus* cells (Somkuti and Steinberg, 1994). Among the different

solvents chloroform, ethanol and toluene are found to be effective (Siso et al., 1992; Flores et al., 1994; Gonzalez-Siso and Suarez-Doval, 1994; Panesar, 2004).

In the present study different techniques like freezing/ thawing, sonication, lyophilization, homogenization, solvent permeabilization and acetone powder preparation have been employed to release *β*-galactosidase of M7-PLsr-$1_{(W)}$. Activity by solvent permeabilization using acetone was maximum (19.03 μMmg^{-1}) when compared to all other techniques employed (Table 3.3).

Table 3.3: *β*-galactosidase activity on permeabilization of M7-PLsr-$1_{(W)}$

Method of permeabilization	**Specific activity (μM mg^{-1})**
Mechanical	
Freezing and Thawing	11.87 ± 0.3
Homogenization	12.26 ± 0.7
Lyophilization	04.87 ± 1.1
Sonication	12.19 ± 1.3
Chemicals-Solvent	
Acetone	19.05 ± 0.6
Chloroform	17.59 ± 1.4
Toluene	18.29 ± 1.0
Enzyme- Lysozyme	12.04 ± 2.0
Detergent	
Triton X-100	10.39 ± 0.9
EDTA	04.21 ± 0.5

3.4.3.2. Optimization of method for disruption to release maximum β-galactosidase enzyme

On employing sonication an activity of 12.19 μM mg^{-1} was observed. With increase in sonication time, a steady increase of activity was observed with maximum (23.6 μM mg^{-1}) at 2 min, after which a decline in the activity was observed (Fig. 3.3). Activity by solvent permeabilization using acetone

was maximum after 60 min of incubation (24.07 μM mg^{-1}). Incubating for a longer time had no significant ($p>0.05$) difference in the activity (Fig. 3.4).

Homogenization of acetone powder resulted in 25.6 μM mg^{-1} activity. Application of solvents (toluene, acetone and chloroform) as permeabilizing agent to acetone cell powder increased the enzyme release (Fig. 3.5). Use of toluene and acetone (1:6 v/v) to disrupt acetone cell powder exhibited maximum release (29.59 μM mg^{-1}). Therefore further experimental steps were carried out with enzyme extract obtained by this method.

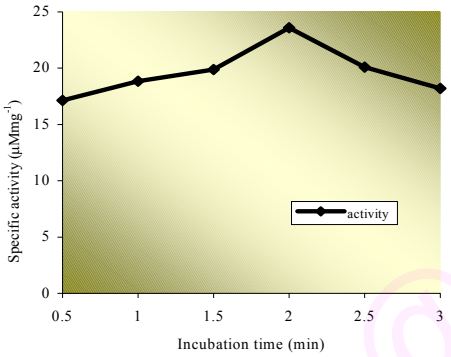


Fig 3.3: Effect of sonication on enzyme release

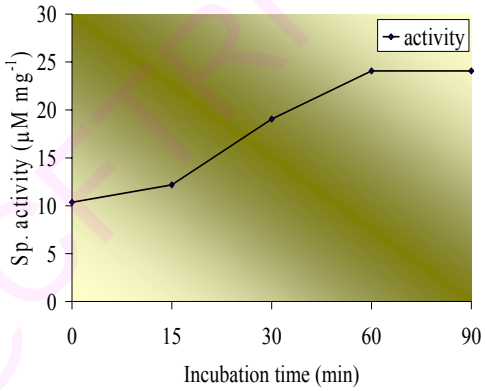


Fig 3.4: Effect of incubation time on release of enzyme from cellular acetone powder

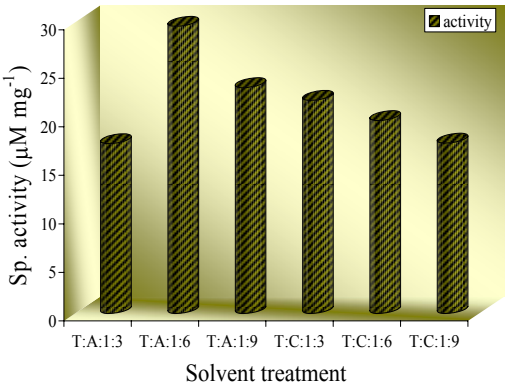


Fig 3.5: Effect of solvents on enzyme release

3.4.4. Application of the response surface methodology for optimizing the culture condition for maximum β-galactosidase activity

The aim of the present study was to optimize the fermentation conditions of mutant strain of *L. mesenteroides* (M7-PLsr-$1_{(W)}$). This statistically designed, multifactorial experiment offers an additional method for potential enhancement of β-galactosidase activity. The response surface methodology (RSM) which includes factorial designs and regression analysis is known to effectively deal with technological optimization studies (Logothetis and Wynn, 1989). Microorganisms differ in their optimum condition for enzyme production. So it is necessary to find methods that are less expensive with higher productivity of the enzyme. Stability of enzyme is an important aspect in biotechnological process that could help in optimization of economic profitability of enzymatic production. The activity of enzyme is influenced by diverse environmental factors like pH, temperature and substrate concentration that strongly affect the spatial confirmation of the protein (Sadana and Henley, 1986; Juradol et al., 2004).

RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effective factors and most importantly, searching for the optimum conditions of factors for desirable response (Montgomery, 1991). In this approach, the physical and chemical parameters (temperature, pH and lactose concentration) are varied in the growth condition of culture isolate to determine the optimum parameter for maximum β-galactosidase activity.

The response quadratic model was obtained as follows with R2 as 91.4%

$$Y = 184.58-21.01X_1 + 6.25X_1^2 + 2.94X_2 + 0.01X_2^2 - 60.38X_3 + 4.65X_3^2 +4.65X_3^2 - 0.57X_1X_2 + 3.64X_1X_3 - 0.38X_2X_3$$

Where Y = Specific activity (μM/ 100 mg protein)

X_1 = Lactose concentration (%)

X_2 = Temperature (°C)

X_3 = pH

The analysis of variance was calculated to check how well the model represented the data and it was found that F value for lactose, temperature and

pH were highly significant (P<0.001). It was concluded that the selected model adequately represented the data for the three parameters. The above model was used to draw the contour plots between the different independent parameters to investigate their effect on the specific activity (Table 3.4).

According to the results obtained, the maximum activity was observed (3.92 μM mg^{-1}) with 7.50 pH MRS broth supplemented with 1.25% lactose (Fig.3.6).

Table 3.4 : Effect of lactose (%), temperature(°C) and pH on β-galactosidase activity in M7-PLsr-1$_{(W)}$

Lactose (%)	Temperature (^{o}C)	pH	Specific activity (μM/ 100 mg protein)
1.125	37.5	7.25	607.6
1.125	37.5	7.75	728.1
1.125	52.5	7.25	565.1
1.125	52.5	7.75	406.4
1.125	37.5	7.25	609.8
1.125	37.5	7.75	719.5
1.125	52.5	7.25	574.1
1.125	52.5	7.75	261.6
1.375	37.5	7.25	60.4
1.375	37.5	7.75	61.4
1.375	52.5	7.25	1617.4
1.375	52.5	7.75	3911.8
1.375	37.5	7.25	614.2
1.375	37.5	7.75	601.0
1.375	52.5	7.25	1801.3
1.375	52.5	7.75	3536.0
1.000	45.0	7.50	1766.6
2.000	45.0	7.50	1385.1
1.250	45.0	7.50	151.75
1.250	45.0	7.50	573.16
1.250	30.0	7.50	383.85
1.250	60.0	7.50	3920.0
1.250	45.0	7.00	2648.2
1.250	45.0	8.00	463.80
1.250	45.0	7.50	549.33
1.250	45.0	7.50	548.40
1.250	45.0	7.50	545.40
1.250	45.0	7.50	558.80
1.250	45.0	7.50	557.50
1.250	45.0	7.50	551.60
1.250	45.0	7.50	550.90
1.250	45.0	7.50	566.40

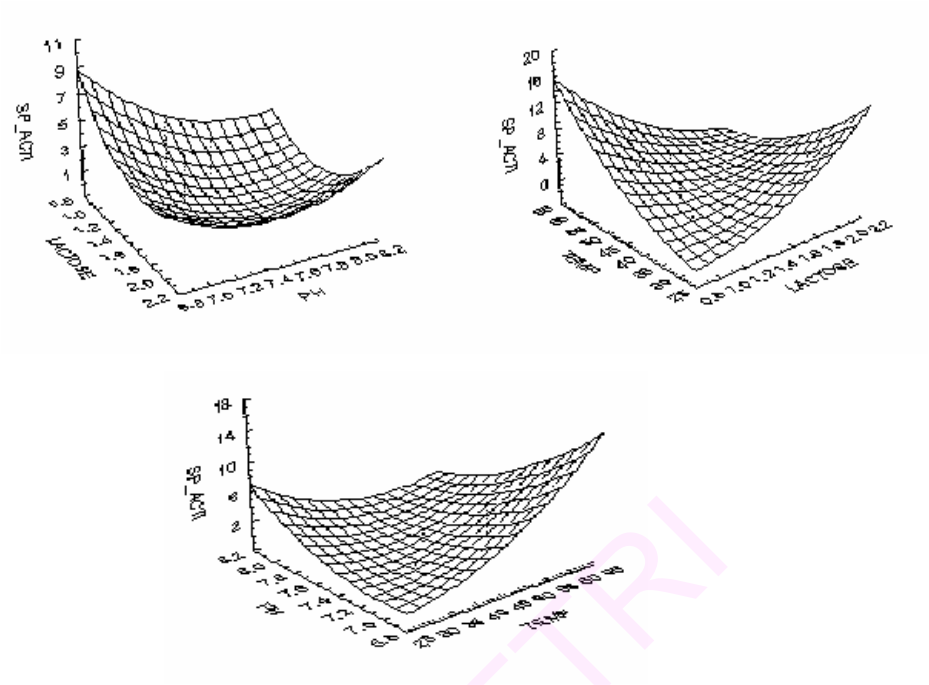


Fig 3.6 : RSM study to optimize the condition for maximum β-galactosidase activity in M7-PLsr-1$_{(W)}$

3.4.5. Ammonium sulphate precipitation of β-galactosidase

In the present study ammonium sulphate precipitation of β-galactosidase from M7-PLsr-1$_{(W)}$ was carried out and studied for its stability under different pH, temperature and substrate concentration.

***Ammonium sulphate precipitation of β-galactosidase*:** The crude enzyme extract obtained on permeabilization with toluene and acetone (1:6) was precipitated using ammonium sulphate at different saturation point (30-90%). The precipitate obtained was dialyzed against Z-buffer. According to results obtained β-galactosidase activity was 187.78 μM mg^{-1} which was 25.04 folds higher as compared to crude extract (Table 3.5). The β-galactosidase after ammonium sulphate precipitation was run for SDS-PAGE which showed a prominent band at 66 KD protein (Fig. 3.7).

Table 3.5 : Enhancement of β-galactosidase production

Steps	Specific activity (μM mg^{-1})	Activity increase (folds)
Crude extract	7.5	1.00
Strain improved (UV-mutant)	15.18	2.03
Acetone powder/T:A permeabilization	29.59	3.94
RSM	39.20	5.23
Ammonium precipitation	187.78	25.04

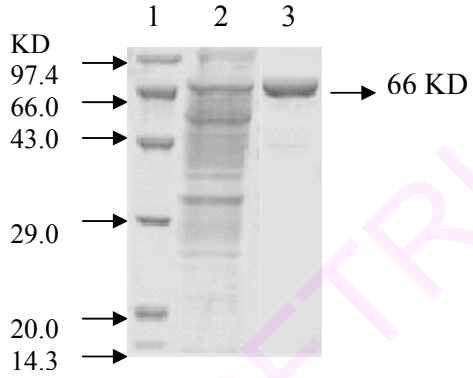


Fig 3.7 : SDS PAGE analysis of β-galactosidase enzyme in M7-PLsr-$1_{(W)}$.

Lane 1: Protein marker, Lane 2: Crude extract, Lane 3: Fraction of Ammonium sulphate precipitated enzyme with a molecular weight of 66 KD.

Effect of pH and temperature: The optimal pH of β-galactosidase was determined to be pH 8.0 (Table 3.6). Preincubation of the enzyme in different buffers (pH 4.0–10.0) had significant ($p<0.05$) effect on enzyme activity. The enzyme activity was 7.85 μM mg^{-1} at low pH (4.0) and maximum (138.86 μM mg^{-1}) at pH 8.0. Optimum temperature for the activity was 20°C (Table 3.7). The enzyme activity was 135.78 μM mg^{-1} at 20°C, whereas only about 7.72 μM mg^{-1} at 50°C.

Table 3.6 : Effect of pH on the β-galactosidase activity of M7-PLsr-$1_{(W)}$

pH	Specific activity (μM mg^{-1})
4.0	007.85 ± 0.12
5.0	013.33 ± 1.20
6.0	116.94 ± 1.11
7.0	122.04 ± 0.02
8.0	138.86 ± 0.31
9.0	126.39 ± 0.15
10.0	013.18 ± 0.02

*Values are mean ± SD (n=3). Initial activity of the enzyme was 187.78 μM mg^{-1}

Table 3.7 : Effect of temperature on the β-galactosidase activity of M7-PLsr-1$_{(W)}$

Temperature (^{o}C)	Specific activity (μM mg^{-1})
20	135.78 ± 1.10
30	123.39 ± 0.21
40	037.59 ± 0.11
50	007.72 ± 0.11

* Values are mean ± SD (n=3). Initial activity of the enzyme was 187.78 μM mg^{-1}

Effect of substrate and enzyme concentration: The enzyme activity increased with the increase of substrate (ONPG) concentration reaching a maximum with 30 μM ONPG. A decline in the enzyme activity was observed with further increase in ONPG concentration (Fig. 3.8). Similarly with increase in enzyme concentration the activity increased reaching a saturation level at 250 μg of enzyme (Fig. 3.9).

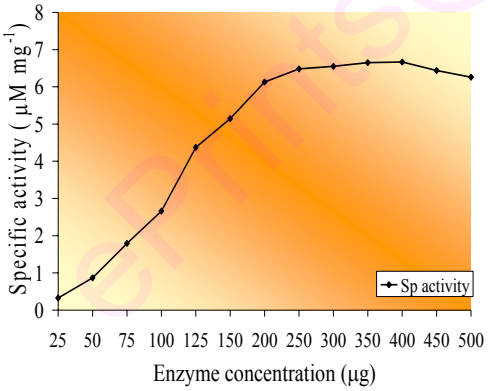


Fig 3.8: Effect of substrate (ONPG) concentration

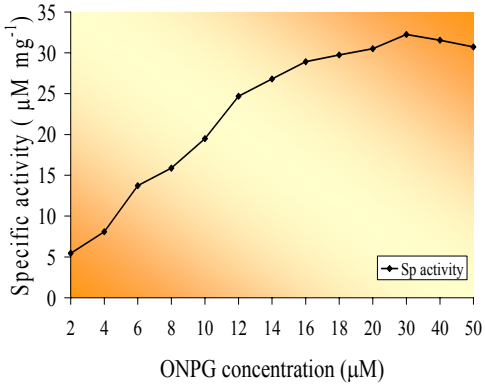


Fig 3.9: Effect of enzyme concentration

Effect of enzyme modulators: The enzyme activity was significantly altered with increasing concentration of EDTA, $HgCl_2$, 1,10-phenanthroline and ascorbic acid. Even at 10 mM concentration of 1,10-phenanthroline, the activity was retained to 81.27% whereas with EDTA and $HgCl_2$ the activity was reduced to 9.54 and 15.24% (Fig. 3.10).

The enzyme was significantly ($P<0.05$) activated by Mn^{2+}. The divalent cations tested markedly inhibited the enzyme activity with increase in concentration to 5 -10 mM except Mn^{2+} wherein relative activity was retained. In the presence of 10 mM Mn^{2+} the relative activity was 157.26% (Fig. 3.11).

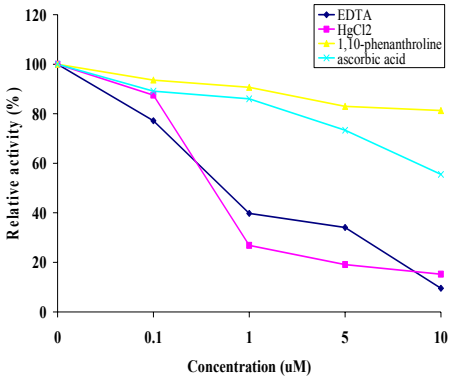


Fig 3.10: Effect of enzyme inhibitors/ modulators on enzyme activity

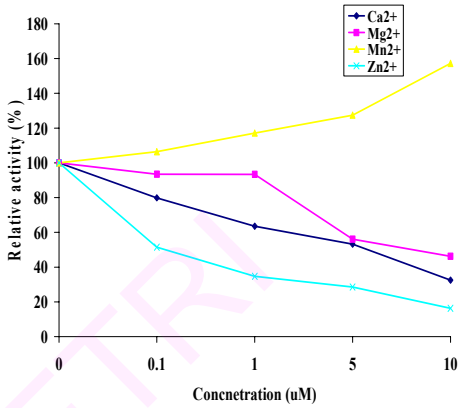


Fig 3.11: Effect of metal ions on enzyme activity

The M7-PLsr-$1_{(W)}$ β-galactosidase was purified to a specific activity of 187.78 µM mg^{-1}, a value greater than that reported for β-galactosidase from *B. longum* CCRC (168.6 µM mg^{-1}; Hsu et al., 2005) and *B. bifidum* (49.8 µM mg^{-1}; Itoh et al., 1980). β-galactosidase activity in the present culture was maximum at pH 8.0 (138.86 µM mg^{-1}). Hung and Lee (2002) have shown maximum β-galactosidase activity at pH 7.5 in *B. infantis*. In another study, Batra et al. (2002) revealed optimum pH 6.0-7.0 in *B. coagulans*. Similar to the present study, Greenberg and Mahoney (1982) have shown optimum pH 8.0 in *S. thermophilus*.

In general, the temperature optimum of β-galactosidase for mesophilic bacteria is found to be 45°C (Nader de Macias et al., 1983; Bhowmik and Marth, 1990). Some reports on thermophilic bacteria shows optimum activity at 55-57^{0}C (Itoh et al., 1980; Hemme et al., 1980). According to Huang et al. (1995) temperature optimum for β-galactosidase was more active at 20^{0}C.

Results obtained in the present study demonstrated that after ammonium sulpahte precipitation the specific activity of *β*-galactosidase increased from 39.2 to 187.78 μM mg^{-1}.

3.5. Conclusion

The probiotic *L. mesenteroides* PLsr-$1_{(W)}$ was improved for *β*-galactosidase by chemical mutagen and UV radiation. The result indicates an increase in enzyme activity in the UV-mutant (M7-PLsr-1) and was taken for all further studies. Conditions for maximum release of enzyme were optimized by different permeabilizing techniques. RSM study revealed the optimum condition for physical and chemical parameter for maximum activity. According to the study maximum activity (39.2 μM mg^{-1}) was observed with the culture grown at 7.50 pH in MRS broth supplemented with 1.25% lactose. Further on ammonium sulphate precipitation the activity increased to 187.78 μM mg^{-1} which was 25 folds higher as compared to the crude extract.

Chapter 4

Enhancement of Culture Shelf Life on Storage

CHAPTER – 4

ENHANCEMENT OF CULTURE SHELF LIFE ON STORAGE

ABSTRACT

Aim of the present work was to preserve the selected culture (M7-PLsr-$1_{(W)}$) for longer shelf life. In this regard, the culture was subjected to different drying methods like spray, vacuum, oven and freeze drying for maximal survival. The results show maximum stability to freeze drying. The content of saturated and unsaturated membrane fatty acid was increased on freeze drying. SDS PAGE analysis of cellular protein showed a prominent protein band at 22.9 and 20.1 KD after freeze drying. Further viability and resistance of the culture to freeze drying was enhanced with supplementation of different adjuvants like polyethylene glycol, lactose and sucrose. Sucrose supplementation enhanced the survival rate of the culture to 72.26% in comparison to control (52.55%). Viability of the freeze dried culture during storage was studied at 30, 4 and -20°C for a period of 6 months. Data determines that the storage temperature of -20°C was optimum for maintaining maximum viability. Even after six months of storage the culture retained its probiotic properties like antimicrobial activity, resistance to low pH and high bile salt concentration. The study shows the importance of cryoprotectants in enhancing the viability and beneficial attributes of culture during storage.

4.1. Introduction

LAB are commonly used in the production of cheese, yogurt, dry sausages, wine, bread and sauerkraut. They contribute to the formation of organoleptic and rheological characteristics of these products and inhibit the growth of undesirable bacteria (Coppola et al., 1998; Caplice and Fitzgerald, 1999). These cultures are added directly to the food matrix either in frozen or dried form. In addition many commercially dried culture products are available in the form of tablets and pharmaceutical preparations to treat large number of diseases and to maintain normal intestinal flora (Shah, 2000).

Interest in ready to use cultures for direct inoculation has placed greater emphasis on starter cultures and preservation methods that promote high cell viability and activity (Broadbent and Lin, 1999). LAB may be preserved and distributed in liquid, spray dried, frozen or lyophilized forms. Spray drying produces dry granulated powder by atomizing the wet product at high velocity within a chamber (Desmond et al., 2002; Corcoran et al., 2004). Freeze drying or lyophilization is a process in which solution of a substance is frozen and then the quantity of water is reduced by sublimation. Further it is subjected to desorption to a value where it will no longer support biological activity or chemical reaction (Jennings, 1990). Microbial survival during these preservation processes depend on many factors like growth conditions (Palmfeldt and Hahn-Hagerdal, 2000), protective medium (Hubalek, 1996; Linder et al., 1997; Fernandez-Murga et al., 1998; Abadias et al., 2001), initial cell concentration (Bozoglu et al., 1987; Costa et al., 2000), freezing temperature (Sanders et al., 1999) and rehydration conditions (Theunissen et al., 1993). Loss in the cell viability can be related to the destruction of cell components including cell membrane, cell wall and DNA (Teixeira et al., 1995; Ray et al., 1971). During the above drying methods the bacterial cultures are also subjected to adverse conditions, such as water crystallization and low temperature, producing a degree of protein denaturation and bacterial membrane injury which consequently decreases the culture viability (Visick and Clark, 1995).

To prevent or reduce all these adverse effects many substances are used as cryoprotectants (Carcoba and Rodriguez, 2000). A good protectant is the one that can be easily dried and gives a good matrix for culture stability (Zhao and Zhang, 2005). These cryoprotectants may be either simple or complex chemical compounds like quickly penetrating compounds (alcohols, amides etc), slowly penetrating compounds (glycerols, triols etc) or non penetrating compounds (mono, di, oligosaccharides, polyalcohols, sugar, proteins etc (Hubalck, 2003). Many compounds have been shown to increase the survival of LAB during freeze drying and the beneficial effect is related to the proteins and membranes of microorganisms (DeValdez et al., 1983; Wiemken, 1990; Leslie et al., 1995).

In the present work freeze drying, spray drying, vacuum drying and oven drying methods were used for the preservation of culture. Stability and viability of the culture was further enhanced on supplementation of different cryoprotectants. The viability of culture during storage at 3 different temperatures (30°, 4° and -20°C) was studied for 6 months after freeze drying. Antimicrobial activity, tolerance to low pH and high bile salt concentration was checked during storage.

4.2. Materials

Chemicals:

1) MRS broth, Bile salt mix, pH 2.0 buffer (as described in chapter 1).
2) Solvents: methanol, chloroform, hexane (HPLC grade: SLR Company, India).
3) Filter sterilized cryoprotectants: polyethylene glycol (PEG), lactose and sucrose (HiMedia Pvt. Ltd, Mumbai, India).

Equipments:

1) Spray drier (Bowen Engineering, Inc, Somerville, New Jersey, USA), Vacuum drier (FJ Stoker Machine Company, Philadelphia, USA), hot air oven (Labline, India) and lyophilizer (Edwards, Freeze drier).
2) Scanning electron microscope (Leo-435 VP, Leo electron microscope, Zeiss ltd, Cambridge, UK).
3) Centrifuge, GC and GCMS (as described in chapter 2).

4.3. Methods

4.3.1. Bacterial culture and growth

M7-PLsr-$1_{(W)}$ was grown in MRS broth (1 L) at 37°C for 16 h. Cell biomass was harvested by centrifugation (10,000 rpm for 15 min) and washed twice in saline (0.8% NaCl). The cell biomass was then suspended in 10 ml of sterile skim milk medium (10% skim milk powder, 0.5% yeast extract and 0.5% glucose).

4.3.2. Different drying methods for maximal survivalibility of the culture

4.3.2.1. Freeze drying of culture

For freeze drying, the culture in skim milk (1g/10 ml) was initially frozen at -50°C in a freezer for 24 h and then subjected to freeze drying using a lyophilizer. The freeze dryer was programmed to operate for initial freezing (10 min) and after the internal pressure is reduced to 10^{-1} Torr, freeze drying was carried out for 48 h. The temperature was maintained at -60°C. After freeze drying samples were stored in a sterile dry glass container properly stoppered until use. Experiments were carried out in triplicate. Stability of the culture was evaluated by determining the bacterial count before and after freeze drying.

4.3.2.2. Spray drying

Sample of culture in skim milk was spray-dried by using a Spray Dryer, the process parameters being as follows: inlet temperature 170°C; outlet temperature 80–85°C; product input flow 500 ml/h. Experiments were carried out in triplicate. Stability of the culture was evaluated by determining the bacterial count before and after spray drying.

4.3.2.3 Vacuum drying

Sample of culture in skim milk was vacuum dried using vacuum drier. Experiments were carried out in triplicate. Stability of the culture was evaluated by determining the bacterial count before and after vacuum drying.

4.3.2.4. Oven drying

Sample of culture in skim milk was oven dried by using a hot air oven at 80^0C until complete evaporation of moisture. Experiments were carried out in triplicate. Stability of the culture was evaluated by determining the bacterial count before and after oven drying.

4.3.3. Enhancement of culture stability during freeze drying using cryoprotectants

Cell biomass of M7-PLsr-$1_{(W)}$ (4 g) was suspended in skim milk (40 ml) and was distributed into four equal parts. One was kept as control (without cryoprotectant) and others were supplemented with filter sterilized sucrose, lactose and polyethylene glycol separately into each fraction. The cell suspension was then aseptically poured into large petriplate, sealed with aluminium foil and then subjected for freeze drying (as described in section 4.3.2.1).

4.3.4. Cellular fatty acid analysis of freeze dried culture

Cellular fatty acids of M7-PLsr-$1_{(W)}$ before and after freeze drying (100 mg each) were extracted according to the method of Bligh and Dyer (1959) and analyzed on GC and GCMS (as described in chapter 2).

4.3.5. Cellular protein analysis by polyacrylamide gel electrophoresis (SDS-PAGE) of freeze dried culture

Cellular protein of fresh cells (cells harvested prior to freeze drying) and freeze dried cells were analyzed on SDS-PAGE by the method of Laemmli (1970) as described in chapter 2.

4.3.6. Culture viability on shelf storage

Viability of culture during storage was evaluated by determining the viable cell count of freeze dried culture incubated at 3 different temperatures (30, 4 and -20°C). Freeze dried sample (100 mg) was rehydrated (1:1) with

sterile saline (0.8% NaCl), serially diluted and plated on MRSA plates. The plates were incubated at 37°C for 24 h. The colonies grown were counted and expressed as cfu/ml. Survival was determined as the viable cell counts obtained during storage in comparison with the viable counts obtained immediately after freeze drying.

4.3.7. Probiotic properties of freeze dried culture M7-PLsr-1$_{(W)}$

4.3.7.1. Tolerance to low pH and high bile salt

Cell viability at pH 2.0 and high bile (4%) was checked every month by suspending (100 mg) freeze dried sample in 1 ml of pH 2.0 buffer (0.2 M HCl-glycine buffer) and 4% bile salt mix separately. The suspension was serially diluted, plated on MRSA and incubated at 37°C. The colonies grown were counted and expressed as cfu/ml.

4.3.7.2. Antimicrobial activity of freeze dried culture

Freeze dried culture (100 mg) stored at -20^{0}C was rehydrated at regular intervals and analyzed for antimicrobial activity against 6 food borne pathogens *E. coli, S. typhi, S. dysenteriae, P. aeroginosa, V. cholerae* and *S. aureus* by the method of agar well diffusion assay (Perez et al., 1990).

4.3.8. Morphological study by scanning electron microscopy

The fresh cells and freeze dried samples were washed twice with phosphate buffer (pH 6.5) and immersed in glutaraldehyde (1%) for 24 h at 4^{0}C. After fixing the cells with glutaraldehyde, the fixative was removed by washing thrice with phosphate buffer (pH 6.5). The cells were subjected to drying in increasing concentration of ethanol (0, 50, 70, 80, 90, 95 and 100%). After complete evaporation of ethanol, cells were mounted on carbon stumps and coated with gold I sputterin device and examined under a scanning electron microscope.

4.4. Results and Discussion

4.4.1. Different drying methods for maximal survivalibility of the culture

In the present study the culture (M7-PLsr-$1_{(W)}$) was subjected to different dehydration techniques for a longer shelf life. According to the results obtained (Fig. 4.1), the culture retained maximum viability on freeze drying (52.55%) followed by spray drying (43.06%), vacuum drying (22.29%) and the least viability was observed in oven drying (6.025%). Selmer-Olsen et al. (1999) has described freeze drying as the most satisfactory method for the long term preservation. Freeze drying preparations exhibit advantages relative to preparations made with other techniques in terms of long term preservation, coupled with convenience in handling, storage, marketing and application (Carvalho et al., 2004).

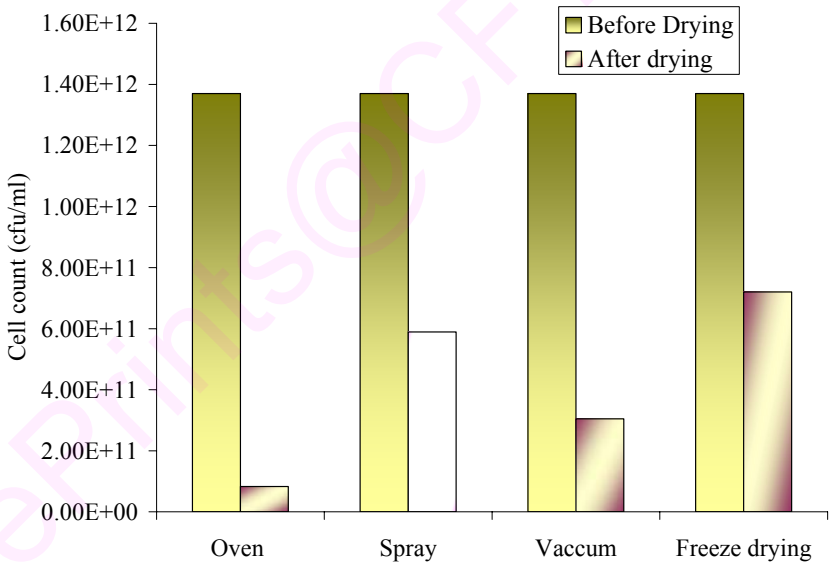


Fig 4.1 : Comparison of preservative methods for stability of M7-PLsr-$1_{(W)}$

Freeze drying was found to be the best method for maintaining the viability of the culture. The stability of freeze dried cultures is known to be dependent on the storage condition (Abadias et al., 2001). In a study Wang et al. (2004) observed that *Bifidobacteria* exhibited 46.2-75.1% survival rate after freeze drying. In the present study M7-PLsr-$1_{(W)}$ exhibited 52.55% survival on freeze drying whereas *S. thermophilus*, the dairy culture showed 49.63% survival (Table 4.1). *L. mesenteroides* M7-PLsr-$1_{(W)}$ exhibited better survival than *S. thermophilus*.

Table 4.1 : Viability of the culture *L. mesenteroides* M7-PLsr-1$_{(W)}$ on freeze drying

Culture	Colony count cfu/ml)		Survival (%)
	Before freeze drying	After freeze drying	
Lsr-1$_{(W)}$	1.37×10^{12} (±0.05)	7.2×10^{11} (±0.08)	52.55
S. thermophilus	1.37×10^{12} (±0.05)	6.8×10^{11} (±0.09)	49.63

*Dairy starter *S. thermophilus* has been used as a positive control
* ±SD- Results are average of three experiments (n=3)
*PEG - polyethyleneglycol

4.4.2. Enhancement of culture stability during freeze drying using cryoprotectants

Previous studies have demonstrated that some of the non-reducing disaccharides such as sucrose and trehalose can be used as cryopreservatives to enhance the viability of the microorganisms (Chavarri et al., 1988). They protect the plasma membrane and protein functionality (Crowe et al., 1988; Leslie et al., 1995). In the present work, three cryopreservatives sucrose, lactose and PEG were used to study their effect in enhancing the stability of culture to freeze drying. To the culture in skim milk filter sterilized cryoprotectants were added at varying concentrations: sucrose (2-8%), lactose (2-8%) and PEG (0.5-2.0%) to determine the optimum concentration of each protectant for maximal survival of the culture. According to the data obtained 7% sucrose, 7% lactose and 1% PEG were found to be the optimum concentration for maximal survival of culture (Table 4.2) and taken for further studies. Survival rate of M7-PLsr-1$_{(W)}$ on freeze drying was enhanced to 72.26% with supplementation of sucrose (7%) as compared to control (52.55%). With supplementation of lactose (7%) and PEG (1%) culture exhibited 62.77 and 56.93% survival (Table 4.3).

Table 4.2 : Effect of varying concentration of cryoprotectants on the viable cell count of M7-PLsr-1$_{(W)}$ on freeze drying

Cryoprotectant	Concentration (%)	Viable count after freeze drying (cfu/ml)
Control	No cryoprotectants	7.20×10^{11} (± 0.11)
PEG	0.5	7.13×10^{11} (± 0.12)
	1.0	7.80×10^{11} (± 0.11)
	1.5	1.20×10^{10} (± 0.31)
Lactose	2.0	1.57×10^{11} (± 0.21)
	4.0	4.34×10^{11} (± 0.15)
	6.0	5.50×10^{11} (± 0.16)
	7.0	8.60×10^{11} (± 0.11)
	8.0	6.40×10^{11} (± 0.11)
Sucrose	2.0	5.23×10^{11} (± 0.11)
	2.0	2.60×10^{11} (± 0.15)
	4.0	4.40×10^{11} (± 0.14)
	6.0	7.07×10^{11} (± 0.14)
	7.0	9.90×10^{11} (± 0.15)
	8.0	2.60×10^{11} (± 0.11)

* Results are average three experiments (mean ± SD)
* PEG-polyethylene glycol

Table 4.3 : Effect of cryoprotectant on cell viability of M7-PLsr-1$_{(W)}$ on freeze drying

Sample	Colony count (cfu/ml)	Survival (%)
Control	7.2×10^{11} (±0.08)	52.55
PEG (1 %)	7.8×10^{11} (±0.05)	56.93
Lactose (7 %)	8.6×10^{11} (±1.66)	62.77
Sucrose (7 %)	9.9×10^{11} (±0.01)	72.26

*initial cell count is 1.37×10^{12} cfu/ml in all cases
* Results are average three experiments
* PEG-polyethylene glycol

De Giulio et al. (2005) have studied the effect of cryoprotective sugars like trehalose, maltose, sucrose, glucose and lactose at a concentration of 32%

on the survival rate of *L. acidophilus, L. delbruekii and S. salivarus*. The study confirms the protective effect of all sugars on cell viability whereas in the present study sucrose at a low concentration of 7% was found to be the best cryoprotectant. The observed survival (72.26%) is much higher than in *B. longum* (40%) as reported by Champagne et al. (1996).

These sugars used as cryoprotectants are known to replace structural water in membrane after dehydration (Clegg, 1986; Crowe and Crow, 1986) and prevent unfolding and aggregation of proteins by hydrogen bonding with polar groups of proteins (Hanafusa, 1985; Carpenter et a., 1990) thus protecting the cells under stressful conditions.

Zhao and Zhang (2005) have shown *L. brevis* and *O. oeni* to exhibit 45.1 and 38.2% survival in 10% sucrose as protective agent respectively and 47.8 and 2.4% survival when supplemented with 10% lactose. Whereas in the present study the culture exhibits 72.26% and 62.77% survival with only 7% sucrose and 7%lactose respectively.

According to Hubalek (2003) protective activity of sugars might be due to the ability of sugars to prevent injurious freezing of cell fluids by trapping salts in a highly viscous like phase. Leslie et al. (1995) postulated that protective effect of sugar may be due to their ability to lower membrane phase transition temperature and protection of protein structure in dry state. Water molecules seem to be trapped between sugar molecules and crystallization of ice is inhibited (Nicolajsen and Hvidt 1994; Chen et al., 2000).

4.4.3. Cellular fatty acid analysis of freeze dried culture

Lonvaud-Funnel and Desens (1990) have observed an increase in cellular membrane unsaturated fatty acid under low temperature. In the present work we observed an increase in both saturated (C16:0 and C18:0) and unsaturated fatty acids (C16:1, C18:2 and C18:3) as compared to respective controls (before freeze drying) (Table 4.4; Fig. 4.2). Gilliland and Speck (1974) have observed a decrease in C18:1 concentration in lactic *Streptococci* in response to freezing and in *L. acidophilus* on spray drying.

Fernandez Murga et al. (1998) determined an increase of C16:0 and C18:2 concentration in *Lactobacillus acidophilus* when grown at low temperature. In the present work there was increase in C16:0, C18:0, C16:1, C18:2 and C18:3 after freeze drying.

Fatty acids are particularly important in the developing neonates. In the present work the freeze dried culture shows increase in palmitic and stearic acid that are known to play an important role in maintaining kidney function by protective cushioning and as a quick energy source (Busconi and Denker, 1997). The concentration of lauric acid was found to increase in freeze dried sample (0.77 % wt) as compared to sample before freeze drying (0.19 % wt). Capric and lauric acid have been found to boost the immune system and also shown to have antiviral, antibacterial, antifungal and antiprotozoal activity (Enig, 1999). Lauric acid and its monoglyceride, monolaurin have shown to reduce the viral load in HIV patients (Enig, 1998; Tayag and Dayrit, 2000). Increase in the concentration of saturated and unsaturated fatty acids in the culture on freeze drying shows its importance in therapeutic and prophylactic use.

Table 4.4 : Cellular fatty acid profile of freeze dried culture

Peak No	Fatty acids (% wt)	*L. mesenteroides* M7-PLsr-1$_{(W)}$		*S. thermophilus*	
		Before freeze drying	After freeze drying	Before freeze drying	After freeze drying
1	Butyric acid (C4)	4.241	1.602	0.488	0.122
2	Capric acid (C8)	0.085	1.051	Nil	Nil
3	Caprilic acid (C10)	0.080	0.102	Nil	Nil
4	Lauric acid (C12)	0.199	0.767	0.794	1.974
5	Myristic acid (C14)	4.964	0.356	0.448	0.122
6	Palmitic acid (C16)	4.538	9.879	9.533	10.80
7	Palmitoleic acid (C16:1)	0.564	1.025	0.992	1.225
8	Stearic acid (C18)	32.802	35.576	33.765	33.904
9	Oleic acid (C18:1)	0.658	0.720	0.686	0.898
10	Linoleic acid (C18:2)	1.222	1.174	1.353	1.474
11	Linolenic acid (C18:3)	22.093	22.916	23.523	23.892
12	Arachidonic acid (C20)	16.034	16.458	16.777	17.592

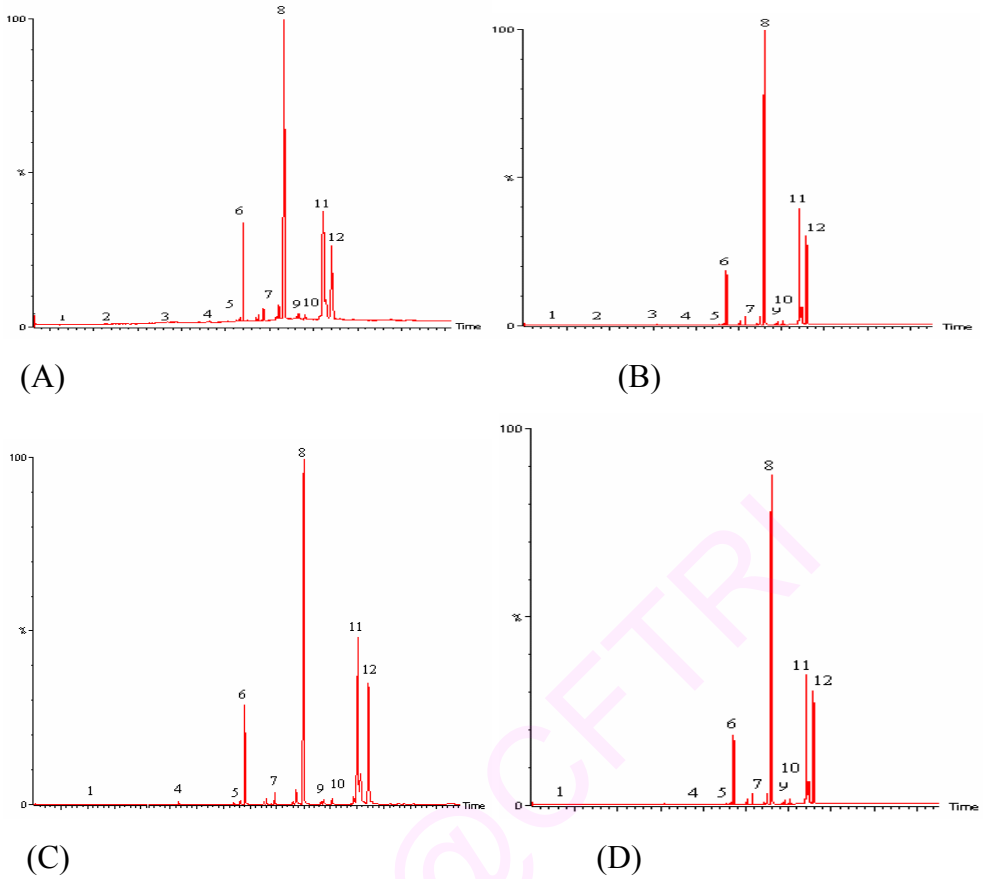


Fig 4.2 : GC profile of cellular fatty acid

(A) *L. mesenteroides* M7-PLsr-1$_{(W)}$ before freeze drying (B) *L. mesenteroides* M7-PLsr-1$_{(W)}$ after freeze drying (C) *S. thermophilus* before freeze drying (D) *S. thermophilus* after freeze drying. Peak number and the corresponding fatty acid are as in table 4.4.

4.4.4. Cellular protein analysis by polyacrylamide gel electrophoresis (SDS-PAGE) of freeze dried culture

Culture has to tolerate the stress of low temperature during freeze drying. This tolerance to stress in lactic acid bacteria varies with each bacterial species. Wang et al. (2004) have observed two types of adaptive response, firstly the synthesis of specific proteins and secondly change in the membrane fatty acids. Wouters et al. (1999) have observed 7 kDa protein which is induced in response to sudden drop in the temperature in *S. thermophilus.* In the present study the freeze dried culture has shown a prominent protein band at 29 and 20.1 KD. Protein band at 14.3 KD is also significant as compared to the control (non freeze dried) sample (Fig. 4.3). Corcoran et al., (2006) have also shown that the overproduction of protein increased the survival of *L. paracasei* NFBC 338 during freeze drying.

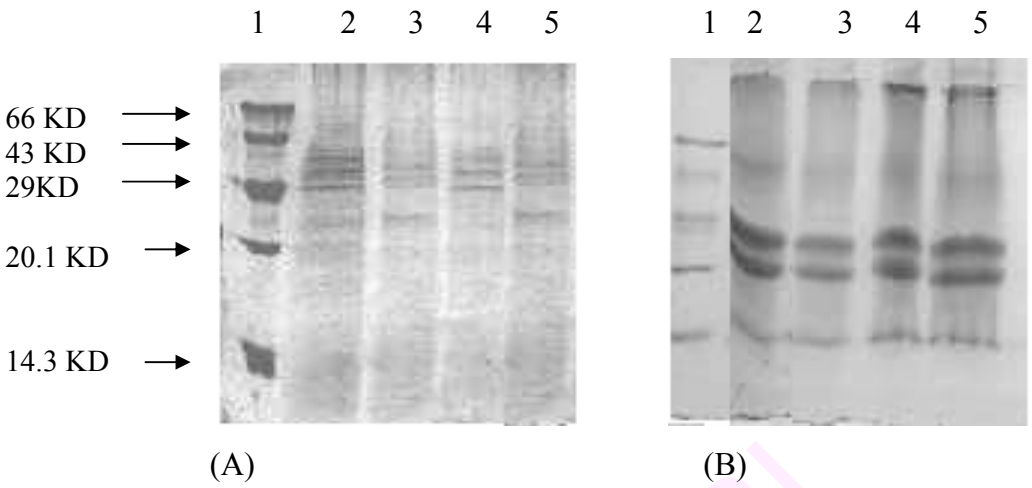


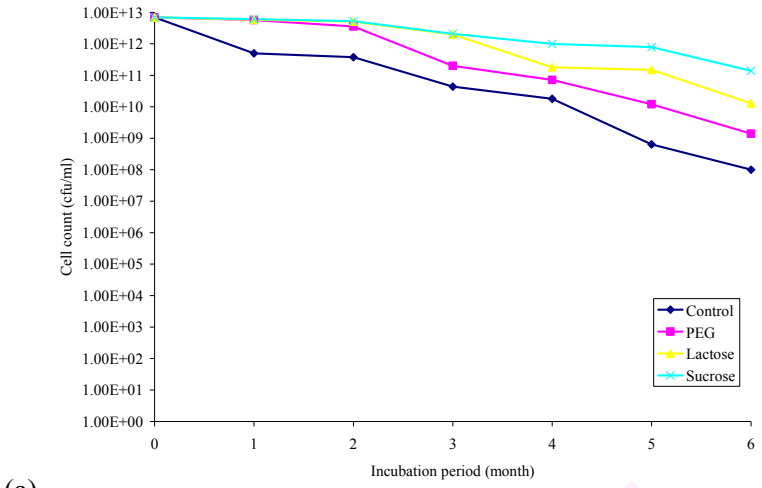
Fig 4.3 : Cellular protein profile of M7-PLsr-1$_{(W)}$

(A) before freeze drying (B) after freeze drying

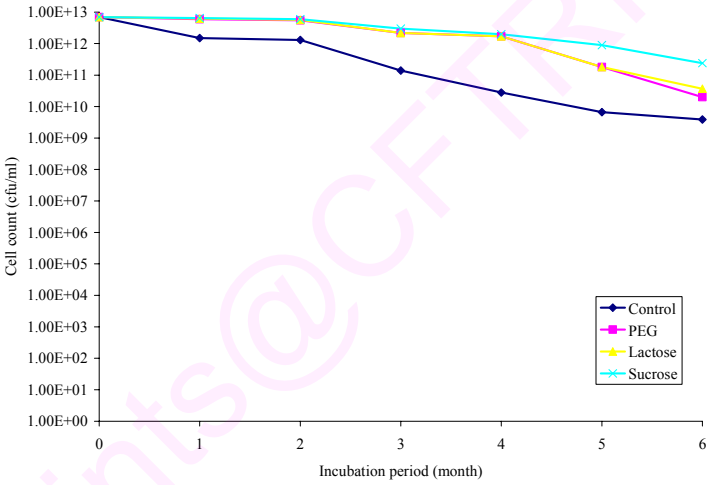
Lane 1: Protein marker
Lane 2: Control without cryoprotectant
Lane 3: Culture supplemented with Polyethylene glycol (1%)
Lane 4: Culture supplemented with lactose (7%)
Lane 5: Culture supplemented with sucrose (7%)

4.4.5. Culture stability on shelf storage

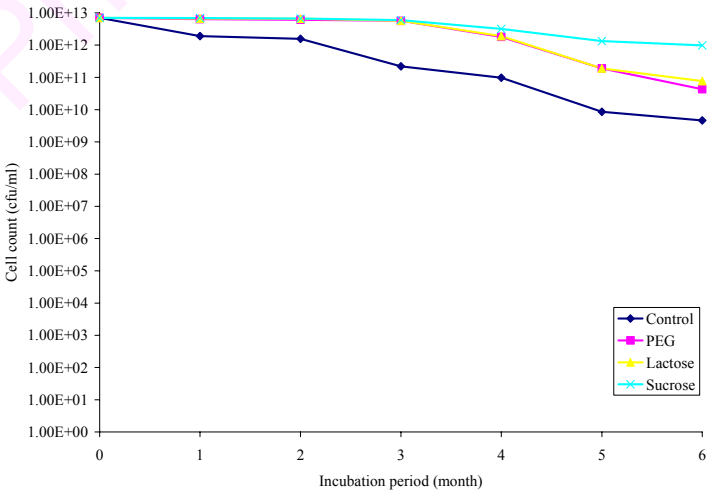
Stability of freeze dried culture was tested during storage at 30, 4 and -20°C upto 6 months (Fig. 4.4). During this time at -20°C, sucrose treated cells had a good survival (9.8×10^{11} cfu/ml) compared with other cryoprotectants. Bruno and Shah (2003) have studied the viability of *Bifidobacterium* in freeze dried probiotic product and have found that the culture viability reduced by 2 logs after 5 months of storage. In the present study the freeze dried culture with sucrose showed only one log reduction after six months. Maximum survival was observed in the sample stored at -20°C till 6 months as compared to sample stored at 30 and 4°C. At -20°C, a cell count of 9.8×10^{11} cfu/ml was observed on supplementation of sucrose as compared to control (with out cryoprotectant) that had a viable cell count of 4.6×10^{9} cfu/ml (Fig. 4.4). According to Hubalek (2003), the protective effect of sugars such as sucrose is due to their ability to prevent injurious effect of freezing by trapping salts in a highly viscous phase. These sugars lower the membrane phase transition temperature and protect protein structure in the dry state.



(a)



(b)



(c)

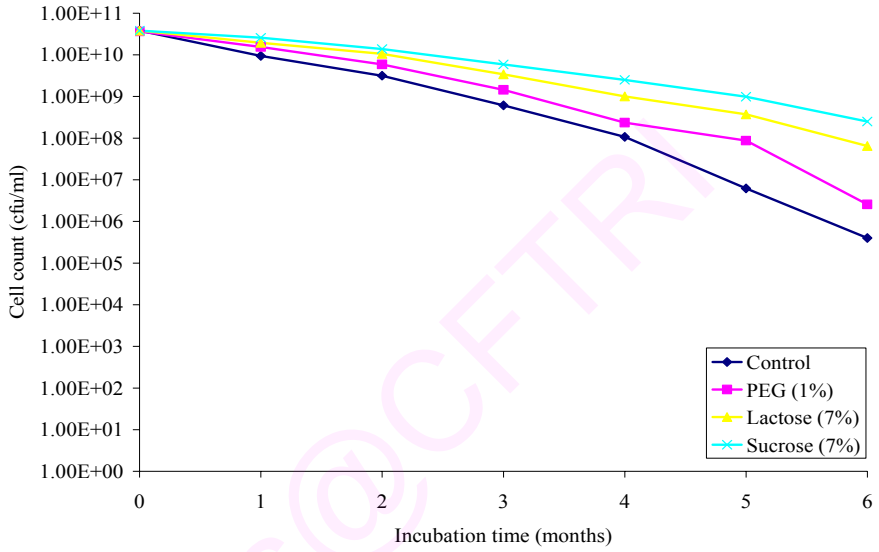
Fig 4.4 : Storage stability of *L. mesenteroides* M7-PLsr-1$_{(W)}$ after freeze drying.

(a) Viability at 30°C (b) Viability at 4°C (c) Viability at -20°C

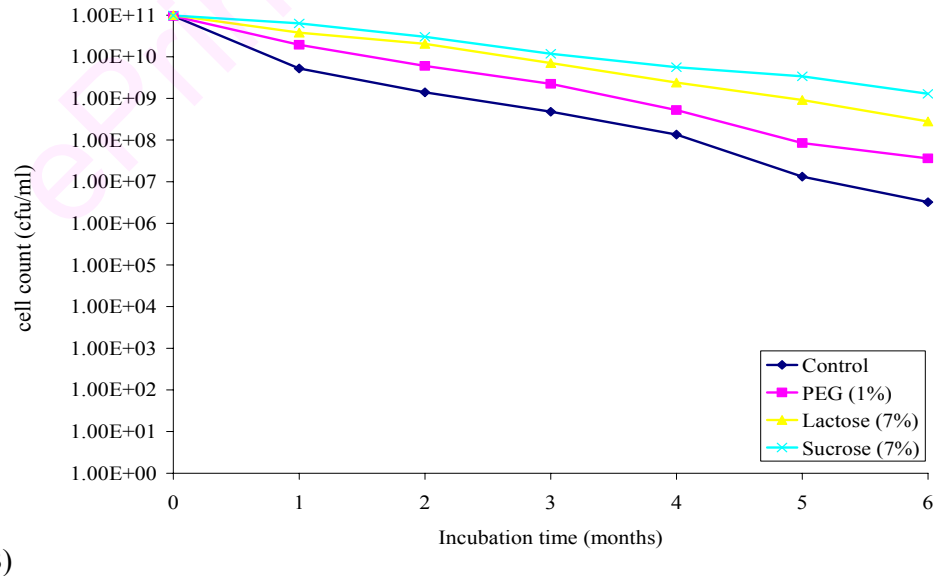
4.4.6. Probiotic properties of freeze dried culture M7-PLsr-1$_{(W)}$

4.4.6.1. Tolerance to low pH and high bile salt

To check the probiotic properties of being resistant to low pH and high bile during storage for six months the freeze dried culture was grown at pH 2.0 and 4% bile salt mix separately. According to the data obtained, the culture retains the ability to resist low pH (2.0) and high bile salt mix concentration (4%) even after a storage period of 6 months (Fig. 4.5).



(A)



(B)

Fig 4.5 : Tolerance of M7-PLsr-1$_{(W)}$ (A) to acidic pH 2.0 (B) to high bile salt concentration (4%)

4.4.6.2. Antimicrobial activity of freeze dried culture

The culture had antimicrobial activity against 6 food borne pathogens namely *E. coli, S. typhi, S. dysenteriae, P. aeroginosa, V. cholera* and *S. aureus* (Table 4.5). On storage of 6 months the activity was maximum with sucrose as cryoprotectant against *E. coli* (16 mm).

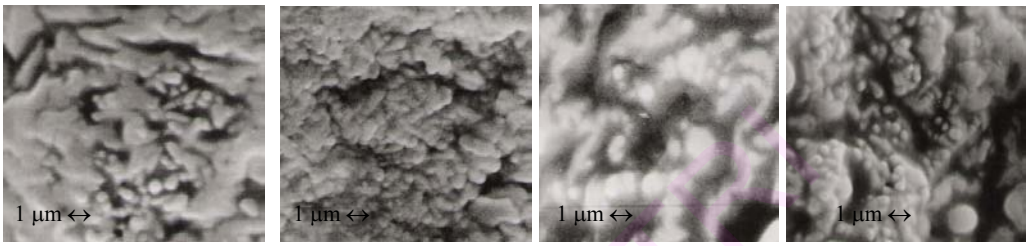
Table 4.5 : Antimicrobial activity of freeze dried cells

Incubation period (months)	Sample	Inhibition zone (mm)					
		E. coli	*S. typhi*	*S. dysenteriae*	*P. aeroginosa*	*V. cholerae*	*S. aureus*
0	Control	20 ± 0.02	23 ± 0.02	23 ± 0.11	23 ± 0.03	10 ± 0.02	18 ± 0.12
	PEG	22 ± 0.10	23 ± 0.03	23 ± 0.02	23 ± 0.03	12 ± 0.02	24 ±0.11
	Lactose	23 ± 0.30	23 ± 0.01	23 ± 0.03	23 ± 0.03	14 ± 0.03	20 ±0.11
	Sucrose	24 ± 0.11	23 ± 0.11	23 ± 0.12	23 ± 0.11	10 ± 0.02	23 ±0.03
1	Control	19 ± 0.22	20 ± 0.21	22 ± 0.12	22 ± 0.22	10 ± 0.11	16 ±0.30
	PEG	22 ± 0.15	22 ± 0.31	22 ± 0.13	22 ± 0.21	12 ± 0.11	20 ±0.03
	Lactose	20 ± 0.16	22 ± 0.44	22 ± 0.15	22 ± 0.15	12 ± 0.05	18 ±0.05
	Sucrose	22 ± 0.17	22 ± 0.33	22 ± 0.16	22 ± 0.17	16 ± 0.15	20 ±0.15
2	Control	18 ± 0.22	20 ± 0.18	20 ± 0.05	20 ± 0.19	10 ± 0.16	16 ±0.15
	PEG	20 ± 0.13	20 ± 0.19	20 ± 0.05	20 ± 0.18	10 ± 0.19	18 ±0.16
	Lactose	18 ± 0.11	20 ± 0.17	20 ± 0.06	20 ± 0.16	12 ± 0.11	16 ±0.17
	Sucrose	20 ± 0.11	20 ± 0.15	20 ± 0.05	20 ± 0.04	14 ± 0.11	20 ±0.01
3	Control	18 ± 0.22	18 ± 0.11	16 ± 0.12	18 ± 0.05	08 ± 0.05	14 ±0.11
	PEG	18 ± 0.11	18 ± 0.11	18 ± 0.11	18 ± 0.05	10 ± 0.05	16 ±0.12
	Lactose	18 ± 0.46	18 ± 0.11	16 ± 0.14	18 ± 0.06	10 ± 0.05	15 ±0.15
	Sucrose	20 ± 0.12	18 ± 0.02	18 ± 0.13	20 ± 0.15	12 ± 0.02	18 ±0.05
4	Control	16 ± 0.11	15 ± 0.02	15 ± 0.01	16 ± 0.16	08 ± 0.12	12 ±0.06
	PEG	18 ± 0.33	16 ± 0.02	15 ± 0.19	18 ± 0.02	08 ± 0.03	16 ±0.06
	Lactose	18 ± 0.18	16 ± 0.13	16 ± 0.12	16 ± 0.02	08 ± 0.13	14 ±0.06
	Sucrose	20 ± 0.19	18 ± 0.30	16 ± 0.11	18 ± 0.02	10 ± 0.13	16 ±0.11
5	Control	16 ± 0.11	14 ± 0.12	12 ± 0.26	14 ± 0.11	06 ± 0.01	12 ±0.12
	PEG	16 ± 0.11	15 ± 0.11	14 ± 0.19	16 ± 0.01	07 ± 0.11	12 ±0.03
	Lactose	18 ± 0.22	16 ± 0.03	12 ± 0.11	14 ± 0.11	08 ± 0.12	10 ±0.09
	Sucrose	18 ± 0.12	16 ± 0.03	15 ± 0.12	16 ± 0.11	08 ± 0.02	14 ±0.01
6	Control	14 ± 0.13	14 ± 0.06	10 ± 0.13	10 ± 0.02	-	08 ±0.12
	PEG	14 ± 0.15	14 ± 0.04	14 ± 0.11	14 ± 0.03	06 ± 0.16	08 ±0.11
	Lactose	15 ± 0.17	14 ± 0.15	10 ± 0.02	12 ± 0.02	07 ± 0.12	-
	Sucrose	16 ± 0.18	14 ± 0.11	10 ± 0.03	12 ± 0.02	07 ± 0.11	10 ±0.01

* Results are average three experiments
* FD = Freeze drying; PEG = polyethylene glycol (1%); Lactose (7%); Sucrose (7%)

4.4.9. Morphological study by scanning electron microscope

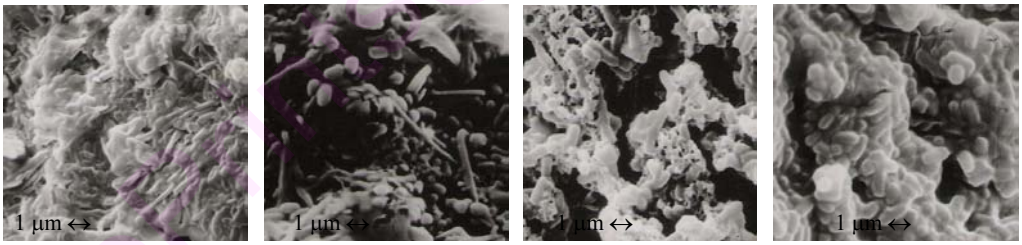
Scanning electron microscopic study shows the surface morphological features of the culture. The results show that the freeze dried cells were protected depending on the nature and type of cryoprotectants used. The cells before freeze drying are in a matrix of skim milk (Fig. 4.6a). After freeze drying with sucrose the cells were found to be protected uniformly whereas it was not so with lactose and PEG (Fig. 4.6b).



(A) (B) (C) (D)

Fig 4.6(a): SEM of *L. mesenteroides* M7-PLsr-1$_{(W)}$ before freeze drying in skim milk

(A) Control sample without any cryoprotectant (B) Culture with PEG (C) Culture with lactose (D) Culture with sucrose. (Magnification 3.00 KX)



(A) (B) (C) (D)

Fig 4.6(b): SEM of *L. mesenteroides* M7-PLsr-1$_{(W)}$ after freeze drying in skim milk

(A) Control sample without any cryoprotectant (B) Culture with PEG (C) Culture with lactose (D) Culture with sucrose (Magnification 3.00 KX)

4.5. Conclusion

Aim of the study was to preserve *L. mesenteroides* M7-PLsr-1$_{(W}$ for longer duration. In this regard, the culture was subjected to different drying methods like spray, vacuum, oven and freeze drying for maximal survival. The result showed maximum stability to freeze drying. *Leuconostoc*

mesenteroides M7-PLsr-$1_{(W)}$ was enhanced for its viability by supplementation of cryoprotectants (PEG 1%; Lactose 7%; sucrose 7%). The percentage of survival was maximum (72.26%) when supplemented with sucrose during freeze drying. Increased expression of 29 and 20.1 KD along with 14.3 KD protein confirms the tolerance of culture towards stress condition. Similarly, culture exhibited tolerance by modification of cellular membrane fatty acids. Fatty acid analysis by GC showed an increase in saturated and unsaturated fatty acid on freeze drying. The study confirms the protective effect of cryoprotectants over a period of 6 months. All the cryoprotectants helped in protecting the culture to resist low pH and high bile salt concentration (4%). Even after 6 months of storage, the freeze dried cells had antimicrobial activity against *E. coli, S. typhi, S. dysenteriae, P. aeroginosa* and *S. aureus*. The above study shows that all the three cryoprotectants have their importance in enhancing beneficial attributes during storage and the consumers can select the cryoprotectant according to the need or the functional property required by the lactic acid bacteria for particular period.

Chapter 5

Functional Food with Leuconostoc: A Native Isolate

CHAPTER – 5

FUNCTIONAL FOOD WITH *LEUCONOSTOC* : A NATIVE ISOLATE

ABSTRACT

In the present work the culture M7-PLsr-$1_{(W)}$ that has the potent probiotic functional properties was used as starter culture in the preparation of fermented milk beverage. The prepared product was found to be rich in proteins, fat, total sugars, fatty acids and minerals like iron, zinc and magnesium. The viability of culture and the nutritional properties of the product were enhanced with supplementation of different adjuvants like tryptone, casein hydrolysate, cysteine hydrochloride and ascorbic acid. After 5 days, maximum viability was observed on supplementation of tryptone (100 mg/L).

5.1. Introduction

Probiotic containing products are gaining popularity and acceptance because of their high therapeutic and health benefits. Fermented dairy products have been regarded as an ideal vector for the delivery of these probiotic bacteria to consumers (Lourens-Hattingh and Viljoen, 2001).

Fermented dairy foods have long been considered safe and nutritious. The health benefits elicited by LAB were the primary reason for Metchnikoff to associate the consumption of yogurt with longevity of Bulgarian peasants. Numerous scientific papers and review articles have been published on the health benefits of fermented dairy products (Kurman et al., 1992; Hughes and Hoover, 1995; Sanders, 1999; Cotter, 2007). Consumption of fermented dairy products is known to have potential in aiding lactose digestion (Shermark et al., 1995; Vesa et al., 1996), preventing traveller's diarrhea (Oksanen et al., 1990), reducing duration of rotavirus diarrhea (Guarino et al., 1998), exert antitumor activity (Kato et al., 1994; Bakalinsky et al., 1996), enhance activity of immune system (Meydani and Ha, 2000) and aid in controlling serum cholesterol (Gilliland et al., 1985; Eichholzer and Stahelin, 1993).

The incorporation of probiotic bacteria in food products has led to the creation of a new and rapidly increasing multi-billion dollar market especially in Europe, Japan and Australia (ADC, 1998; Sanders, 1998; Stanton et al., 2001). There is a tremendous increase in the world sale of cultured products containing probiotic bacteria in the form of yogurt, fermented milk, ice-creams and pharmaceutical products because of their health effects (Mattila-Sandholm, 1999; Ostlie, 2005). Products containing *Bifidobacteria* are also produced in Canada, Italy, Poland, Czechoslovaskia, US and Brazil.

Foods containing these bacteria are often referred to as functional foods because of its potential benefits beyond being as a source of nutrient (Gilliland et al., 2002). Fermented milk is a product obtained by controlled fermentation to produce desirable acidity and flavor (Thapa, 2000). Owing to the nutritional and therapeutic significance, an increasing trend towards consumption of yogurt is observed world wide (Sarkar, 2006). The

consumption has been considerably increased in UK (Kowalska et al., 2000) and in USA. In 1995 sales figure was $ 516 million US dollars (Sivak, 2000).

Probiotic culture cannot exert beneficial effect unless their population reaches a certain minimum level. For instance there is no general agreement on minimum concentration of probiotic to achieve therapeutic benefits. While some researchers suggest a concentration level above 10^6 cfu/ml (Kurmann and Rasic, 1991; Samona and Robinson, 1991; Vinderola, et al., 2000) others stipulate $>10^7$ and 10^8 cfu/ml as satisfactory levels (Davis et al., 1971; Kailasapathy and Rybka, 1997). Oksanen et al. (1990) have shown that 10^9 cfu/ml of *L. bulgaricus GG* is required to reduce the occurrence of traveller's diarrhea. In Japan, fermented milk and lactic acid beverage association has specified that there has to be atleast 10^7 cfu/ml of viable cells in fermented drinks (Lourens-Hattingh and Viljoen 2001). The international standard of federation / International Dairy federation (FIL/IDF) suggests 10^7 cfu/g of *L. acidophilus* and 10^6 cfu/g of *Bifidobacteria* in fermented milk (IDF, 1992). The number of live cells as well the organoleptic qualities play a major role in the acceptability of any functional fermented foods.

The present culture *L. mesenteroides* (M7-PLsr-$1_{(W)}$), a strain that was found to have potential probiotic functional characters was used as a starter culture for the preparation of fermented milk beverage. Viability of culture in the fermented milk was enhanced with supplementation of different adjuvants and studied for the nutritional aspect like protein, fat, sugars, minerals, titrable acidity and fatty acid profile.

5.2. Materials:

Pasteurized cow milk (KMF, Nandini Mysore, India).

Chemicals: All the chemicals purchased were of analytical grade.

1) MRSA media.
2) Potassium oxalate, potassium hydroxide, sodium hydroxide, ammonia, tryptone, casein hydrolysate, cysteine hydrochloride and ascorbic acid (HiMedia Pvt Ltd, Mumbai, India).

3) *Solvents*: Formalin, peroxide free diethyl ether, petroleum ether, methanol, chloroform, and hexane (SRL Company, India).
4) Phenolphthalein indicator: Phenolphthalein (1 g) is mixed in ethyl alcohol (110 ml). Further 80 ml of water is added and mixed with 0.1 M NaOH solution until one drop gives a faint pink coloration. Make up to 200 ml with distilled water.

Equipments: Centrifuge, Spectrophotometer, Incubator (37°C), Muffle furnace (as described in chapter 1 and 2).

5.3. Methods

5.3.1. Preparation and quality analysis of fermented milk beverage

A commercial homogenized and pasteurized milk (1 L) was sterilized at 121°C for 5 min and cooled to room temperature. It was inoculated with the starter culture *L. mesenteroides* (M7-PLsr-$1_{(W)}$) at a concentration of 1×10^5 cfu/ml and incubated at 37°C for 24 h. After the fermentation period, the product was stored at 4°C for 5 days.

5.3.1.1. Viability of bacterial culture

Bacterial growth was estimated using colony plate count method (Martinez-Villaluenza et al., 2005). An aliquot of sample was taken on 1, 3 and 5^{th} day of storage, serially diluted and the appropriate dilution was plated on MRSA plates. Plates were incubated at 37°C for 24 h. Colonies grown were counted and expressed as cfu/ml.

5.3.1.2. Protein estimation

Total protein content was determined by formal titration method using phenolphthalein as an indicator (Bennenberg et al., 1949; Saha et al., 2003). Sample (10 ml) was mixed with phenolphthalein (0.5 ml; 0.5 % w/v) and neutral saturated potassium oxalate (0.4 ml). The mixture was then neutralized with sodium hydroxide (0.1 M) till it turns pink. Further formalin (2 ml) was added and mixed well. The mixture was then allowed to stand for few minutes to form clear colorless solution and then titrated against 0.1M NaOH until pink. Protein content was then calculated by following formula

% Protein content = 1.7 (a-b) × 100

wherein, a = volume of 0.1 M NaOH used for sample

b = volume of 0.1M NaOH used for blank

Blank value: Titrate separately 2 ml formalin with 10 ml water against 0.1 M NaOH.

5.3.1.3. Titrable acidity

Titrable acidity was determined according to the procedure of Hughes and Hoover (1995) by titrating against 0.1N NaOH using phenolphthalein as an indicator. Results are reported as equivalent of lactic acid.

5.3.1.4. Estimation of total sugar

Total sugar was estimated by phenol-sulphuric acid method as described by Dubois et al. (1956). Weighed sample (0.1 ml) was mixed with 2.5 N HCl (5 ml) and boiled for 3 h. After cooling, the mixture was neutralized with sodium carbonate until effervescence ceases. Further the mixture was made upto 20 ml using double distilled water. An aliquot of this mixture (0.1 ml) was mixed with 900 µl of distilled water. Phenol solution (0.5%; 1 ml) and H_2SO_4 (96%; 5 ml) was added to the mixture and vortexed. Mixture was then incubated at 30°C for 20 min and then absorbance was measured at 490 nm. Total sugar content was calculated by comparing the standard graph (Fig. 5.1).

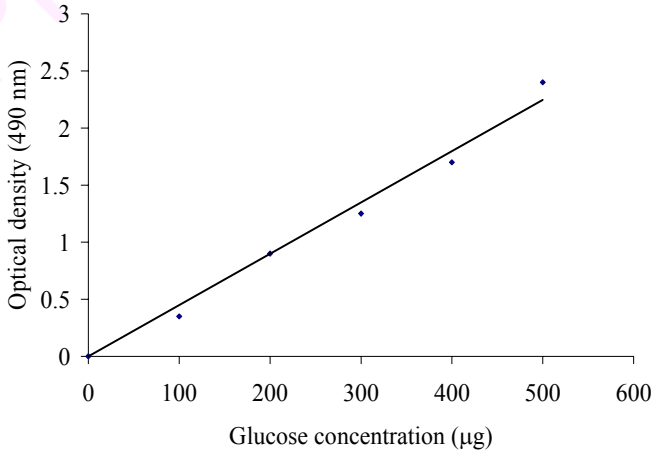


Fig 5.1 : Standard graph for the estimation of total sugars

Graph was prepared by using glucose solution of different concentration (100-500 µg).

5.3.1.5. Fat estimation

Fat content was determined by Rose-Gottlieb method (IDF 1996). The sample (10 g) was mixed with ammonia (1 ml) and 95% alcohol (10 ml). Peroxide free diethyl ether (25 ml) was added to this mixture and vigorously shaken for 1 min. Further, petroleum ether (25 ml) was added and shaking was continued for 30 sec. Solvent layer was then carefully transferred to previously weighed flask. After extracting twice the solvent layer was collected in the weighed flask. The flask was then dried for 1 h at 100°C, cooled and weighed again. Difference in the weight of the flask was calculated and expressed as g/100 ml or percentage.

5.3.1.6. Mineral estimation

Total and soluble mineral content of the fermented milk was analyzed by using atomic absorption spectra according to the method described by Jacob (1958) and modified by Miller-Ihli (1996). Sample (50 ml) was taken out on 1, 3 and 5^{th} day and the soluble fraction was separated by high speed ultra centrifugation at 100,000 g. The supernatant fluid was carefully removed and filtered through whatman-40 paper. Samples were dried at 100°C in a crucible for 6 h. After charring, samples were incinerated in a muffle furnace at 460°C for 24 h. The ash obtained was dissolved in concentrated H_2SO_4 (2 ml) and warmed for 5 min at 40°C in a water bath. The mixture was then madeup to 30 ml with double distilled water and analyzed by atomic absorption spectral studies.

5.3.1.7. Fatty acid analysis

Fatty acid analysis was carried out by the method of Bligh and Dyer (1959). (operation condition as described in chapter 2).

5.3.2. Enhancement of nutritional property by adjuvant supplementation

The sterilized milk (4.5 L) was inoculated with M7-PLsr-$1_{(W)}$ at a concentration of 1×10^5 cfu/ml and the mix was divided into 18 equal portions (250 ml/flask). Filter sterilized adjuvants (tryptone, casein hydrolysate, cysteine hydrochloride and ascorbic acid) were added to achieve a final concentration of 50, 100, 250 and 500 mg/L each in a different conical flask (one portion was kept as control without adjuvant). Incubation was

carried out at 37°C for 24 h. After the fermentation period, the product was stored at 4°C for 5 days. Cell count, protein, titrable acidity, total sugar, fat, minerals and fatty acids were estimated as described earlier.

5.4. Results and discussion

5.4.1. Quality analysis of probiotic fermented milk beverage

The study was carried out to analyze the quality of fermented milk beverage prepared by using the present probiotic culture M7-PLsr-$1_{(W)}$. The culture exhibits an increase in cell count from 1×10^5 to 1.5×10^6 cfu/ml after 5 days of incubation.

Table 5.1 represents the proximate composition of the fermented product. Protein content in milk is 3.4% which increases to 3.57 ± 0.21% after 24 h of fermentation. On storage protein content slightly increased to 3.6 ± 0.11 and 3.62 ± 0.16% at day 3 and 5 respectively. Kroger and Weaver (1983) reported 3.29% protein in commercial yogurt which is lower than analyzed in our product. The traditional fermented products like Roub and Nanu have a protein content of 3.48 and 3.26% respectively (El Zubeir et al., 2005). Fermented milk prepared with a combination of *L. acidophilus, S. thermophilus* and *B. bifidus* contain 4% protein, whereas milk fermented with *B. bifidus* and *L. acidophilus* show 4.8% protein. It appears that the protein content of the fermented milk depends on the starter culture used. Total sugars in the fermented milk prepared (1 d) was 5.36 ± 0.21%. On storage of 5 days it reduces to 4.92 ± 0.11% indicating the utilization of sugar by culture for its growth. The fat content of the product was 4.5 ± 0.17% which is much higher as compared to the finding of Athar (1986) who reports 3.5% fat in yogurt. Gambelli et al. (1999) have reported the fat content of the fermented milk in the range of 3.5-3.8%. The present fermented milk was also found to be rich in mineral content like iron (0.941 ppm), zinc (1.962 ppm) and magnesium (1.191 ppm). Fuente et al. (2003) have examined mineral content of 16 commercial yogurts and have determined soluble fraction of zinc and magnesium in the range of 63-77% and 87-96% respectively. In the present fermented milk, soluble form of zinc and magnesium was found to be much higher (90 and 87% respectively).

Table 5.1 : Chemical and microbial composition of fermented milk beverage

Parameter	Milk	Fermented milk			
		0 d	1 d	3 d	5 d
Cell count (cfu/ml)	Nil	1×10^5 (± 0.02)	1.2×10^6 (± 0.11)	1.3×10^6 (± 0.15)	1.5×10^6 (± 0.15)
Protein (%)	3.40 ± 0.21	3.40 ± 0.21	3.57 ± 0.21	3.60 ± 0.11	3.62 ± 0.16
Total sugar (%)	5.40 ± 0.12	5.40 ± 0.12	5.36 ± 0.21	5.21 ± 1.00	4.92 ± 0.11
Fat (%)	4.70 ± 0.13	4.70 ± 0.13	4.50 ± 0.17	4.30 ± 0.15	4.00 ± 0.15

Mineral content of fermented milk (ppm)

Mineral (ppm)	Incubation time (d)								
	0			1			5		
	Total	Soluble	Bioavailable (%)	Total	Soluble	Bioavailable (%)	Total	Soluble	Bioavailable (%)
Iron	0.769 ± 0.11	0.523 ± 0.21	68.01	0.941 ± 0.15	0.828 ± 0.63	87.99	0.941 ± 0.21	0.812 ± 0.11	86.29
Zinc	1.724 ± 0.15	1.496 ± 0.19	86.77	1.962 ± 0.15	1.766 ± 0.10	90.01	1.993 ± 0.19	1.812 ± 0.11	90.92
Magnesium	1.038 ± 0.16	0.912 ± 0.11	87.86	1.191 ± 0.11	1.036 ± 0.11	86.98	1.211 ± 1.21	1.121 ± 0.23	92.57

*Values are mean ± SD (n=3)

5.4.2. Enhancement of nutritional property by adjuvant supplementation

In any functional fermented food it is important that the cell number of live probiotic microorganism should be high till the end of the shelf life of the product. For this, many workers have reported addition of adjuvants into the product to increase the viability of the culture. Dave and Shah (1998) have shown the improvement of probiotic *Bifidobacterium* viability in yogurt with supplementation of cysteine, whey protein concentrate, acid casein hydrolysate and tryptone wherein improvement in 3 logs was observed as compared to control (without cryoprotectant). In the present study also an increase in cell count was observed on adjuvant supplementation (Table 5.2). After 24 h of fermentation, maximum viability was found with supplementation of tryptone (100 mg/L). On shelf storage of 5 days it increased to 9×10^7 cfu/ml. Increase in the cell viability was also observed with supplementation of other adjuvants like casein hydrolysate, cysteine hydrochloride and ascorbic acid as compared to control.

5.4.3. Effect of adjuvants on protein content of fermented milk

Protein content of fermented milk prepared was 3.57%. On incorporation of adjuvants higher protein content was recorded. Fermented milk supplemented with tryptone (100 mg/L) exhibited highest protein content (3.91%) after 24 h of fermentation (Table 5.3). On storage for 5 days, the protein content gradually increased to 4.0%.

Table 5.2 : Effect of adjuvant supplementation on cell viability

Sl No	Sample Cell count (cfu/ml)	Storage period (days)		
		1	3	5
1	Milk as such	Nil	Nil	Nil
2	Milk+Culture	1.2×10^6 (± 0.11)	1.3×10^6(± 0.15)	1.5×10^6 (± 0.15)
3	Milk+Culture+Tryptone (50mg/l)	1.4×10^6 (± 0.15)	9.5×10^6 (± 0.21)	1.0×10^7 (± 0.15)
4	Milk+Culture+Tryptone(100mg/l)	2.0×10^6 (± 0.11)	1.0×10^7 (± 0.11)	9.0×10^7 (± 1.12)
5	Milk+Culture+Tryptone(250mg/l)	1.9×10^6 (± 0.11)	7.7×10^6 (± 2.11)	2.1×10^7 (± 1.11)
6	Milk+Culture+Tryptone(500mg/l)	1.6×10^6 (± 0.12)	3.7×10^6 (± 2.06)	4.5×10^6 (± 0.16)
7	Milk+Culture+Casein hydrolysate(50mg/l)	2.0×10^6 (± 1.11)	2.1×10^6 (± 0.31)	2.9×10^6 (± 0.15)
8	Milk+Culture+Casein hydrolysate(100mg/l)	3.0×10^6 (± 0.09)	4.9×10^6 (± 0.16)	1.3×10^7 (± 0.31)
9	Milk+Culture+Casein hydrolysate(250mg/l)	2.2×10^6 (± 0.15)	3.2×10^6 (± 0.15)	1.1×10^7 (± 0.11)
10	Milk+Culture+Casein hydrolysate(500mg/l)	2.0×10^6 (± 0.21)	2.7×10^6 (± 0.19)	1.0×10^7 (± 0.11)
11	Milk+Culture+Cysteine hydrochloride(50mg/l)	1.2×10^6 (± 0.15)	4.0×10^6 (± 0.11)	8.0×10^6 (± 0.06)
12	Milk+Culture+Cysteine hydrochloride(100mg/l)	1.9×10^6 (± 0.14)	6.9×10^6 (± 0.15)	1.7×10^7 (± 0.05)
13	Milk+Culture+Cysteine hydrochloride(250mg/l)	1.7×10^6 (± 0.16)	6.0×10^6 (± 0.18)	6.2×10^6 (± 0.05)
14	Milk+Culture+Cysteine hydrochloride(500mg/l)	1.3×10^6 (± 0.15)	1.5×10^6 (± 0.15)	4.3×10^6 (± 0.19)
15	Milk+Culture+Ascorbic acid (50mg/l)	2.0×10^6 (± 0.11)	2.1×10^6 (± 0.16)	2.7×10^6 (± 1.11)
16	Milk+Culture+Ascorbic acid(100mg/l)	1.8×10^6 (± 0.12)	2.2×10^6 (± 0.11)	3.0×10^6 (± 1.19)
17	Milk+Culture+Ascorbic acid(250mg/l)	1.7×10^6 (± 0.08)	1.8×10^6 (± 0.21)	2.0×10^6 (± 0.05)
18	Milk+Culture+Ascorbic acid(500mg/l)	1.5×10^6 (± 0.17)	1.6×10^6 (± 0.03)	1.6×10^6 (± 0.02)

* Values are mean ± SD (n =3).

Table 5.3 : Protein content of fermented milk on adjuvant supplementation during storage

Sl No	Sample	Protein Content (%)		
		1 d	3 d	5 d
1	Milk as such	3.40 ± 0.21	3.40 ± 0.21	3.40 ± 0.21
2	Milk+Culture	3.57 ± 0.21	3.60 ± 0.11	3.62 ± 0.16
3	Milk+Culture+Tryptone(50mg/l)	3.61 ± 0.82	3.64 ± 0.14	3.66 ± 1.40
4	Milk+Culture+Tryptone(100mg/l)	3.91 ± 0.60	3.96 ± 0.29	4.00 ± 0.93
5	Milk+Culture+Tryptone(250mg/l)	3.74 ± 0.63	3.75 ± 0.17	3.79 ± 0.21
6	Milk+Culture+Tryptone(500mg/l)	3.67 ± 0.58	3.71 ± 0.14	3.75 ± 0.79
7	Milk+Culture+Casein hydrolysate(50mg/l)	3.58 ± 0.61	3.59 ± 0.01	3.65 ± 0.24
8	Milk+Culture+Casein hydrolysate(100mg/l)	3.66 ± 0.17	3.71 ± 0.61	3.74 ± 0.17
9	Milk+Culture+Casein hydrolysate(250mg/l)	3.62 ± 0.14	3.64 ± 0.35	3.70 ± 0.45
10	Milk+Culture+Casein hydrolysate(500mg/l)	3.60 ± 2.08	3.63 ± 0.01	3.68 ± 0.19
11	Milk+Culture+Cysteine hydrochloride(50mg/l)	3.59 ± 0.17	3.62 ± 0.60	3.69 ± 0.19
12	Milk+Culture+Cysteine hydrochloride(100mg/l)	3.69 ± 0.14	3.72 ± 0.11	3.75 ± 0.21
13	Milk+Culture+Cysteine hydrochloride(250mg/l)	3.65 ± 0.60	3.67 ± 0.21	3.71 ± 0.11
14	Milk+Culture+Cysteine hydrochloride(500mg/l)	3.62 ± 2.01	3.65 ± 0.14	3.70 ± 0.11
15	Milk+Culture+Ascorbic acid (50mg/l)	3.58 ± 0.60	3.61 ± 0.14	3.63 ± 0.21
16	Milk+Culture+Ascorbic acid(100mg/l)	3.66 ± 0.70	3.69 ± 0.17	3.73 ± 0.93
17	Milk+Culture+Ascorbic acid(250mg/l)	3.63 ± 0.12	3.67 ± 0.29	3.69 ± 0.11
18	Milk+Culture+Ascorbic acid(500mg/l)	3.60 ± 0.25	3.64 ± 0.21	3.69 ± 0.21

* Values are mean ± SD (n =3). Initial protein content of the product was recorded to be 3.4 %

5.4.4. Effect of adjuvant supplementation on titrable acidity during storage

The titrable acidity of the fermented milk is shown in the table 5.4. After 24 h of fermentation highest titrable acidity (0.684 ± 0.11%) was recorded on supplementation of tryptone (100 mg/L). On storage, acidity further increased reaching a maximum 0.864 ± 0.11% after 5 d.

Table 5.4 : Effect of adjuvant supplementation on titrable acidity of fermented milk during storage

Sl No	Sample	Titrable acidity (% lactic acid)		
		1 d	3 d	5 d
1	Milk as such	0.324 ± 0.02	0.324 ± 0.02	0.324 ± 0.02
2	Milk+Culture	0.432 ± 0.02	0.540 ± 0.08	0.576 ± 0.02
3	Milk+Culture+Tryptone(50mg/l)	0.648 ± 0.03	0.648 ± 0.04	0.792 ± 0.14
4	Milk+Culture+Tryptone(100mg/l)	0.684 ± 0.35	0.720 ± 0.05	0.864 ± 0.05
5	Milk+Culture+Tryptone(250mg/l)	0.576 ± 0.12	0.648 ± 0.02	0.756 ± 0.04
6	Milk+Culture+Tryptone(500mg/l)	0.468 ± 0.80	0.612 ± 0.32	0.756 ± 0.01
7	Milk+Culture+Casein hydrolysate(50mg/l)	0.576 ± 0.02	0.648 ± 0.03	0.754 ± 0.12
8	Milk+Culture+Casein hydrolysate(100mg/l)	0.648 ± 0.13	0.684 ± 0.04	0.764 ± 0.13
9	Milk+Culture+Casein hydrolysate(250mg/l)	0.576 ± 0.08	0.612 ± 0.02	0.720 ± 0.11
10	Milk+Culture+Casein hydrolysate(500mg/l)	0.504 ± 0.41	0.504 ± 0.23	0.648 ± 0.05
11	Milk+Culture+Cysteine hydrochloride(50mg/l)	0.638 ± 0.04	0.648 ± 0.05	0.666 ± 0.14
12	Milk+Culture+Cysteine hydrochloride(100mg/l)	0.648 ± 0.14	0.720 ± 0.05	0.792 ± 0.11
13	Milk+Culture+Cysteine hydrochloride(250mg/l)	0.612 ± 0.01	0.622 ± 0.14	0.648 ± 0.21
14	Milk+Culture+Cysteine hydrochloride(500mg/l)	0.540 ± 0.05	0.576 ± 0.12	0.612 ± 0.19
15	Milk+Culture+Ascorbic acid (50mg/l)	0.576 ± 0.05	0.598 ± 0.06	0.620 ± 0.05
16	Milk+Culture+Ascorbic acid(100mg/l)	0.612 ± 0.13	0.648 ± 0.03	0.692 ± 0.02
17	Milk+Culture+Ascorbic acid(250mg/l)	0.566 ± 0.08	0.582 ± 0.03	0.594 ± 0.06
18	Milk+Culture+Ascorbic acid(500mg/l)	0.540 ± 1.12	0.556 ± 0.05	0.578 ± 0.11

* Values are mean ± SD (n =3). Initial titrable acidity of fermented milk was found to be 0.324 ± 0.32 %

5.4.5. Effect of adjuvants on total sugar content of fermented milk

Table 5.5 reports the total sugar content of the fermented milk. As the cell count increased the total sugar content reduced. During the first hour of fermentation the lowest sugar content (4.14 ± 0.11%) was recorded where the fermented milk was supplemented with tryptone (100 mg/L).

Further experiments were carried out with this optimum concentration of each **adjuvant (100 mg/L)**, as this particular concentration gives maximum viability with high protein and titrable acidity.

Table 5.5 : Effect of adjuvants on total sugar content of fermented milk

Sl No	Sample	Total sugar (%)		
		1 d	3 d	5 d
1	Milk as such	5.42 ± 0.12	5.42 ± 0.12	5.42 ± 0.12
2	Milk+Culture	5.36 ± 0.21	5.05 ± 1.00	4.92 ± 0.11
3	Milk+Culture+Tryptone (50mg/l)	5.00 ± 0.14	4.81 ± 0.02	4.18 ± 0.15
4	Milk+Culture+Tryptone(100mg/l)	4.39 ± 0.17	4.24 ± 0.01	4.14 ± 0.11
5	Milk+Culture+Tryptone(250mg/l)	5.07 ± 0.05	4.88 ± 0.02	4.40 ± 0.02
6	Milk+Culture+Tryptone(500mg/l)	5.17 ± 0.45	4.72 ± 0.01	4.66 ± 0.05
7	Milk+Culture+Casein hydrolysate(50mg/l)	4.59 ± 1.12	4.31 ± 0.01	4.30 ± 0.02
8	Milk+Culture+Casein hydrolysate(100mg/l)	4.50 ± 0.24	4.28 ± 0.03	4.20 ± 0.01
9	Milk+Culture+Casein hydrolysate(250mg/l)	4.61 ± 0.21	4.51 ± 0.04	4.48 ± 0.03
10	Milk+Culture+Casein hydrolysate(500mg/l)	4.88 ± 1.17	4.86 ± 0.02	4.82 ± 0.12
11	Milk+Culture+Cysteine hydrochloride(50mg/l)	5.12 ± 0.09	4.37 ± 0.03	4.25 ± 0.15
12	Milk+Culture+Cysteine hydrochloride(100mg/l)	4.45 ± 0.08	4.25 ± 0.03	4.18 ± 0.02
13	Milk+Culture+Cysteine hydrochloride(250mg/l)	5.17 ± 0.18	4.71 ± 0.10	4.63 ± 0.16
14	Milk+Culture+Cysteine hydrochloride(500mg/l)	5.26 ± 0.19	4.84 ± 0.07	4.83 ± 0.11
15	Milk+Culture+Ascorbic acid(50mg/l)	5.02 ± 0.11	4.64 ± 0.01	4.87 ± 0.03
16	Milk+Culture+Ascorbic acid(100mg/l)	4.90 ± 0.21	4.53 ± 0.01	4.47 ± 0.01
17	Milk+Culture+Ascorbic acid(250mg/l)	5.18 ± 0.12	4.69 ± 0.01	4.85 ± 0.03
18	Milk+Culture+Ascorbic acid(500mg/l)	5.22 ± 0.11	4.76 ± 0.06	4.90 ± 0.02

*Values are mean ± SD (n =3). Initial total sugar content was 6.42% in all the cases

5.4.6. Effect of adjuvant supplementation on fat content of fermented milk

In the present study supplementation of adjuvants was found to reduce the fat content of fermented milk (Table 5.6). Maximum reduction was observed on supplementation of tryptone. Reduction of lipid could be attributed to their utilization by fermenting organism. This is an advantage for keeping quality of fermented product as the chances of rancidity would be greatly reduced (Sanni et al., 1999; Sunny-Roberts et al., 2004).

Table 5.6 : Effect of adjuvant supplementation on fat content of fermented milk

Sample (100mg/L)	Fat content (%) during storage		
	1 d	3 d	5 d
Control	4.5 ± 0.17	4.3 ± 0.15	4.0 ± 0.15
Tryptone	3.9 ± 0.18	3.6 ± 0.01	3.0 ± 0.11
Casein hydrolysate	4.2 ± 0.06	4.0 ± 0.08	3.8 ± 0.18
Cysteine hydrochloride	4.0 ± 0.16	3.7 ± 1.11	3.2 ± 0.12
Ascorbic acid	4.3 ± 0.08	4.0 ± 0.02	3.8 ± 1.20

*Values are mean ± SD (n =3)

5.4.7. Effect of adjuvants on mineral content of fermented milk

Total and soluble mineral content of the fermented milk is reported in table 5.7. The mineral absorption does not solely depend on the amount of element present in dairy product but on solubility (Delisle et al., 1995). Hence the fermented milk prepared was analyzed for minerals in soluble form. The prepared fermented milk was rich in iron, zinc and magnesium which were enhanced with supplementation of adjuvants. Iron, zinc and magnesium bioavailability enhanced to 90-92%, 91-95% and 89-92% respectively on supplementation of adjuvants as compared to control. The noticeable increase in the levels of soluble minerals could favor the absorption of these nutrients in the gastrointestinal tract.

Table 5.7: Effect of adjuvant supplementation of soluble mineral content of fermented milk

Sample (100 mg/L)	Concentration (ppm)								
	Iron			Zinc			Magnesium		
	Total	Soluble	Bioavailable (%)	Total	Soluble	Bioavailable (%)	Total	Soluble	Bioavailable (%)
Control	0.941 ±0.11	0.828 ±0.63	88.00	1.962 ±0.15	1.766 ±0.10	90.00	1.191 ±0.16	1.036 ±0.11	86.98
Tryptone	1.346 ±0.49	1.239 ±0.07	92.05	1.929 ±0.28	1.833 ±0.08	95.02	1.131 ±0.10	1.041 ±0.12	92.04
Casein hydrolysate	1.308 ±0.38	1.191 ±0.40	91.05	1.907 ±0.30	1.774 ±0.18	93.03	1.144 ±0.08	1.041 ±0.05	90.99
Cysteine hydrochloride	1.125 ±0.05	1.013 ±0.06	90.00	1.971 ±0.20	1.814 ±0.07	92.00	1.154 ±0.05	1.039 ±0.40	90.03
Ascorbic acid	1.571 ±0.37	1.430 ±0.08	91.02	2.019 ±0.20	1.838 ±0.07	91.04	1.165 ±0.17	1.037 ±0.20	89.01

*Values are mean ± SD (n =3)

Table 5.8 : Effect of fatty acid composition of fermented milk on adjuvant supplementation

Sample (100 mg/L)	Concentration (%)							
	Butyric	Capric	Lauric	Myristic	Palmitic	Stearic	Oleic	Linolenic
Control	4.700±0.1	1.560±0.2	3.880± 0.1	11.830 ± 0.1	31.010 ± 0.2	09.250 ± 0.1	18.730 ± 0.1	0.560 ± 0.1
Tryptone	7.262±0.3	1.587 ± 0.1	4.683 ± 0.1	10.514 ± 0.1	27.920 ± 03	12.330 ± 0.2	22.301 ± 0.2	0.607 ± 0.3
Casein hydrolysate	2.600±0.1	ND	4.220 ± 0.1	07.412 ± 0.1	26.140 ± 0.2	11.682 ± 0.1	34.560 ± 0.2	0.713 ± 0.2
Cysteine hydrochloride	2.514±0.1	0.983 ± 0.2	2.560 ± 0.1	07.354 ± 0.1	22.230 ± 0.2	11.460 ± 0.1	39.390 ± 0.1	0.647 ± 0.2
Ascorbic acid	6.399 ± 0.4	1.871 ± 0.1	4.016 ± 0.1	06.482 ± 0.1	16.070 ± 0.4	14.250 ± 0.3	27.080 ± 0.2	0.993 ± 0.1

*Values are mean ± SD. ND-not detected.

5.4.8. Effect of adjuvant on fatty acid composition of fermented milk

Fatty acid analysis of the fermented milk is presented in table 5.8. Supplementation of tryptone and ascorbic acid increased the content of both saturated (butyric, capric, lauric and stearic acid) and unsaturated fatty acids (oleic and linolenic acid) compared to the control. On supplementation of cysteine hydrochloride and casein hydrolysate an increase in unsaturated fatty acid was observed. Maximum butyric and lauric acid content was estimated in fermented milk supplemented with tryptone which was 1.54 and 1.21 folds higher than the control. These fatty acids are known to have anticarcinogenic activity (Rabizadeh et al., 1993). Maximum capric, stearic and linolenic acid was found in samples supplemented with ascorbic acid which was 1.19, 1.54 and 1.77 folds higher than the control sample. Maximum oleic acid (39.39%) was found with cysteine hydrochloride supplementation. Oleic acid inhibited the mutagenic activity of food pyrolysate mutagens, polycyclic aromatic hydrocarbons, and nitrosamines (Hayatsu et al., 1981; 1983).

All these fatty acids formed in the product are very advantageous from nutritional point of view. These fatty acids also contribute significantly to flavor production and act as precursors for the formation of other aroma components such as esters, aldehydes and alcohols (Fuente et al., 1993).

5.5. Conclusion

This study determines the high nutritional quality of fermented milk prepared from the present culture *L. mesenteroides* M7-PLsr-$1_{(W)}$. For health conscious consumers, the demand for the functional product with health benefits are increasing in the market. It is likely that the present target is for specific products containing well characterized bacteria with specific health enhancing characteristics. In this regard *L. mesenteroides* M7-PLsr-$1_{(W)}$ having potential probiotic functional properties was used for the preparation of fermented milk and analyzed for the nutritional benefits. The prepared

fermented milk beverage was found to be rich in proteins, fats, total sugars, minerals and fatty acids content providing nutritional advantages to the consumers. Further the nutritional properties and the viability of the culture in the product were enhanced with supplementation of different adjuvants. Maximum viability was observed on supplementation of tryptone (100 mg/L). The noticeable increase in the minerals confirms the role of the fermented milk as a source of essential nutrients.

Chapter 6

Preservation of Fermented Milk over Shelf Storage

CHAPTER - 6

PRESERVATION OF FERMENTED MILK OVER SHELF STORAGE

ABSTRACT

This chapter addresses the preservation of fermented milk beverage during shelf storage. The functional fermented milk beverage prepared with M7-PLsr-$1_{(W)}$ was studied for the spoilage bacterial cultures wherein *Pseudomonas sp* was identified as dominant spoilage bacteria. The signal molecule for spoilage was identified as hexanoyl homoserine lactone (HHSL) and butryl homoserine lactone (BHSL) through TLC, GC and GCMS. Initially inhibition of *Pseudomonas sp* was standardized by using furanones (bromofuranone and 2(5H)-furanone) in LB broth and further taken up for preservation of fermented milk for longer shelf life. With addition of 2(5H)-furanones the shelf life was increased upto 9 days by preventing the pathogenic load. Because of its structural similarity furanones specifically interfere with signal molecule of spoilage bacterial culture without any adverse effect on the beneficial bacteria.

6.1. Introduction

A nutritive fermented milk prepared from *L. mesenteroides* (M7-PLsr-$1_{(W)}$) (as described in chapter 5) is a good source of protein (3.57%) and also iron and zinc (0.941 and 1.962 ppm). A fat content of 4.5%, the fatty acid composition and acceptable titrable acidity makes the product a delicious and refreshing beverage. On longer shelf storage the product was found to be spoilt with whey separation. This may cause significant loss and provoke the opportunistic microorganisms to flourish and spread diseases. Nutrients and the storage environment of the product create a selective condition for the growth of these spoilage bacteria. The traditional preservative methods by physical and chemical means are known to be less effective (Nebedum and Obiakor, 2007) as these spoilage organisms exhibit high intrinsic resistance to variety of stress conditions (Beales, 2004). These bacterial cultures are known to release some signal molecules in a density dependent manner for synchronizing expression of particular sets of genes and coordinating cellular activities (Dong et al., 2002). This phenomenon is described as quorum sensing (Fuqua et al., 1994).

The concept of quorum sensing has encouraged us to engage in the development of a novel non-antibiotic, anti-bacterial therapy using quorum sensing inhibitor compound. Traditional treatment of using antibiotics to kill or inhibit the growth of bacteria has created a major global concern for antibiotic resistant strains (Geddes, 2000; Hentzer et al., 2003). Hence the method of regulating bacterial virulence by interfering with quorum sensing system has afforded a novel opportunity to control infectious diseases and will not create a selection pressure for development of resistant strains.

Keeping this in mind, the predominant bacterial culture responsible for spoilage of fermented milk on storage was isolated and characterized. The signal molecule produced by the culture was identified by TLC and GC and GCMS. Further we attempted to attenuate bacterial growth and virulence by interfering with quorum sensing system. Our approach was based on using furanones that acts as a competitive inhibitor are natural and approved by FDA to be used in foods. The ultimate aim of the present work was to preserve the fermented milk for longer shelf life.

6.2. Materials

Fermented milk using M7-PLsr-$1_{(W)}$ as starter culture.

Bacterial culture and growth: *Agrobacterium tumefaciens* (kindly provided by Dr. Stephen K. Farrand, , Department of Microbiology, University of Illinois at Urbana-Champaign Illinois 61801 USA).

Chemicals: All the chemicals purchased were of analytical grade.

a) De Mann Rogosa Sharpe Agar (MRS), Luria-Bertani media (LB), Braid Parker media (BP), Salmonella-Shigella agar (SS), Listeria Oxford Media (LM) and Pseudomonas agar (PA) were purchased from HiMedia Pvt Ltd, Mumbai, India.
b) Skimmed milk media: Skimmed milk (10 g) was suspended in 100 ml of distilled water and homogenized. It was supplemented with 1.5% of agar and sterilized at 121°C for 15 min at 15 lb pressure.
c) Bromofuranone (BF) and 2(5H)-furanone [2(5H)-F] (Fluka, Buchs, Switzerland).
d) Standards of hexanoyl homoserine lactone and butryl homoserine lactone: N-acyl homoserine lactone (AHL) standards were purchased from Fluka (Buchs, Switzerland). The standards were dissolved in methanol at a concentration of 1 mg/ml and stored at -20°C. For GC and GCMS analysis the stock solutions were mixed and diluted with methanol at 100 µg/ml.
e) Rhamnose, X-gal, orcinol, HCl and H_2SO_4 (HiMedia Pvt Ltd, Mumbai, India).

Solvents: Dichloromethane, diethyl ether, methanol (HPLC grade, SRL Laboratories, India).

Equipments: Gas chromatograph, Gas chromatograph and Mass spectrometer, Centrifuge, Spectrophotometer (as described in chapter 2 and 3).

6.3. Methods

6.3.1. Isolation and characterization of bacterial cultures from fermented milk

Bacterial cultures were isolated from spoilt fermented milk stored at 4 and 30°C by serial dilution and plating method. The colonies grown were purified by repeated streaking and stored at 4°C under paraffin until use. The predominant culture was further characterized by biochemical assays according to Bergey's manual of systematic bacteriology (Krieg 1984).

6.3.2. Identification of signal molecules

6.3.2.1. *Thin layer chromatography (TLC)*

The predominant spoilage culture (*Pseudomonas sp*) was grown in LB media (100 ml) for 20 h at 28°C. Cell free supernatant was collected by centrifugation at 8000 rpm for 15 min. The supernatant was then extracted twice with equal volume of ethyl acetate and the extract was filtered and dried.

Synthetic AHL standards (1 mg/ml) and the ethyl acetate culture extract were taken in HPLC grade ethyl acetate and spotted onto TLC plate (20 × 20 cm). The chromatogram was developed using a solvent system of methanol:water (60:40 v/v) as described by Shaw et al. (1997). After development the solvent was evaporated and the dried plate was overlaid with monitor strain (*A. tumefaciens*). A 10 ml overnight culture of *A. tumefaciens* was inoculated into 150 ml of LB medium containing X-gal and the culture was spread over the surface of the developed TLC plate. The plate was incubated overnight at 30°C in a sterilized closed plastic container. AHL were visualized as blue spots.

6.3.2.2. *Gas chromatographic technique*

Extraction: A simple method was standardized by using GC and GCMS for the identification and quantification of AHL. Cell free supernatant (5 ml) was extracted twice with an equal volume of dichloromethane (DCM), dried over anhydrous sodium sulphate and filtered. The solvent layer was carefully

separated and dried. The dry residue was redissolved in 1 ml of DCM for GC and GCMS analysis.

GC conditions: The column used was OV-17 with an oven temperature programming from 70^0C to 250^0C ($70\text{-}150^0$C; 6^0C/min, $150\text{-}250^0$C; 10^0C/min, 250^0C with 10 min holding time).

GCMS conditions: Analysis were performed using a model Turbomass Gold (Perkin Elmer International, Switzerland) interfaced to a single quadrapole mass selective detector, both of which were controlled by a computer equipped with turbomass version 4 software. Sample was injected in the split mode (40:1) in an ELITE-1 column, 30 m × 0.25 mm id and 0.25 µm film thickness coated with 100% poly dimethoxy siloxane. Pure Helium was used as the GC carrier gas at a flow rate of 1 ml/min. The GC injector temperature was set at 100°C and oven temperature at 70°C. The detector was adjusted to 250°C. Mass spectrometry conditions were as follows: electron ionization source set to 70 eV, emission current 100 µA, MS Quad 150°C, MS source 150°C. The mass spectrometer was run in full scan mode.

6.3.3. Optimization and effect the of furanone to inhibit spoilage organism in LB culture medium

To study which of the furanone was effective against spoilage bacterial culture (*Pseudomonas sp*) two commercially available furanones, Bromofuranone and 2(5H)-furanone were tested for their efficacy in reducing the growth of spoilage bacterial culture and its virulence expression.

6.3.3.1. ***Effect of furanones on bacterial growth*****:** Selected bacterial strain (*Pseudomonas sp*) was cultured in 100 ml LB broth supplemented with furanones (Bromofuranone and 2(5H)-Furanone) at different concentrations (100, 200 and 300 µM) separately and incubated at 4 and 30°C for 5 days. Cultured broth without furanone was used as control. At regular interval of time (1^{st}, 3^{rd} and 5^{th} day) an aliquot (1 ml) of sample was drawn, serially diluted and plated on selective media (*Pseudomonas* agar) to enumerate the number of surviving bacterial cells.

6.3.3.2. *Effect of furanone on rhamnolipid content of spoilage bacterial culture (Pseudomonas sp)*: Rhamnolipid in culture supernatant was detected as previously described by Koch et al. (1989). The culture (*Pseudomonas sp*) was grown in LB broth supplemented with two furanones [Bromofuranone and 2(5H)-furanone] separately at varying concentrations (100-300 µM). After 24 h of incubation at 30°C, culture broth was centrifuged (8000 rpm for 10 min) and the supernatant was filtered through 0.2 µm filter. Filtrate was extracted thrice with 2 volumes of diethyl ether. The pooled ether extract was further extracted with 20 mM HCl and the ether phase was evaporated to dryness. The residue was dissolved in water. Rhamnose content in each sample was determined by orcinol assay (Ochsner, 1993) and compared with rhamnose standards (Fig. 6.1). Rhamnolipid was determined as 1 mg of rhamnose corresponding to 2.5 mg of rhamnolipid.

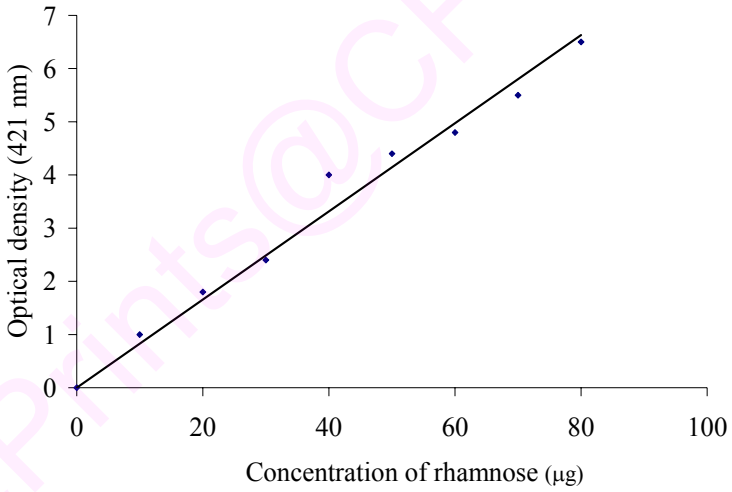


Fig 6.1 : Standard graph for rhamnose estimation

Standard graph was prepared by using rhamnose. Different concentrations of rhamnose solution was prepared (20-100 µg) and assayed by orcinol assay. Absorbance was measured at 421 nm in a spectrophotometer. The values of absorbance (y-axis) were then plotted against the concentration of rhamnose (x-axis) to obtain a standard graph.

6.3.3.3. *Effect of furanone on Motility of spoilage bacterial culture (Pseudomonas sp)*: To the petriplates containing LB media (0.5% agar) with 2(5H)-furanone (100 and 300 µM separately) an overnight grown culture of *Pseudomonas sp* was spotted. The plates were then incubated at 28°C for 48 h and observed for the size of the colony. LB media plate without furanone was used as control.

6.3.3.4. *Effect of furanone on Exoprotease activity/casein hydrolysis of spoilage bacterial culture (Pseudomonas sp)*: Bacterial culture grown in the presence of different concentrations of 2(5H)-furanone (100, 300 and 500 μM) were centrifuged separately and the cell free supernatant was analyzed for the exoprotease activity or the casein hydrolysis using skimmed milk media (Chancey et al., 1999). Wells of 4 mm diameter were made in the media plates and inoculated with cell free supernatant. After the incubation period for 24 h at 28°C, plates were observed for the clear zone around the wells.

6.3.4. Growth inhibition of spoilage bacterial culture (*Pseudomonas sp*) in fermented milk by using furanone

Fermented milk was prepared by inoculating M7-PLsr-$1_{(W)}$ at a concentration of 1×10^5 cfu/ml. After 24 h of fermentation, *Pseudomonas sp* as spoilage bacterial culture was added (1% v/v) into the fermented milk and was divided into 6 equal portions. Two fractions were supplemented with 500 μM of 2(5H)-furanone and other two with 1000 μM of 2(5H)-furanone. Two were kept as control. One set (each of control and fraction supplemented with 500 μM and 1000 μM) was incubated at refrigerated condition (4°C) and other at room temperature (30°C). At regular intervals (0-3 days) an aliquot of sample (1 ml) was drawn, serially diluted and plated. The colonies grown were enumerated and expressed as cfu/ml.

6.3.5. Preservation of fermented milk over shelf life by supplementation of furanone

Fermented milk was prepared by adding M7-PLsr-$1_{(W)}$ at a concentration of 1×10^5 cfu/ml along with supplementation of 2(5H)-furanone (1000 μM). The fermented milk was incubated at 30 and 4°C to study the stability of the product. At regular intervals (0-10 days) an aliquot of sample (1 ml) was drawn, serially diluted and plated on MRSA and other selective media. The colonies grown were enumerated and expressed as cfu/ml.

6.4. Results and Discussion

6.4.1. Isolation and characterization of bacterial cultures from spoilt fermented milk

The nutritive probiotic fermented milk beverage prepared using the potent probiotic isolate M7-PLsr-$1_{(W)}$ was studied for its shelf life. Although the product was good upto 5 days of storage, spoilage was observed after the 6^{th} day of storage with whey separation. The bacterial cultures responsible for the spoilage were isolated by plating an aliquot of spoilt fermented milk on different selective media (Braid Parker media, Salmonella-Shigella agar, MacConkey agar, Pseudomonas agar and deMann Rogosa Sharpe agar).

Totally 22 bacterial cultures were isolated from spoiled fermented milk. About 50% of the isolates were gram positive, catalase negative, non-spore forming and non-haemolytic. Of the other 11 bacterial strains 9 isolates were identified as *Pseudomonas sp* according to Bergey's manual (Table 6.1; Fig. 6.2).

Huis In't Veld (1996) has also reported *P. fluorescens* as the specific spoilage organism in refrigerated milk. They impart off-flavor in milk due to extracellular proteinases and lipases (Sorhaug and Stepaniak, 1997; Dogan and Boor, 2003). Jaspe et al. (1995) have isolated a number of *Pseudomonas spp* (80 strains) from raw milk. *Pseudomonas spp* are known to be predominant spoilage flora in proteinaceous raw foods stored under aerobic refrigerated condition (Gennari and Dragotto, 1992; Ternstrom et al., 1993) particularly in beef, fish, chicken, raw milk and fermented milk (Kraft, 1992; Labadie, 1999; Tryfinopoulou et al., 2002; Jay et al., 2003). They have significant spoilage potential by virtue of their ability to elaborate heat stable enzymes (proteases and lipases) which can survive pasteurization and even UHT heat treatment.

Table 6.1 : Characteristics of spoilage bacterial culture

Character	***Pseudomonas sp***
Structure	G-ve rod
Arginine dihydrolase	+
Motility	+
Acid production	+
Gas from glucose	+
Sucrose	-
D-Sorbitol	-
L-Arabinose	-
L-Rhamnose	-
D-Xylose	+
Trehalose	-
D-Mannose	-
D-Ribose	+
D-Galactose	-
D-Fructose	+

(+) – Positive; (-) - negative

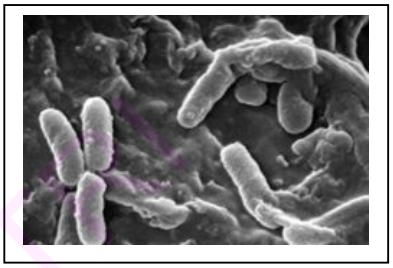


Fig. 6.2: SEM of *Pseudomonas sp.*

6.4.2. Identification of signal molecules

Spoilage of milk by *P. fluorescens* is correlated with its ability to produce AHLs and extracellular protease (Liu and Griffiths, 2003).

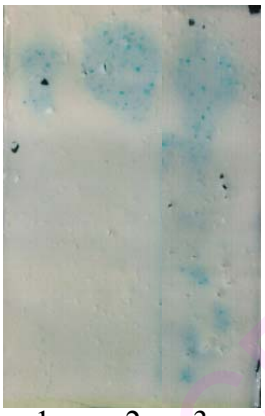
Pseudomonas spp are ubiquitous gram negative bacteria known to be opportunistic pathogen causing nosocomial pneumonia, catheter and urinary tract infections, sepsis in burns, wound in immunocompromised patients and chronic pulmonary inflammation in cystic fibrosis patients (Pruitt et al., 1998; Takeyama et al., 2002; Hart and Winstanley, 2002; Kang et al., 2003).

Pseudomonas spp regulate virulence gene expression in a cell density dependent manner. After reaching a threshold concentration, autoinducer (AI) activates a lux R-type transcriptional activators to induce specific genes (Fuqua et al., 1996) for the release of N-acyl homoserine lactones as signal molecules. These quorum sensing signal molecules regulate the synthesis of elastase, alkaline protease, exoenzymes, haemolysin, lectins, pyocyanin, rhamnolipids, HCN or oxidative stress responsive enzymes, catalase and superoxide dismutase (Pearson et al., 1997; Hasset et al., 1999; Pessi and Haas, 2000; Winzer et al., 2000). Quorum sensing also regulates competence development, sporulation and virulence factor induction along with other physiological events in pathogenic bacterial infections (Cvitkovitch et al., 2003; Greenberg, 2003; Yarwood and Schlievert, 2003). Hence it is very essential to identify the signal molecule produced by these bacterial cultures to prevent its growth and virulence.

A large number of researchers have applied different methods for identifying these signal molecules like green fluorescent protein technology (Hentzer et al., 2002), semi-preparative HPLC, coupled with MS and NMR spectroscopy (Eberl et al., 1996), colourimetric method (Hendricks et al., 2004) and PCR (Nakayama et al., 2003) which are time and resource consuming techniques.

Simple and convincing method for separation and tentative identification of AHL molecules in extract of whole cell cultures has been developed by Shaw et al. (1997). It consists of TLC followed by detection of AHL molecule by means of agar overlay with sensor bacteria (*A. tumefaciens*). Another method based on conversion of AHL molecule to pentafluorobenzyloxime derivatives and identification on GCMS was developed by Charlton et al. (2000) but it is a resource consuming method and the derivatization may be hazardous to health. Capillary separation with mass spectrometric detection was followed by Frommberger et al. (2004). As AHL are actually cyclic esters, analysis of this compound is possible by GCMS without derivatization (Cataldi et al., 2004).

In the present work initially TLC was performed with AHL extract from representative strain (*Pseudomonas sp*) followed by agar overlay with *A. tumefaciens* (Fig. 6.3). TLC results indicate the presence of two kinds of AHL in the extract of *Pseudomonas sp* which was tentatively identified as butryl homoserine lactone (BHSL) and hexanoyl homoserine lactone (HHSL) by comparing with AHL standards.



Lane 1. BHL
Lane 2.HHSL
Lane 3.*Pseudomonas sp* extract

Fig 6.3 : TLC Overlay assay for identification of signal molecule

In the present study a simple method for the identification of these signal molecule was optimized using GC and GCMS. As these N-AHL molecules result from enzymatic condensation of HSL with 3–hydroxy, 3–oxo or an un substituted fatty acid (Schaefer et al., 1996) mass spectrum can be used as a quantitative tool for identification of AHL molecules (Cataldi et al., 2004; Gould et al., 2006). Thus, under the optimized experimental conditions a series of sample extract from supernatant of bacterial cultures were investigated. *Pseudomonas sp* when assayed showed the presence of HHSL and BHSL molecule which were confirmed by comparing the retention time of standard AHL and spiking with the standards (Fig. 6.4). The best results in terms of selectivity and analysis time was obtained using a temperature programme of 70^0C to 250^0C ($70\text{-}150^0$C; 6^0C/min, $150\text{-}250^0$C; 10^0C/min, 250^0C with 10 min holding time).

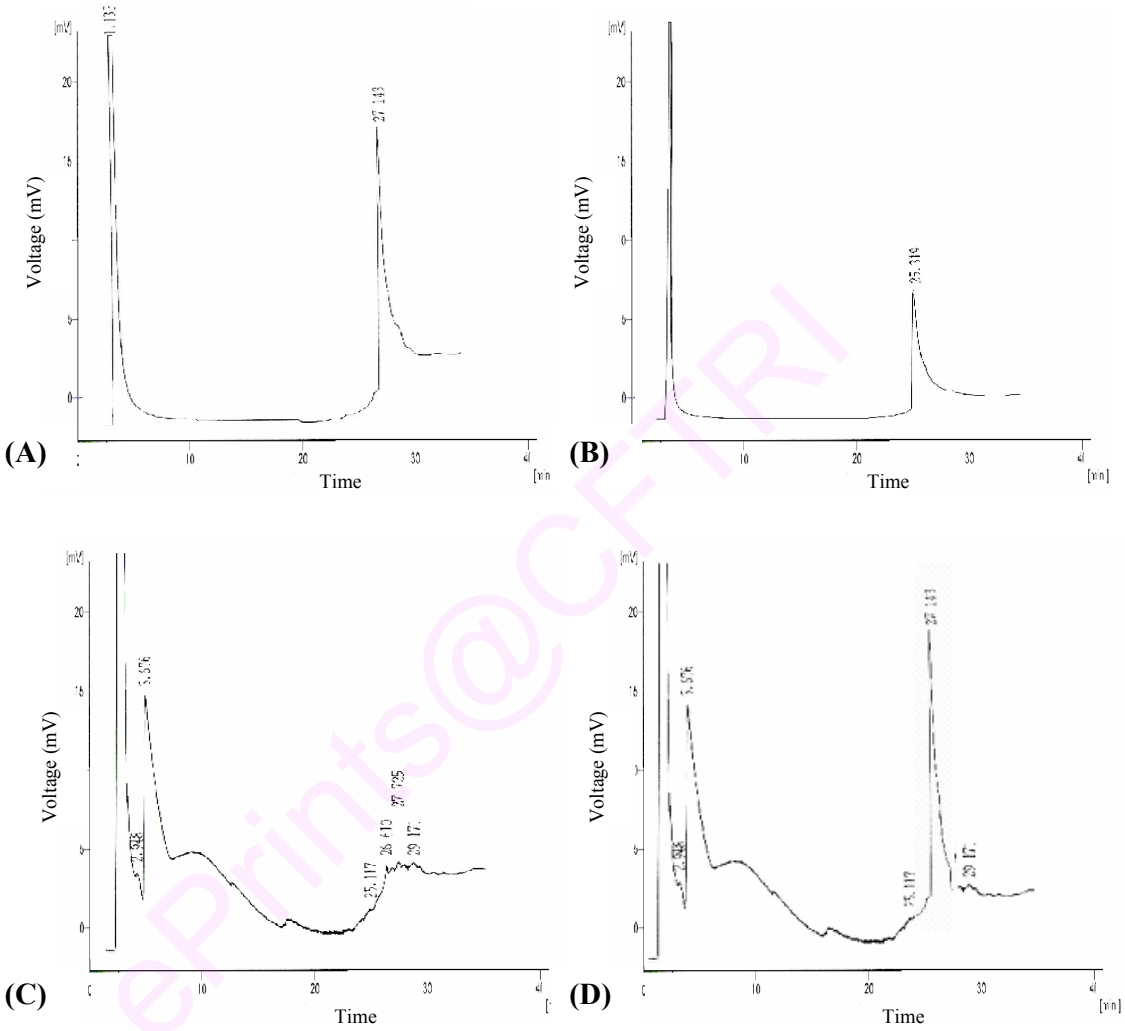
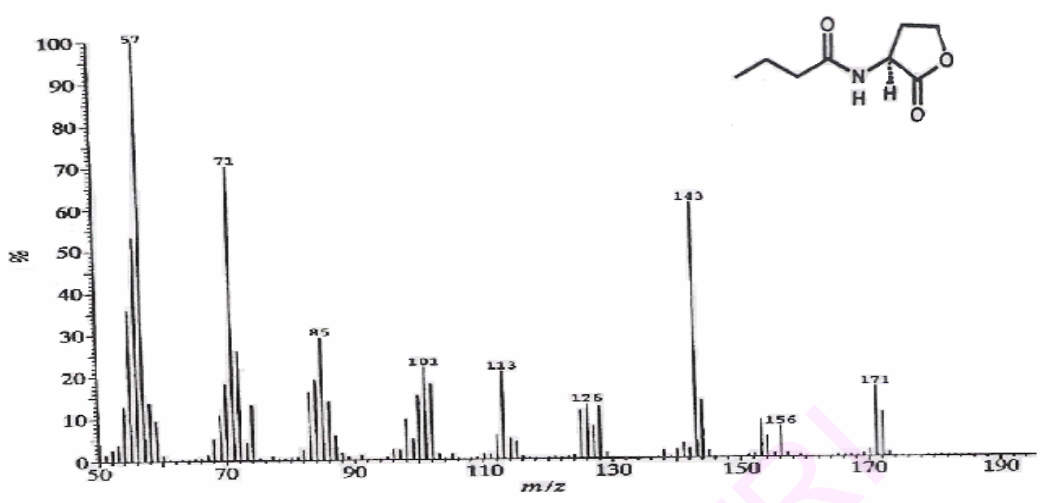


Fig 6.4 : GC chromatograph

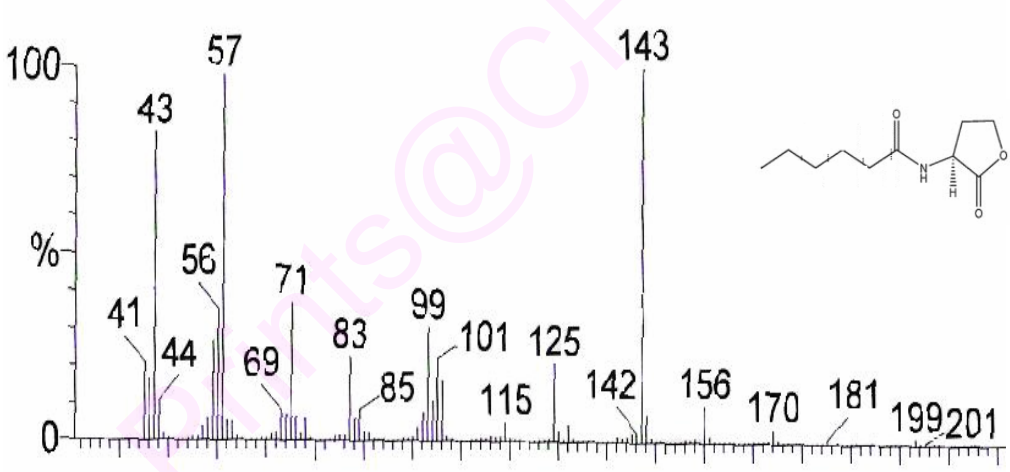
(A) standard HHSL (B) standard BHSL and (C) *Pseudomonas sp* extract (D) *Pseudomonas sp* extract spiked with standard HHSL

Further GCMS analysis gave a direct and effective indication of signal molecule present in the culture supernatant extract. The mass spectra of HHSL and BHSL standard exhibited a characteristic molecular ion $[M]^+$ that was identified as m/z 143 and m/z 171 respectively. A similar mass spectrum was also reported by Pearson et al. (1995) and Cataldi et al. (2004) for N-butryl HSL (molecular weight; 171) and hexanoyl homoserine lactone (molecular weight; 199).

In the present study, the MS fragmentation pattern maximum peaks were found at 143, 57, 43, 71, 99 for HHSL and 57, 71, 143, 85, 101 for BHSL (Fig. 6.5). A comparison with the chromatogram recorded for sample with that of a standard allowed for the identification of signal molecule. Accordingly, HHSL and BHSL were identified in the extract of *Pseudomonas sp* found to be associated with fermented milk spoilage. Similarly Winson et al. (1995) have also identified these signal molecules in the spent culture supernatant of *P. aeroginosa* by HPLC and EI-MS and have detected *VsmI* gene responsible for their production.



GCMS fragmentation pattern of BHSL



GCMS fragmentation pattern of HHSL

Fig 6.5 : GCMS fragmentation pattern of AHL produced by *Pseudomonas sp*

6.4.3. Optimization and the effect of furanone to inhibit spoilage organism in LB culture medium

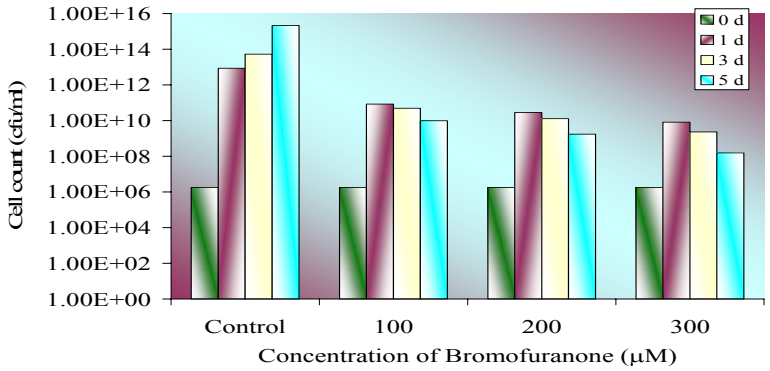
Inhibition of bacterial growth in general is followed by using biocides, antibiotics and bacteriophages (Costerton et al., 1999). But these methods have failed due to non-penetration of biofilm. Cells in a biofilm are in starved state and hence are not susceptible to these antimicrobial agents (Mah and O'Toole, 2001; Stewart and Costerton, 2001; Gilbert et al., 2002). Hence, it is necessary to apply alternative methods for disruption of this biofilm for bacterial inhibition. In this regard signaling pathway has become an important target for inhibition of bacteria. Disruption of quorum sensing may be accomplished by blocking the AHL synthesis, AHL signal molecule degradation and inhibition of AHL receptor activation (Juhas et al., 2005). Hence application of drug that inhibit/ even prevent biofilm formation seems to be a promising approach.

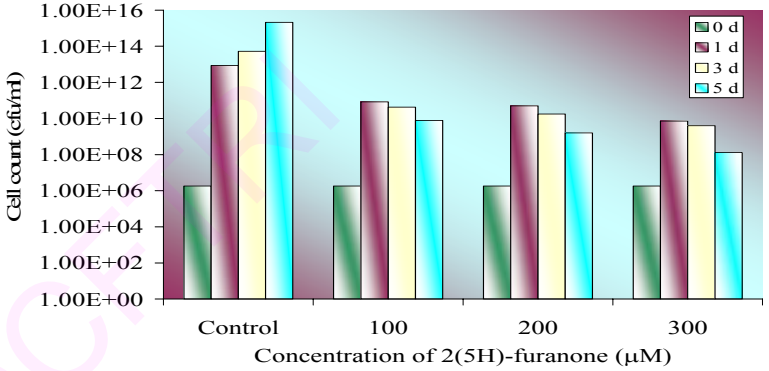
Use of lactonase enzyme (Dong et al., 2002; Reimmann et al., 2002; Park et al., 2003) that cleaves the lactone ring is one of the possible approach for abolishing their activity. But one of the draw back of this approach is the difficulty in delivering active enzyme to the site of infection. In case of las R/las I and rhlR/rhlI expression, the deletion of quorum sensing gene virtually diminishes the expression of las R and reduces the production of virulence factor (Chugani et al., 2001). Another alternative approach is the use of antisense oligonucleotides that specifically binds to las R/las I or rhl R/rhl I transcripts (Kurreck, 2003). Due to time consumtion and other obstacles such as cell wall permeability, specificity and efficacy of mode of delivery these methods are not popular.

Out of all these methods, use of furanone compounds is most significant because of its small size and ease of delivery. Furanones that are produced by *D. pulchra*, a marine alga functions as competitive inhibitors (Givskov et al., 1996; Manefield et al., 2000). They bind to lux R in a mode similar to binding of signal molecule thus inhibiting the transcription of virulence genes (Zhang et al., 2002; Vannini et al., 2002; Koch et al., 2005).

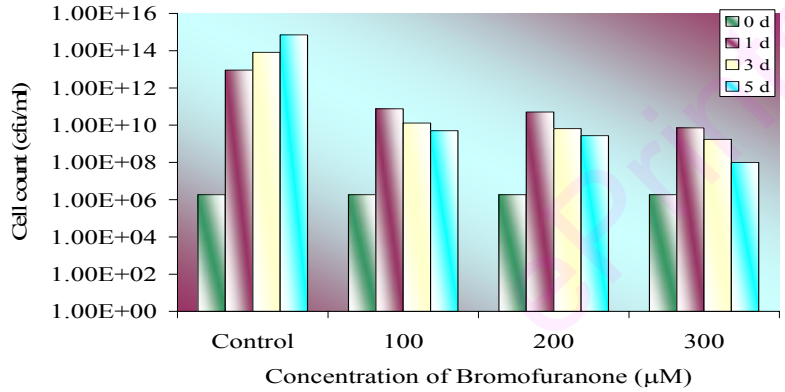
In the present experiment two commercially available furanones Bromofuranone and 2(5H)-furanone were tested to study their efficacy in reducing the growth of spoilage bacterial culture and its virulence expression in LB culture medium.

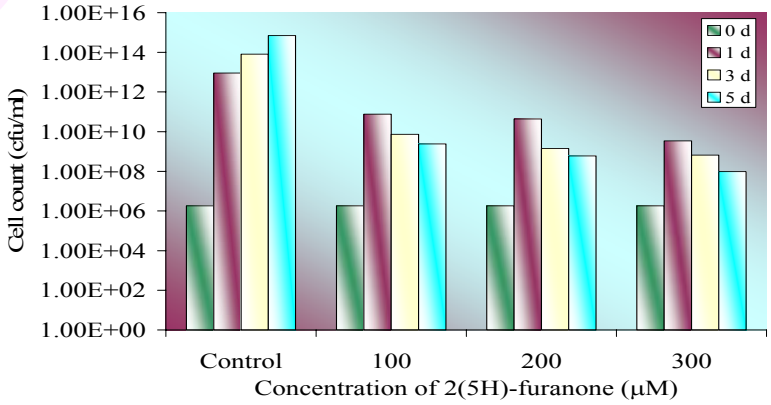
6.4.3.1. ***Effect of furanones on bacterial growth:*** The *Pseudomonas sp* grown in LB broth supplemented with furanones showed reduction in cell count as compared to control grown in absence of furanone (Fig. 6.6). After 5 days of incubation at 4°C, culture supplemented with 2(5H)-furanone (300 µM) reduced from 2.1×10^{15} to 1.3×10^{8} cfu/ml as compared to 2.1×10^{15} to 1.56×10^{8} cfu/ml in case of bromofuranone supplementation (300 µM). At room temperature cell decline was observed from 7.03×10^{14} to 9.8×10^{7} cfu/ml with 2(5H)-furanone and from 7.03×10^{14} to 1.0×10^{8} cfu/ml with bromofuranone. In the present work 2(5H)-furanone is found more effective than bromofuranone in inhibiting *Pseudomonas sp* growth.





Growth of *Pseudomonas sp* at 4°C





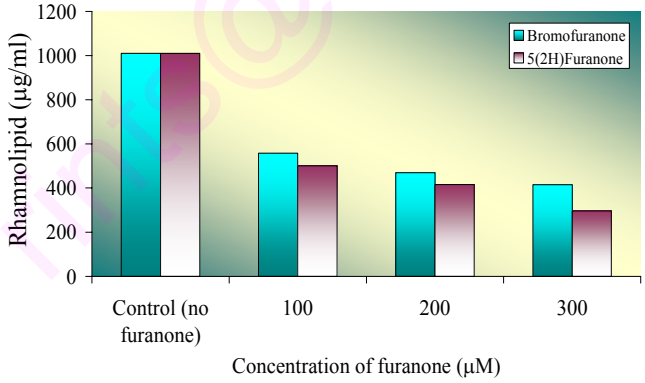
Growth of *Pseudomonas sp* at room temperature (30°C)

Fig 6.6 : Effect of furanone on *Pseudomonas sp* growth in culture media

Further the effect of furanone was confirmed by reduction of rhamnolipid content, motility and exoprotease activity.

6.4.3.2. *Effect of furanone on rhamnolipid content of spoilage bacterial culture (Pseudomonas sp)*: Both las I and las R homologues in *Pseudomonas sp* are required for transcription of rhlA which is responsible for the synthesis of rhamnolipid (Pearson et al., 1997). Therefore the rhamnolipid estimation assay is an indirect method to know the efficacy of furanone in inhibiting las regulator. In our work production of rhamnolipid was significantly ($P<0.05$) inhibited by furanone in a dose depended manner (Fig. 6.7). At 4°C, 2(5H)-furanone (300 µM) inhibited 70.71% of rhamnolipid as compared to the control where as with bromofuranone (300 µM) only 58.9% reduction was observed. At room temperature 2(5H)-furanone (300 µM) exhibited 73.81% reduction where as bromofuranone 64.79% reduction. This particular experiment confirms that 2(5H)-furanone is more efficient than bromofuranone in inhibiting bacterial growth.

(A)



(B)

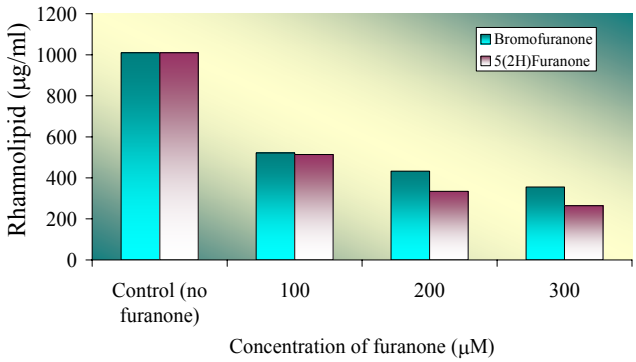
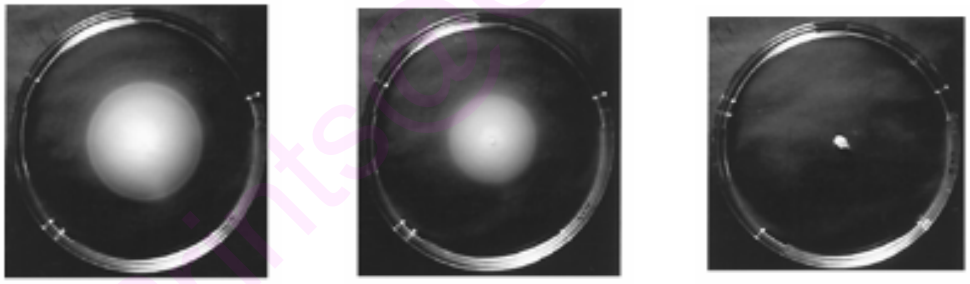


Fig 6.7 : Effect of furanone on rhamnolipid content of *Pseudomonas sp*

(A) *Pseudomonas sp* incubated at 4°C (B) *Pseudomonas sp* incubated at 30°C

6.4.3.3. ***Effect of furanone on Motility of spoilage bacterial culture (Pseudomonas sp)*:** Motility/swarming is a multicellular, density dependent behavior that is induced in response to recognition of surface with a certain viscosity (Givskov et al., 1996). Once the bacteria recognize the appropriate environmental signals, they differentiate into swarming cells (Allison and Hughes, 1991). Hence the effect of 2(5H)-furanone on *Pseudomonas sp* motility was studied using LB agar plates. Results demonstrate the inhibitory activity of *Pseudomonas sp* with furanone (Fig. 6.8). The motility was affected markedly ($P<0.05$) on supplementation of 300 µM furanone (3 mm) as compared to control (20 mm). In a report, Givskov (1996) have demonstrated that furanone inhibits swarming by suppression of the AHL autoinduction circuit. Our results show that furanone inhibits the motility of *Pseudomonas sp* it is highly possible that the furanones may be displacing cell signal from receptor.



No furanone 100 µM furanone 300 µM furanone

Fig 6.8 : Inhibition of *Pseudomonas sp* motility by 2(5H)-furanone

6.4.3.4. ***Effect of furanone on Exoprotease activity/casein hydrolysis of spoilage bacterial culture (Pseudomonas sp)*:** Extracellular proteinase secretion is another important quorum sensing regulated expression in pathogenic cultures. Hobden (2002) and Thibodeaux et al. (2005) have found *P. aeroginosa* to produce protease that is virulent for mice which indicate pathogenesis of *Pseudomonas sp* and that of protease as important factor contributing to the virulence of organism. Hence in the present experiment, the effect of 2(5H)-furanone on exoprotease (casein hydrolase) of isolated

Pseudomonas sp was studied. The ability of the culture to hydrolyze casein on a skimmed milk media plates by forming a clear zone indicated the secretion of protease enzyme. According to the results obtained the zone of clearance was reduced on a dose dependent manner (Fig. 6.9). At 500 μM concentration of 2(5H)-furanone, the inhibition zone was 8 mm as compared to control with 18 mm. Some of the earlier reports have indicated that protease activity in *P. fluorescence* is responsible for food spoilage (Liu and Griffiths, 2003; Jaspe et al., 1995; Picot et al., 2004) therefore the inhibition of its activity with furanone throws light towards the prevention of food spoilage.

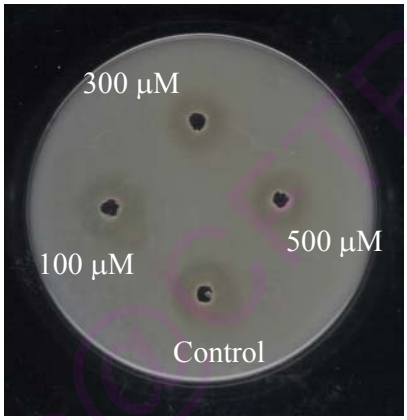


Fig 6.9 : Effect of furanone on exoprotease activity of *Pseudomonas sp*

6.4.4. Growth inhibition of spoilage bacterial culture (*Pseudomonas sp*) in fermented milk by using furanone

As 2(5H)-furanone was found to be effective against *Pseudomonas sp* growth in LB broth the same was used to inhibit its growth in fermented milk. Fermented milk was initially spoilt by adding *Pseudomonas sp* and then supplemented with 2(5H)-furanone (500 and 1000 μM) to study the effect on its growth. *Pseudomonas sp* in the absence of furanone increased from 1×10^6 to 2.2×10^{10} cfu/ml after 3 days of incubation at 4°C and from 1×10^6 to 4×10^{11} cfu/ml at 30°C. When 2(5H)-furanone (1000 μM) was supplemented reduction from 2.2×10^{10} to 5.2×10^6 cfu/ml was observed at 4°C whereas from 4×10^{11} to 4.9×10^5 cfu/ml at 30°C (Table 6.2).

Table 6.2: Effect of 2(5H)-furanone on *Pseudomonas sp* growth in fermented milk

Incubation period (days)	Furanone (μM)	4°C		30°C	
		MRS	PA	MRS	PA
0		1.0×10^5	1.0×10^6	1.0×10^5	1.0×10^6
1	No furanone	1.0×10^5	1.2×10^9	7.5×10^6	7.6×10^9
	500	9.2×10^5	4.0×10^8	7.3×10^6	6.7×10^7
	1000	9.0×10^5	1.0×10^8	7.1×10^6	5.8×10^7
2	No furanone	1.2×10^6	1.3×10^{10}	9.2×10^6	3.4×10^{10}
	500	1.7×10^6	1.4×10^8	9.6×10^6	4.5×10^8
	1000	2.0×10^6	6.1×10^5	9.9×10^6	2.2×10^7
3	No furanone	9.2×10^6	2.2×10^{10}	1.5×10^7	4.0×10^{11}
	500	9.6×10^6	6.6×10^5	2.2×10^7	2.3×10^6
	1000	9.9×10^6	5.2×10^5	2.5×10^7	4.9×10^5

6.4.5. Preservation of fermented milk over shelf life by supplementation of furanone

Effect of furanone in increasing the shelf life of fermented milk was analyzed by incubating the product supplemented with 2(5H)-furanone (1000 μM) at 4 and 30°C for 10 days. In the absence of furanone the product was found to spoil from the 6th day. The spoilage bacterial cultures took over the probiotic culture (M7-PLsr-1$_{(W)}$) on the 9th day. On supplementation of furanone (1000 μM), no spoilage bacterial growth was observed upto 8 and 9 days at 30° and 4°C respectively (Table 6.3). This shows that furanone could enhance the shelf life of fermented milk by 72 h.

Table 6.3 : Preservation of fermented milk with 2(5H)-furanone for longer shelf life

Fermented milk incubated at 4^{o}C

Fermented milk	Media	Incubation time (days)/ colony count (cfu/ml)										
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
No furanone	MRS	1.0×10^5	1.2×10^6	1.2×10^6	1.3×10^6	1.6×10^6	2.1×10^6	2.8×10^6	5.2×10^6	2.4×10^6	1.6×10^5	8.2×10^4
	Selective media	-	-	-	-	-	-	2.0×10^3	3.3×10^5	3.3×10^6	1.7×10^7	6.2×10^8
Furanone (1000 μM)	MRS	1.0×10^5	1.2×10^6	1.2×10^6	1.3×10^6	1.6×10^6	2.1×10^6	2.8×10^6	3.5×10^6	9.8×10^6	2.2×10^7	1.0×10^7
	Selective media	-	-	-	-	-	-	-	-	-	-	<100

Fermented milk incubated at 30^{o}C

Fermented milk	Media	Incubation time (days)/ colony count (cfu/ml)										
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
No furanone	MRS	1.0×10^5	7.2×10^6	1.2×10^7	2.9×10^7	3.0×10^7	3.1×10^7	3.2×10^7	1.8×10^6	8.4×10^5	2.2×10^5	8.2×10^3
	Selective media	-	-	-	-	-	-	4.2×10^3	8.7×10^5	7.8×10^6	3.8×10^7	8.2×10^8
Furanone (1000 μM)	MRS	1.0×10^5	7.2×10^6	1.2×10^7	2.9×10^7	3.0×10^7	3.1×10^7	4.8×10^7	8.5×10^7	2.8×10^8	6.2×10^7	8.6×10^6
	Selective media	-	-	-	-	-	-	-	-	-	<100	2.0×10^2

* (-) indicate no growth. MRS (deMann Rogosa Sharpe media) is for LAB count. Count of the selective media is the total cell count obtained in different media plates (LB, BP, SS, LM and PA).

6.5. Conclusion

The present chapter demonstrates a method for the preservation of fermented milk on shelf storage. *Pseudomonas sp* associated with spoilage of fermented milk was characterized and the signal molecules produced were identified as hexanoyl and butryl homoserine lactone by GC and GCMS method. Further furanones that are having structural similarity to that of AHL was used to inhibit the *Pseudomonas sp* growth. 2(5H)-furanone was found effective in inhibiting the growth and virulence factor expression of *Pseudomonas sp* (rhamnolipid, exoprotease and motility). With addition of 2(5H)-furanone (1000 μM) the shelf life of fermented milk was increased upto 9 days.

Use of such method of inhibiting growth of pathogenic/ spoilage bacteria by using furanone may lead to the development of novel and non-antibiotic method which aims in inhibiting bacterial virulence.

Chapter 7

In-vivo Studies using Leuconostoc for Functional Attributes

CHAPTER - 7

IN-VIVO STUDIES USING *LEUCONOSTOC* FOR FUNCTIONAL ATTRIBUTES

ABSTRACT

Lactose intolerance, a clinical problem associated with unpleasant abdominal discomfort. Novel approaches have been tried as alternative to antibiotics in treating this problem. In the present work *Leuconostoc mesenteroides* M7-PLsr-$1_{(W)}$ has been studied in growing rats for its safety and as a source to treat lactose intolerance. M7-PLsr-$1_{(W)}$ was administered to a group of male rats at a dose of 10^6-10^{16} cfu/ml in the single dose study and the effective dose was found to be 10^8 cfu/ml. In long term study for 90 days, initially the rats were fed with lactose rich diet to induce lactose intolerance. Further induced rats were fed with diet supplemented with effective dose of M7-PLsr-$1_{(W)}$ and four days of probiotic feeding was found good with disappearance of diarrhea. No change in morphology, behavior and treatment related toxicity/ bacterial translocation was observed on probiotic feeding. Transit tolerance and adherence ability of M7-PLsr-$1_{(W)}$ was checked by counting the cell number in caecum and large intestine. Reduction of *E. coli* counts in caecum shows the antimicrobial activity of the culture.

7.1. Introduction

The potential of probiotic lactic acid bacteria has been given a novel approach to functional foods and pharmaceuticals (Kuipers et al., 2000; Renault, 2002). It is important to carefully document the efficacy of the strain for its potential application and safety. Several aspects (functional and technological) are to be considered in the selection of probiotic strains for food application. The functional aspects include survival and adhesion to intestinal epithelium, antimicrobial activity and influence on metabolic activities (Sanders and Huis in't Veld, 1999).

In our laboratory, a lactic acid bacterium *L. mesenteroides* M7-PLsr-$1_{(W)}$ has been selected with high β-galactosidase activity, an enzyme which is responsible for the break down of non-reducing disaccharide lactose into simple sugars for easy absorption (chapter 3).

Lactase/β-galactosidase is normally present in human intestinal epithelial cells and thereby contributes to the digestion of lactose. In persons who are deficient in lactase suffer from unpleasant abdominal pain (Sieber et al., 1997). Temporary lactase deficiency may result from damage of the intestinal lining or alterations in genetic expression of lactase phlorizin hydrolase and is of great concern. Symptoms of lactose intolerance/ maldigestion include loose stools, abdominal bloating and pain, flatulence, nausea, gas production and cramps (Hammer et al., 1996; Sieber et al., 1997). The osmotic pressure of the lactose causes secretion of fluid and electrolyte into intestinal lumen causing diarrhea (Launiala, 1968; Christopher and Bayless, 1971). According to Swagerty et al. (2002) about 70-90% of adults are known to be lactose intolerant.

Approaches have been tried as an alternative to antibiotics in treating lactose intolerance because of the growing antibiotic resistance and side effects caused due to regular consumption of antibiotics (Fuller, 1992). The common therapeutic approach would be to exclude milk and dairy products with lactose from the diet but this will have nutritional disadvantage as it reduces intake of calcium, phosphorus and vitamins (Di Stefano et al.,

2002). Use of lactase enzyme may be less effective probably due to enzyme gastric inactivation (Suarez et al., 1995). Hence the use of probiotic culture may be the alternative for treating lactose intolerance.

In the present work efficacy of M7-PLsr-$1_{(W)}$, its survival in gastrointestinal transit, adherence to the mucosa of gut and as a source for treating lactose intolerance were studied. It is generally accepted that rat is a good animal model for studying interactions between the gut microbes and the host. Although there are some anatomical differences in the gastrointestinal tracts of rat and men, the fecal bacterial populations of the major groups of bacteria is found to be similar (Tannock, 1999) and hence in the present study albino Wister rats were selected as *in-vivo* model. Aims of the present study are to evaluate the safety of culture and to determine digestion of lactose in the intestine for clarifying possible involvement of the culture in reducing lactose intolerance.

7.2. Materials

1) *Animals and diet*: Male albino rats [OUTB-Wister, IND-cft (2c)] weighing 40 ± 2g, were obtained from animal house facility of Central Food Technological Research Institute, Mysore. They were used for experimenting safety and functionalities of M7-PLsr-$1_{(W)}$. They were fed on basal pellet diet (Amrut diet, Sangli, India) and had free access to tap water, *ad libitum*. Institutional animal ethical committee approved the experimental protocol.
2) *Chemicals*: Glucose estimation kit (ERBA diagnostic, Germany), cholesterol estimation kit (Agappe diagnostic, Ernakulam, Kerala), urea estimation kit (Agappe diagnostics, Ernakulam, Kerala), ONPG (HiMedia Pvt Ltd, Mumbai, India), MRS media (as described in chapter 1)
3) *Equipments*: pH meter, Balance, Centrifuge, Spectrophotometer (as described in chapter 2 and 3).

7.3. Methods

7.3.1. Single dose study

The objective of study was to evaluate the safety aspects of culture and to study its functional attributes. Further, study was carried out to determine the dosage required for maximum production of β-galactosidase. Rats (n=6/group) were statistically grouped by randomized design and assigned to various groups. They were housed individually in stainless steel cages. A dose of probiotic culture (M7-PLsr-$1_{(W)}$) suspended in skim milk (2 ml) was administered intragastrically at a concentration of 10^6, 10^8, 10^{10}, 10^{12}, 10^{14} and 10^{16} cfu/ml. Rats (n=6) receiving only skim milk without culture was considered as control group. Animals were observed for morphological, behavioral changes if any and body weight was recorded. The maximum tolerated dose and LD_{50} values were calculated according to the method of Litchfield and Wilcox (1949).

7.3.2. Long term studies

The objective of the study was to evaluate the efficiency of the culture in reducing lactose intolerance. Rats (n=36) were housed individually in stainless steel cages in a room maintained at 25 ± 2°C, a relative humidity of 45-70% and under 12:12 h light and dark cycle. They were fed with 40% lactose with basal diet for 7 days to induce lactose intolerance. Appearance of diarrhea was monitored during lactose induction period. Occurrence of 100% diarrhea was considered as lactose intolerant rats. A separate group (n=6) was killed at 0 day for baseline data and considered as control. Lactose intolerance induce rats were statistically divided into 5 groups (n=6/group). Group one was killed at 0 day after induction of lactose intolerance. The other groups were fed with fresh basal diets supplemented with effective dose (high β-galactosidase activity) of culture isolate (10^8 cfu/ml). During the experimental run rats were monitored for the disappearance of diarrhea and weekly gain in body weight was recorded. During the course of the experiment all the animals were carefully monitored for any signs of change in morphology and behavior. At the end of each experiment (15, 30, 60 and 90 days) animals were fasted overnight

and humanly sacrificed under ether anesthesia. Blood was drawn by cardiac puncture and serum was separated by centrifugation at 2,500 rpm for 10 min. Tissues (liver, kidney, spleen, heart, ceacum, small and large intestine) were collected, rinsed with ice-cold saline, weighed, blotted and stored at -20^{0}C until analyzed.

(1) Clinical chemistry

(a) Estimation of serum glucose

Serum glucose was estimated by using commercially available glucose estimation kit. The principle behind the reaction is the action of glucose oxidase and peroxidase enzyme which gives an intensive pink color to reaction mixture which is proportional to glucose concentration and can be measured spectrophotometrically at 510 nm.

The reaction is as follows

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O$$

$$H_2O_2 + \text{4HBA} + \text{4AAP} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 2H_2O$$

4HBH: 4-hydroxy benzoic acid

4AAP: 4-Amino antipyrine

Calculation

$$\text{Glucose (mg/dL)} = \frac{\text{(absorbance of sample)}}{\text{(absorbance of standard)}} \times \text{concentration of standard (mg/dL)}$$

(b) Estimation of urea

Serum urea was determined by using commercially available kit. Enzymatic determination of urea is according to the following reaction

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{Salicylate} \xrightarrow{\text{Nitro prusside hypochlorite}} \text{2,2-dicarboxyindophenol}$$

The dye 2,2-dicarboxylindophenol gives a light green color product which can be measured spectrophotometrically at 600 nm.

Calculation

$$\text{Urea (mg/dL)} = \frac{\text{(absorbance of sample)}}{\text{(absorbance of standard)}} \times 40$$

*(C) Estimation of cholesterol***:** Blood samples were collected from rats by arterial puncture and the serum was separated by centrifugation. Serum cholesterol was estimated by the modified method of Roeschlau's method using commercially available cholesterol estimation kit. The reaction is as follows-

$$\text{Cholesterol ester} \xrightarrow{\text{CE}} \text{Cholesterol + fatty acid}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\text{CHOD}} \text{Cholest-4-en-3-one + } H_2O_2$$

$$2H_2O_2 + \text{4AAP + phenol} \xrightarrow{\text{POD}} 4H_2O + \text{quenoneimine}$$

where, CE: Cholesterol esterase
CHOD: Cholesterol oxidase
4AAP: 4-Aminoantipyrine

Calculation

$$\text{Cholesterol (mg/dL)} = \frac{\text{(absorbance of sample)}}{\text{(absorbance of standard)}} \times \text{Concentration of standard (mg/dL)}$$

(2) Measurement of pH and cell count

Transit tolerance of the culture through gastrointestinal tract condition was determined by the cell count in caecum, feces and large intestine samples. Approximately, one gram each sample was suspended separately in saline (0.85% NaCl), serially diluted and plated for LAB on MRS agar and for *E. coli* on MacConkey's agar media. After 24 h of incubation period at 37°C, colonies grown were counted and expressed as colony forming unit/ g of sample. pH of urine, caecum and feces were measured using pH meter.

(3) Urine analysis

In the last week of experiment urine samples were collected over a period of 24 h from each rat and examined for pH, glucose and urea using standard procedure as described earlier.

(4) *β*-galactosidase activity

Small and large intestines were brought to room temperature and were cut to required size (60 and 5 cm respectively), washed thoroughly by flushing saline into the intestinal lumen using a syringe. Carefully intestine was cut open and scrapped for the intestinal content and suspended in 5 ml saline. The suspension was homogenized and centrifuged (8000 rpm for 15 min) to collect a clear supernatant. An aliquot (100 µl) of this supernatant was used for the estimation of *β*-galactosidase enzyme using ONPG as substrate (Bhowmik and Marth 1989) and expressed as specific activity (µM/mg protein). Enzyme activity in caecum was estimated by suspending caecum (1 g) in 5 ml saline and determining specific activity as described elsewhere. Protein was estimated by Lowry's method (Lowry et al., 1951).

(5) Bacterial translocation

Bacterial translocation was analyzed in blood, liver and spleen. Blood (100 µl) was cultured on MRS agar and incubated at 37°C to observe for the bacterial growth. Liver and spleen (1 g each) were homogenized in saline (1 ml) and 100 µl of the resulting homogenate was plated on MRS media as mentioned above. After 24 h of incubation at 37°C, colonies grown were counted and the results were expressed as incidence of translocation i.e., number of rats where translocation was detected/ total number of rats.

7.4. Results and Discussion

Probiotic are viable microbes which beneficially influence the health of the host (Schrezenmeir and de Vrese, 2001). Several beneficial effects of these organism in gut has been reported which include growth promotion, protection from pathogens, alleviation of lactose intolerance, relief of constipation, anticholesterolaemic effect and immunostimulation (Casas and Dobrogosz, 2000; Bertazzoni et al., 2001; Aattouri et al., 2002). To exert all these functional properties, the probiotic culture should be able to survive in GIT, produce antimicrobial compounds and must adhere to the intestinal cells of the host. *In-vivo* studies are a prerequisite to confirm the safety and health benefits of M7-PLsr-$1_{(W)}$.

General health status

On feeding *L. mesenteroides* (M7-PLsr-$1_{(W)}$), no noticeable abnormal activity or behavioural changes were observed in the rats and no mortality occurred. There was no difference in animals' aggressivity between treatment and control groups. Similar reports on general health status of rats have been mentioned earlier by Choi et al. (2005) and Lara-Villoslada et al. (2007).

7.4.1. Single dose study

Oral administration of culture has no adverse effect ($P>0.05$) on food intake as compared to the control. The LD_{50} and maximum tolerable dose of culture were worked out to be $>10^{12}$ cfu/ml and $>10^{14}$ cfu/ml respectively (Table 7.1). The effective dose for higher β-galactosidase was determined to be 10^8 cfu/ml. Mild diarrhea was observed only at higher doses (10^{16} cfu/ml) which was transient and disappeared within 4-6 h. No mortality and clinical sign of toxicity was observed during the experimental period. Food consumption was normal and comparable with that of control animals. Zhou et al. (2000) have reported administration of *L. rhamnosus, L. acidphilus* and *B. lactis* at a rate of 10^{11} cfu/day to be safe. Larsen et al. (2006) have determined the tolerable dose (10^{11} cfu/ml) of *B. animalis* and *L. paracasei* in young adults.

Table 7.1 : Toxicity of M7-PLsr-$1_{(W)}$ in experimental rats

Mortality (%)	LD_{50}	Maximum tolerable dose	Remarks
Nil	$> 10^{12}$ cfu/ml	$> 10^{14}$ cfu/ml	Mild diarrhea was observed only at higher doses and was transient and disappeared after 4-6 h of post treatment

(a) Serum glucose and urea

Probiotic treatment induced a dose dependent increase in urea concentration in the serum (Table 7.2) indicating the protein digestion. Maximum serum glucose (60 mg/dL) was observed in group fed with 10^8 cfu/ml of M7-PLsr-$1_{(W)}$. On further increase of culture concentration (>10^8 cfu/ml), the glucose concentration reduced in the serum. This can be correlated with that of elevated β-galactosidase activity ($P<0.05$) in the caecum compared with those of other concentrations (Fig. 7.1).

Table 7.2 : Glucose and Urea in serum of experimental rats fed with M7-PLsr-$1_{(W)}$

Group	Increase (%)	
	Glucose	**Urea**
Group I (10^6 cfu/ml)	29.46 ± 7.46	29.23 ± 0.81
Group II (10^8 cfu/ml)	58.43 ± 5.00	38.36 ± 2.33
Group III (10^{10} cfu/ml)	28.52 ± 1.36	38.84 ± 0.58
Group IV (10^{12} cfu/ml)	23.23 ± 4.98	39.74 ± 0.34
Group V (10^{14} cfu/ml)	19.17 ± 5.02	61.24 ± 1.08
Group VI (10^{16} cfu/ml)	08.45 ± 1.22	64.72 ± 2.12

*values are mean ± SD (n=6) after baseline corrected. Control group had a serum glucose concentration of 37.87 mg/dL and urea was 12.28 mg/dL.

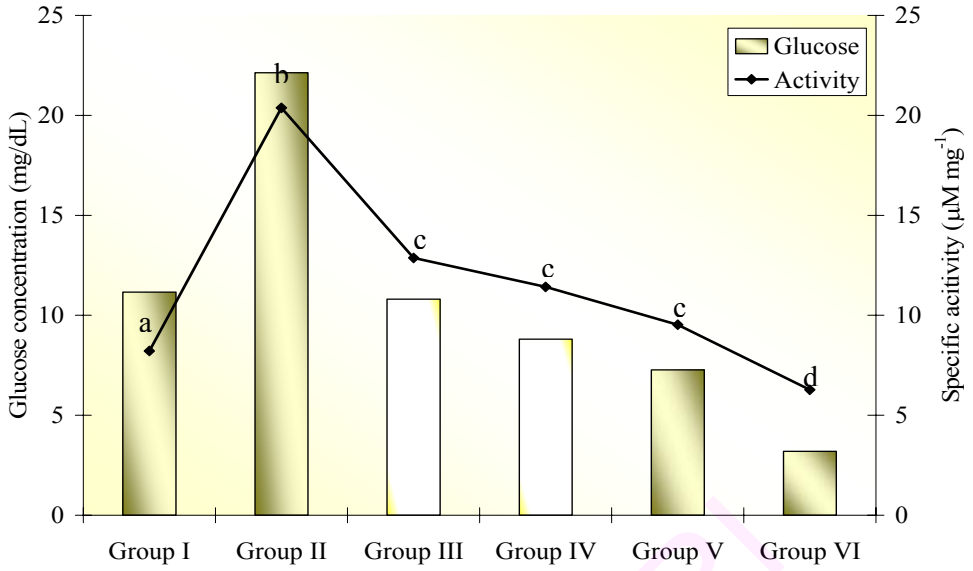


Fig 7.1 : Serum glucose concentration and β-galactosidase activity in caecum of M7-PLsr-1$_{(W)}$ fed rats.

Group I, II, III, IV, V and VI are rats fed with probiotic culture at a concentration of 10^6, 10^8, 10^{10}, 10^{12}, 10^{14} and 10^{16} cfu/ml respectively. Values are mean ± SD (n=6). The values not sharing a common letter are significantly different ($p<0.05$) between groups as determined by ANOVA.

(b) Probiotic influence on serum cholesterol

The effect M7-PLsr-1$_{(W)}$ feeding on serum cholesterol of rats was evaluated in the present work. Maximum reduction (40.59%) of serum cholesterol was observed in group fed with 10^8 cfu/ml as compared to control group (Fig. 7.2). The dose exhibiting higher β-galactosidase activity is relatively lower than dose used by Hashimoto et al. (1999) wherein they have reported hypocholesterolemic action of *L. casei* with a dose of 10^{11} cfu/ml. Usman and Hosono (2001) have reported *L. gasseri* to exert hypocholesterolemic activity with 10^9 cfu/ml. Du Toit et al. (1998) observed a decrease in serum cholesterol after administration of mixture of *L. johnsonii* and *L. reuteri* at dose (10^{12} cells/ day) in pigs whereas Taranto et al. (1998) have reported the effect of *L. reuteri* at a dose of 10^4 cells/day in mice. Variation in the effective doses may be due to difference in strains of lactic acid bacteria and animal model. The present culture is able to reduce serum cholesterol in rats from 51.01 mg/dL to 30.31 mg/dL on feeding a dose of 10^8 cfu/ml. This shows the potential hypocholesterolemic activity of the culture which imparts its importance to reduce cholesterol level.

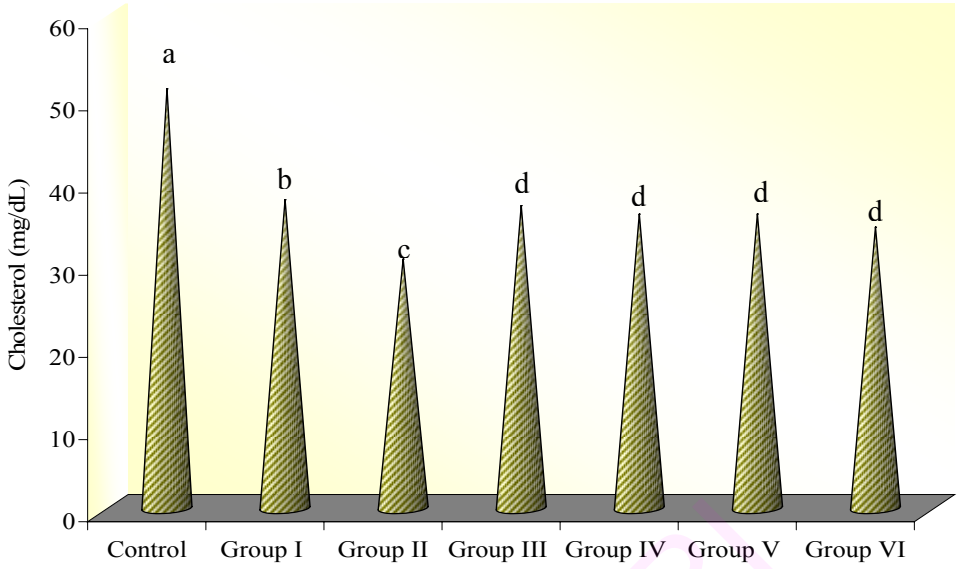


Fig 7.2 : Effect of *L. mesenteroides* (M7-PLsr-1$_{(W)}$) on serum cholesterol of experimental rats.

Group I, II, III, IV, V and VI are rats fed with probiotic culture at a concentration of 10^6, 10^8, 10^{10}, 10^{12}, 10^{14} and 10^{16} cfu/ml respectively. Values are mean ± SD (n=6). The values not sharing a common letter are significantly different ($p<0.05$) between groups as determined by ANOVA

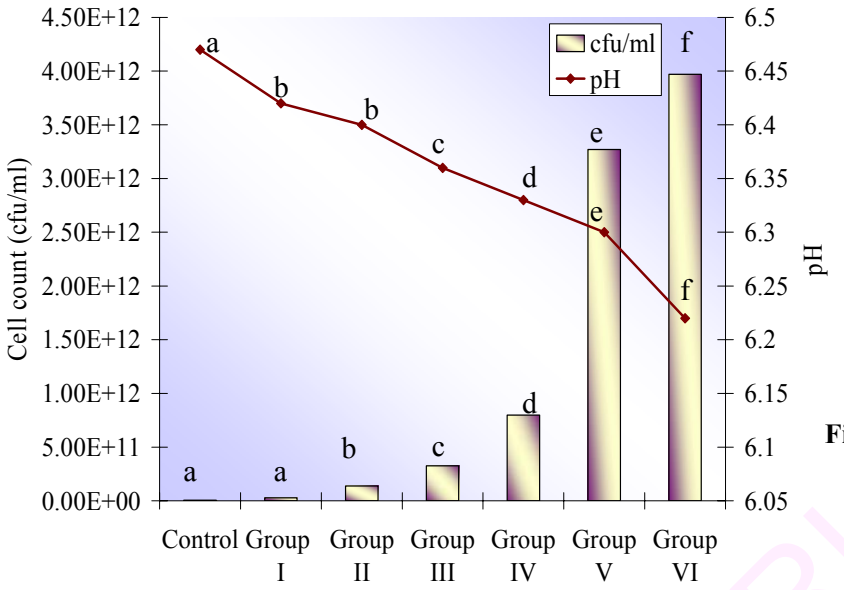
(c) Probiotic transit tolerance and adherence

The increased microbial load in caecum is directly proportional to the concentration of culture ingested and increase in the caecum weight (Table 7.3). This determines that the culture was able to survive the transit condition and reach the intestine to maintain microbial balance. The reduction in pH also signifies the growth of culture in the caecum (Fig. 7.3). Adherence is also evident from the SEM preparation of intestinal section of probiotic fed rats (Fig. 7.4).

Table 7.3: Caecum analysis of experimental rats

Group	*Weight of caecum (g)	$^{\delta}$Cell count (cfu/ml)	*pH
Control (no probiotic)	8.80 ± 0.07	6.63×10^9	6.47 ± 0.11
Group I (10^6 cfu/ml)	9.00 ± 0.12	2.80×10^{10}	6.42 ± 0.12
Group II (10^8 cfu/ml)	9.20 ± 0.04	1.40×10^{11}	6.40 ± 0.12
Group III (10^{10} cfu/ml)	9.70 ± 0.04	3.27×10^{11}	6.36 ± 0.18
Group IV (10^{12} cfu/ml)	10.2 ± 0.02	8.00×10^{11}	6.33 ± 0.11
Group V (10^{14} cfu/ml)	10.5 ± 0.04	3.27×10^{12}	6.30 ± 0.11
Group VI (10^{16} cfu/ml)	10.6 ± 0.06	3.97×10^{12}	6.22 ± 0.15

*Values are mean ± SD (n=6). $^{\delta}$Values are mean of duplicate analysis



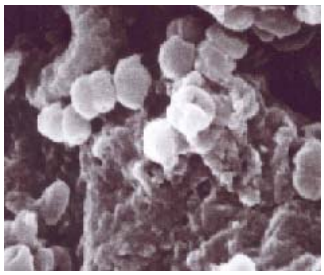


Fig 7.4: SEM photograph of probiotic fed rat intestine showing adhesion of probiotic culture

Fig 7.3 : pH change and LAB count in caecum on feeding *L. mesenteroides* (M7-PLsr-1$_{(W)}$).

Group I, II, III, IV, V and VI are fed with probiotic culture at a concentration of 10^6, 10^8, 10^{10}, 10^{12}, 10^{14} and 10^{16} cfu/ml respectively. Values are mean ± SD (n=6). The values not sharing a common letter are significantly different ($p<0.05$) between groups as determined by ANOVA

(d) β-galactosidase activity in caecum

Table 7.4 represents the β-galactosidase activity in caecum of probiotic fed rats. Maximum enzyme activity (48.54 ± 2.02 µM/mg protein) was observed in the group fed with 10^8 cfu/ml of probiotic culture Lsr-1$_{(W)}$ which was 1.7 folds more as compared to control group (no probiotic fed). With further increase of culture concentration, a reduction in enzyme activity was observed. This may be due to increase of digestible glucose concentration that can act as a repressor for β-galactosidase synthesis.

Table 7.4 : β-galactosidase activity in caecum on feeding *L. mesenteroides* (M7-PLsr-1$_{(W)}$)

Group	Specific activity (µM/mg protein)
Group I (10^6 cfu/ml)	36.38 ± 5.95
Group II (10^8 cfu/ml)	48.54 ± 2.02
Group III (10^{10} cfu/ml)	41.04 ± 5.06
Group IV (10^{12} cfu/ml)	39.59 ± 0.32
Group V (10^{14} cfu/ml)	37.69 ± 0.45
Group VI (10^{16} cfu/ml)	34.44 ± 1.25

*values are mean ± SD (n=6). Control group (no probiotic fed) had an enzyme activity of 28.17 µM/mg protein.

7.4.2. Long term study

General conditions of rats were normal and no animals died during 90 days feeding with M7-PLsr-$1_{(W)}$.

Initially for induction of lactose intolerance, rats were fed with lactose rich diet (40%) for one week. Occurrence of diarrhea was found from the day 4 and it remained till one week. All the lactose intolerance induced rats were subsequently fed with basal diet supplemented with effective dose as determined from the single dose study (10^8 cfu/ml). Diarrhea was observed to disappear within 4 days of probiotic feeding.

(a) Gain in body weight of experimental rats

During lactose induction period there was 5.7% reduction in body weight. The weight of the vital organs like lung, heart and spleen slightly decreased ($p>0.05$) whereas weight of kidney increased by 0.9 g/Kg body weight (Table 7.5). However significant ($P<0.05$) increase in the caecum weight of rats was observed (2.9-9.0 g/kg body weight) which can be attributed for enhanced growth of the microbial flora and their fermentation activity in presence of lactose. On subsequent feeding of the culture an increase in the body weight was observed. Hadani et al. (2002) have observed gain in the body weight of pig (21g) on probactrix administration. Similarly, Aboderin and Oyetayo (2006) have observed gain in the body weight with 10^7 cfu/ml of probiotic culture, whereas no significant ($p>0.05$) difference was observed in growth rate and weight gain of rats by Choi et al. (2005) and Lara-Villoslade et al. (2007) on probiotic administration.

Table 7.5 : Weight range of experimental rats on probiotic feeding

Incubation time (days)	Gain in body weight (g)	Gain in the weight of vital organs (g/kg body weight)					
		Lungs	Kidney	Heart	Liver	Spleen	Caecum
Lactose induction (0 day)	-2.6 ± 1.0	-0.2 ± 1.7	0.9 ± 0.2	-0.4 ± 1.5	0.0	-0.1 ± 1.5	9.0 ± 1.7
15	3.20 ± 0.2	-0.1 ± 0.1	0.9 ± 0.3	-0.2 ± 1.8	1.0 ± 0.3	0.1 ± 0.6	5.2 ± 5.7
30	6.23 ± 0.4	0.1 ± 0.6	1.0 ± 0.5	-0.1 ± 1.5	1.8 ± 0.6	0.2 ± 0.7	3.1 ± 1.4
60	6.57 ± 0.4	0.3 ± 0.7	1.2 ± 0.1	0.1 ± 2.3	3.1 ± 0.7	0.25 ± 0.4	2.9 ± 0.4
90	7.60 ± 0.9	0.4 ± 1.8	1.5 ± 0.6	0.6 ± 1.5	0.9 ± 0.4	0.25 ± 1.3	2.9 ± 1.2

*Values are mean ± SD after baseline corrected. Negative reading indicates the loss in the weight in comparison with the control group. Weight (g) of control group: body weight (45 ± 1.1); lungs (4.6 ± 0.7); Kidney (7.3 ± 0.8); Heart (3.2 ± 0.4); Liver (12.5 ± 1.8); Spleen (2.1 ± 0.3); Caecum (20.8 ± 1.7).

(b) Caecum, feces and urine *pH*

Table 7.6 represents the change in the pH of urine, feces and caecum during experimental period. There is a reduction in the pH in all the three cases on probiotic feeding with respect to their control groups. This may be due to increase of lactic acid producing microflora in the GIT. Similarly, Liong and Shah (2006) have observed a gradient decrease in pH value of caecum (6.43 to 6.28) and feces (8.13 to 7.8) on feeding *L. casei*. They have attributed the change in the pH as a cause of lactic acid bacterial growth.

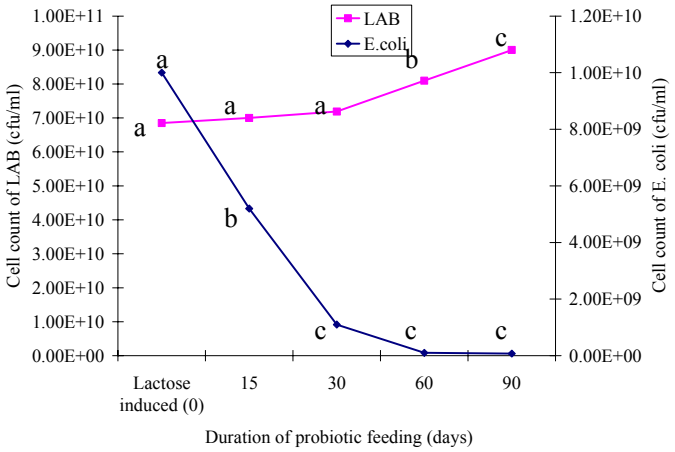
Table 7.6 : pH change during probiotic feeding for 90 days

Incubation time (days)	pH		
	Urine	Feces	Caecum
Control	8.65 ± 1.1	7.04 ± 0.2	6.52 ± 1.2
Lactose induced (0)	8.28 ± 0.2	6.74 ± 2.0	6.35 ± 0.2
15	8.12 ± 0.5	6.50 ± 1.2	6.32 ± 0.2
30	7.97 ± 0.6	6.60 ± 0.6	6.31 ± 0.1
60	7.71 ± 1.6	6.35 ± 0.5	6.20 ± 0.3
90	6.49 ± 1.2	6.15 ± 0.6	6.05 ± 0.2

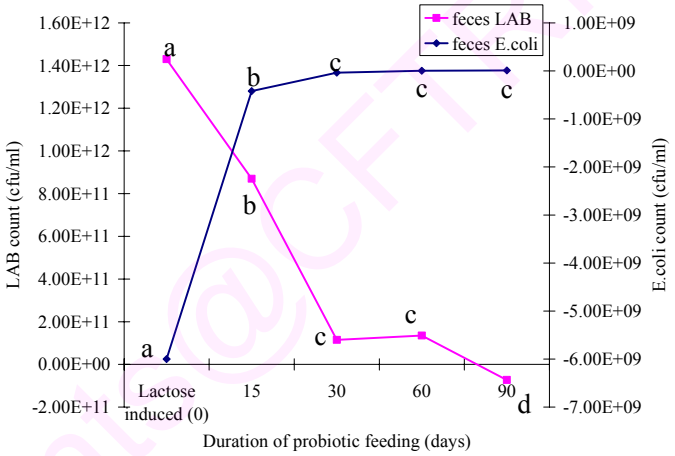
* Values are Mean ± SD (n=6).

(c) Probiotic adhesion and influence on intestinal E. coli

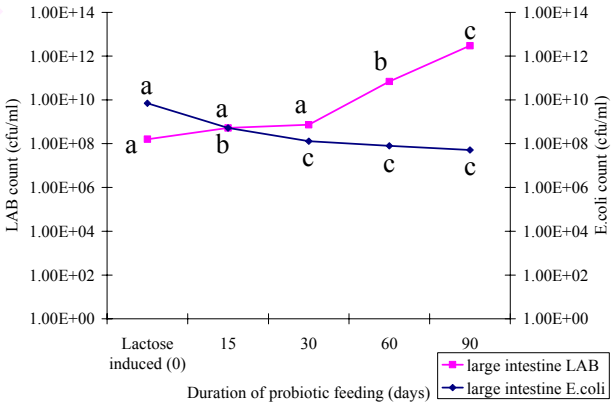
Administration of probiotic culture increased LAB count with the reduction of *E. coli* count in caecum as well as in large intestine in a time dependent manner (Fig. 7.5) whereas in feces the *E. coli* count was higher indicating the excretion of strain from intestine. These results suggest the ability of *L. mesenteroides* M7-PLsr-$1_{(W)}$ to survive the gastrointestinal tract and compete with other microorganisms within the gut environment. Scanning electron microscopic examination showed the culture adhered to intestine of rats (Fig. 7.6). Higher microbial count in caecum and large intestine show *in-vivo* adhesion ability of the probiotic strain. Lesniewska et al. (2006) and Frece et al. (2005) have also observed one log reduction in *Enterobacteriaceae* on feeding probiotic lactic acid bacteria. In the present work a 3 log reduction in the *E. coli* was observed in the caecum on probiotic feeding.



(A)



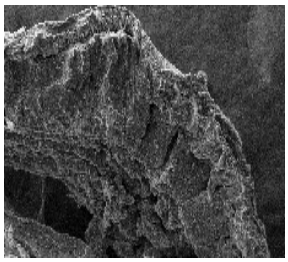
(B)



(C)

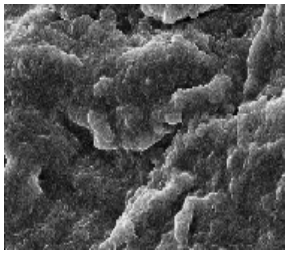
Fig 7.5 : Probiotic influence on *E. coli*

(A) Caecum, (B) Feces, (C) Large intestine. Values are mean ± SD (n=6). Baseline corrected. The values at each time point not sharing a common letter are significantly different ($p<0.05$) as determined by ANOVA. Negative reading indicates a lesser cell count than the control group.

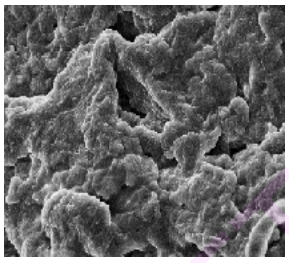


LI section (control group)

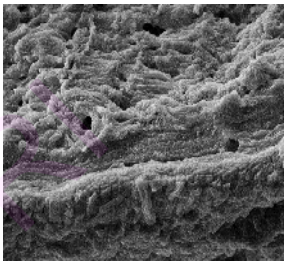
Lactose intolerance induced rat LI section



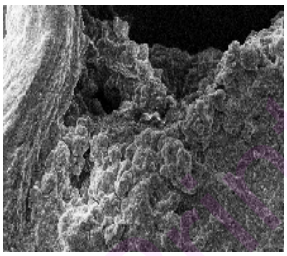
LI-control (30 d)



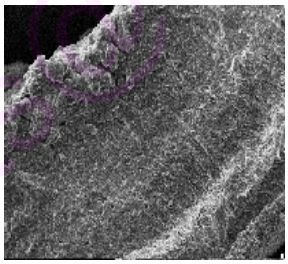
LI-probiotic fed (30 d)



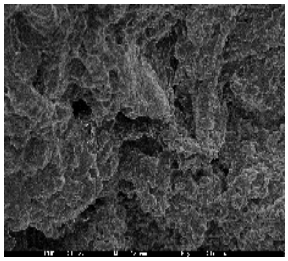
Caecum- probiotic fed (30 d)



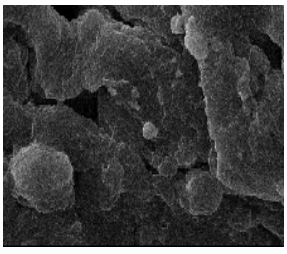
LI-control (60 d)



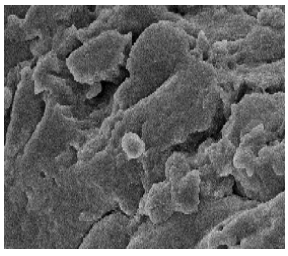
LI-probiotic fed (60 d)



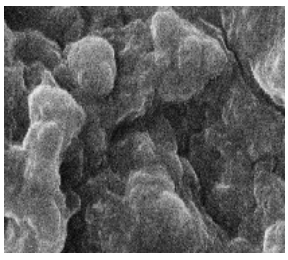
Caecum- probiotic fed (60 d)



LI-control rat (90 d)



LI-pro fed rat (90 d)



Caecum- probiotic fed (90 d)

Fig 7.6 : SEM photograph showing adhesion of probiotic culture to large intestine (LI) and caecum of experimental rats

(d) Glucose and urea concentration in serum and urine

Results of serum glucose in rats fed on probiotic are given in table 7.7. It could be seen that lactose fed animals had 57.39% increase in serum glucose concentration as compared to control which may be attributed to the action of lactase enzyme present in intestinal lining. This is in accordance with the work of Patrick et al. (2000) who has observed *S. thermophilus* release substantial amount of glucose when grown in presence of lactose. Leichter et al. (1984) have also observed an increase in serum glucose in rats fed on lactose diet. On administration of probiotic feed there was a gradual reduction in the glucose concentration. This confirms the emptying of lactose content in the intestine through the action of probiotic lactase activity which is in accordance with increase in lactase activity in the caecum (Fig. 7.7). Later after 90 days, the glucose concentration of probiotic fed rats was far with control group. This indicates complete hydrolysis of lactose.

Urea concentration in serum and urine was increased during lactose induction period which shows the protein utilization by animal system (Table 7.7) as suggested by Morens et al. (2003). On further feeding with probiotic culture, there was a reduction in urea content because rats at this stage may be able to utilize the carbon source for metabolism.

Table 7.7 : Glucose and urea concentration in serum and urine of M7-PLsr-1$_{(W)}$ fed rats

Probiotic feeding (days)	Increase in glucose concentration (mg/dL)		Increase in urea concentration (mg/dL)	
	Serum	Urine	Serum	Urine
Lactose induction	29.9 ± 1.6	8.3 ± 0.7	4.6 ± 1.8	62.0 ± 2.5
15	15.5 ± 1.3	4.6 ± 0.5	5.2 ± 1.4	59.2 ± 0.7
30	05.9 ± 1.3	3.3 ± 0.3	6.6 ± 1.9	53.7 ±1.9
60	04.2 ± 1.5	1.9 ± 1.5	8.0 ± 2.4	44.0 ± 1.4
90	04.0 ± 2.0	1.8 ± 0.9	8.7 ± 2.0	22.0 ± 2.4

*Values are mean ± SD (n=6).
Values are obtained by subtracting experimental values with that of control. Control group: serum (glucose-52.16; urea-27.6 0.7) and urine (glucose-61.6 0.7; urea-102 0.3)

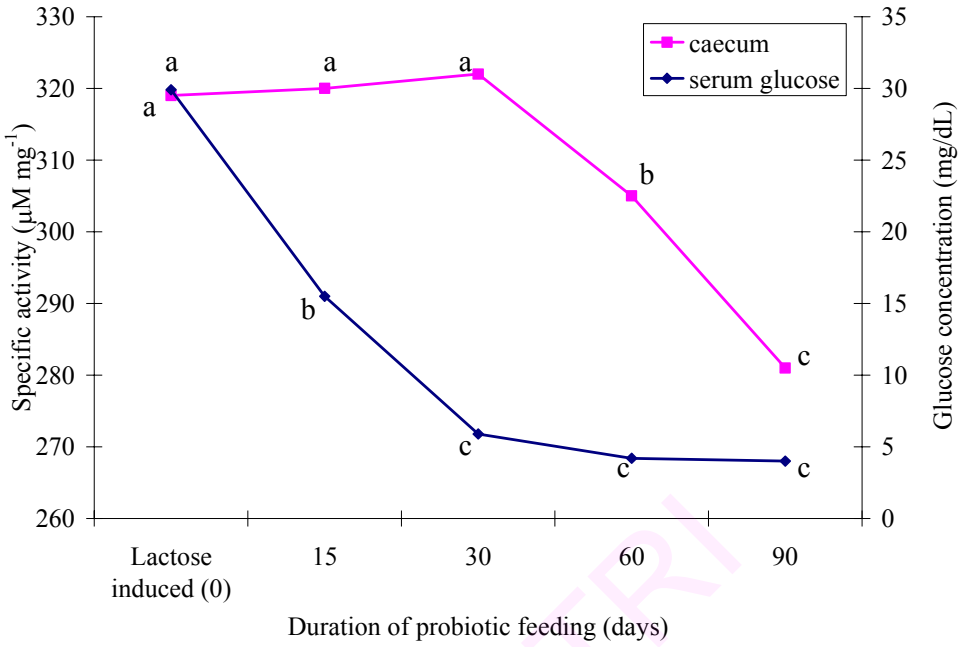


Fig 7.7 : Serum glucose concentration and the enzyme activity in the caecum of experimental rats.

Values are obtained by subtracting experimental values with that of control. The values at each time point not sharing a common letter are significantly different ($p<0.05$) as determined by ANOVA

(*e) β-galactosidase activity*

Figure 7.8 represents the *β*-galactosidase activity in small intestine and caecum of control and experimental animal. This enzyme activity is directly proportional to the lactose hydrolysis. Similarly Leichter et al. (1984) have observed a linear regression between lactase activity and lactose absorption. In the present work, during lactose induction period an increase in enzyme activity was observed in caecum which is evident from the increase in probiotic microflora. In small intestine, decrease in enzyme activity may be due to reduction in intestinal lactase activity as compared to control group. On further feeding (90 days), the enzyme activity was comparatively similar with that of control group indicating complete hydrolysis of lactose. In small intestine a significant increase ($p<0.01$) in the activity was observed after 30 days of probiotic feeding. This indicates emptying of lactose and regeneration of lactase activity in the intestinal lining.

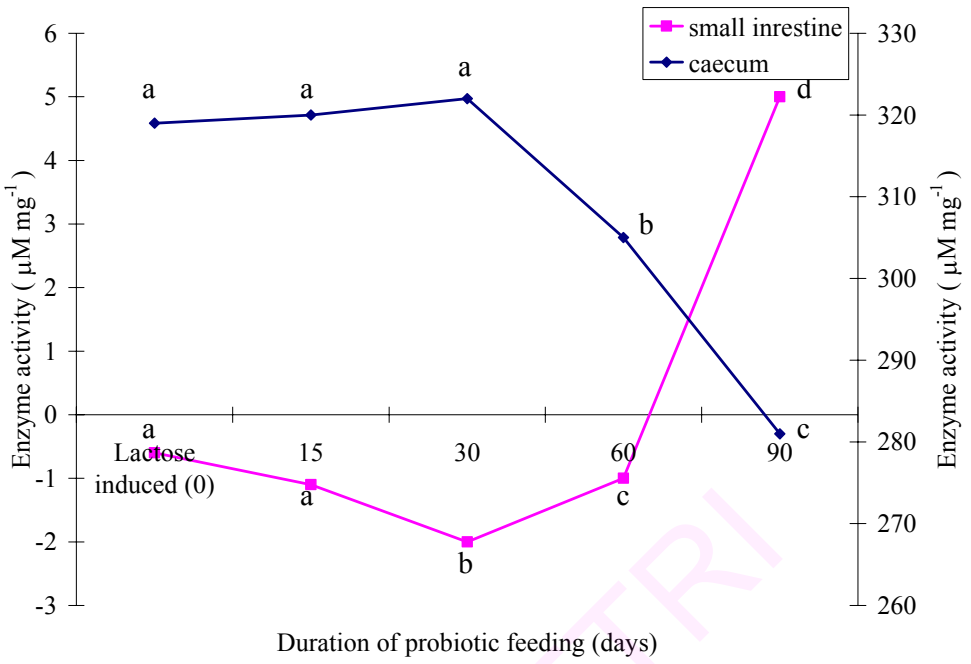


Fig 7.8: *β*-galactosidase activity in caecum and small intestine of experimental rats.

Values are obtained by subtracting experimental values with that of control**.** The values at each time point not sharing a common letter are significantly different ($p<0.05$) as determined by ANOVA.

(f) Bacterial translocation

The incidence of translocation of bacteria from gut to different tissues was determined by culturing the samples of liver, blood and spleen on MRS-media. No growth was seen in any of the samples. Bacterial translocation is known to be potential indicator of culture toxicity as it is the first step in the pathogenesis process (Duffy et al., 1999; Zhou et al., 2000). In the present study, inspite of high doses of probiotic administration there was no bacterial growth in any of the tested organs. These data suggest that oral administration of the present probiotic culture M7-PLsr-$1_{(W)}$ does not increase the bacterial translocation either to blood, spleen or liver which confirms the safety of the culture.

Some of the earlier reports have assayed the safety and probiotic potential of certain lactic acid bacterial species (Frece et al., 2005; Lar-Villoslada et al., 2007). This is the first *in-vivo* report with probiotic *L. mesenteroides* for the analysis of safety and functional properties.

Additionally the culture has been evaluated for its ability to reduce lactose intolerance problem. Earlier experiments in animals have indicated that kefir cultures play important role in improving lactose digestion (de Vrese et al., 1992). Goodenough and Kleyn (1976) have studied the influence of viable yogurt culture on digestion of lactose by the rats. Rats were fed on yogurt supplemented with lactose and sucrose for 7 days. Assay showed that lactase activity was more in animals fed with yogurt culture. Studies in lactase deficient humans confirm that yogurt can reduce lactose intolerance (Savaiano and Levitt, 1987).

Shah and Jelen (1991) studied the lactose absorption by post weaning rats and found that lactose digestion from yogurt and guarg was facilitated and the assay confirmed the presence of viable cultures and *β*-galactosidase activity after feeding. A study was designed to determine the effect of oral feeding of *L. acidophilus* on lactose tolerance by Saltzman et al. (1999). The culture was fed twice a day upto 7 days to 42 patients with lactose intolerance but they found that there was no significant change in breath hydrogen excretion. In the present study there was reduction in diarrhea on probiotic feeding. After 4 days of probiotic feeding to lactose intolerant induced rats there was disappearance of diarrhea. This shows that the present probiotic isolate Lsr-$1_{(W)}$ has potential in treating lactose intolerance.

7.5. Conclusion

From the study it can be concluded that oral feeding of probiotic culture exerts beneficial effect on experimental rats. Feeding probiotic culture *L. mesenteroides* (M7-PLSr-$1_{(W)}$) has no harmful effect on the animals either morphologically or behaviorally. Hence, the culture is safe and is able to resist the gastrointestinal conditions. The culture shows transit tolerance in gastrointestinal tract. The culture is able to adhere and has antimicrobial activity against the intestinal *E. coli*. Reduction in the number of *E. coli* counts in caecum and large intestine supports that the culture maintains healthy intestinal microflora. High *β*-galactosidase activity of the culture reduces the accumulation of lactose as observed from the experimental results.

Summary & Conclusions

SUMMARY AND CONCLUSION

- Bacterial cultures were isolated from milk and milk products and screened for lactic acid bacteria and were adapted to tolerate gastrointestinal conditions. The strain that was able to survive under such environment was characterized and identified through biochemical assays and molecular methods as *Leuconostoc mesenteroides* (PLsr-$1_{(W)}$).
- The selected culture (PLsr-$1_{(W)}$) exerts antimicrobial activity against seven toxic food pathogens and was resistant to three common antibiotics. The antioxidative activity, serum cholesterol reducing ability, adherence ability, production of therapeutically important volatile compound and β-galactosidase activity shows potential functional characteristics of the probiotic culture isolate.
- The culture strain was improved by using UV irradiation where the β-galactosidase activity increased by 2.03 folds. This was coded as M7-PLsr-$1_{(W)}$ and all further studies were conduced on this improved strain. On ammonium sulphate precipitation M7-PLsr-$1_{(W)}$ exhibited 25 folds higher activity as compared to crude extract of PLsr-$1_{(W)}$.
- The culture M7-PLsr-$1_{(W)}$ was preserved by freeze drying and its viability was further enhanced with supplementation of cryoprotectants during 6 months shelf storage. During the storage period the culture retained its antimicrobial activity and resistance to low pH and high bile salt concentration.
- The fermented milk beverage prepared with the M7-PLsr-$1_{(W)}$ was found to be rich in protein, total sugar, fatty acids and minerals like iron, zinc and magnesium. The viability of the culture and nutritional properties of the product was further enhanced with supplementation of adjuvants so that it can be used by all ages for its beneficial effects.
- This fermented milk beverage was preserved over a storage time by interrupting the signal molecules produced by of spoilage bacteria using 2(5H)-furanone.
- The *in-vivo* experiments conducted with albino Wister rats gave a clear evidence for the potential probiotic functional properties and its importance in reducing lactose intolerance problem.

Bibliography

Aattouri, N., Bouras, M., Tome, D., Marcos, A. and Lemonnier, D. (2002). Oral ingestion of lactic acid bacteria by rats increases lymphocytic proliferation and γ interferon production. Br *J Nutr* **87:** 367–373.

Abadias, M., Teixido, N., Usall, J., Benabarre, A. and Vinas, I. (2001). Viability, efficacy, and storage stability of freeze-dried biocontrol agent *Candida sake* using different protective and rehydration media. *J Food Prot* **64 (6):** 856–861.

Abdelali, H., Cassand, P., Soussotte, V., Daubeze, M., Bouley, C. and Narbonne, J.F. (1995). Effect of dairy products on initiation of precursor lesions of colon cancer in rats. *Nutr Cancer* **24:** 121-132.

Aboderin, F.I. and Oyetayo, V.O. (2006). Haematological studies of rats fed different doses of probiotic, *Lactobacillus plantarum,* isolated from fermenting corn slurry. *Pak J Nutr* **5(2):** 102-105.

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

ADC (Australian Dairy Corporation). (1998). Dairy compendium. Melbourne: Australian Dairy Corporation, pp. 11-13.

Agarwal, K.N. and Bhasin, S.K. (2002). Feasibility studies to control acute diarrhoea in children by feeding fermented milk preparations Actimel and Dahi. *Eur J Clin Nutr* **56:** S56–S59.

Agrawal, R., Rati, E.R., Vijayendra, S.V.N., Varadaraj, M.C., Prasad, M.S. and Krishnanand. (2000). Flavor profile of idli batter prepared from defined microbial starter cultures. *World J Microbiol Biotechnol* **16:** 687-690.

Ahn, Y.T., Kim, G.B., Lim, K.S., Baek, Y.J. and Kim, H.U. (2003). Deconjugation of bile salts by *Lactobacillus acidophilus* isolates. *Int Dairy J* **13:** 303–311.

Akahoshi, A., Goto, Y., Murao, K., Miyazaki, T., Yamasaki, M., Nonaka, M., Yamada, K. and Sugano, M. (2002). Conjugated linoleic acid reduces body fats and cytokine levels of mice. *Biosci Biotechnol Biochem* **66(4):** 916-920.

Alander, M., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Matilla-Sandholm, T. and Wright, A. (1997). Recovery of *Lactobacillus rhamnosus GG* from human colonic biopsies. *Lett Appl Microbiol* **24:** 361–364.

Albayrak, N.A. and Yang, S.T. (2002). Production of galacto-oligosaccharides from lactose by *Aspergillus oryzae β*-galactosidase immobilized on cotton cloth. *Biotechnol Bioeng* **77:** 8 – 19.

Aleljung, P., Shen, W., Rozalska, B., Hellman, U., Ljungh, A. and Wadstrom, T. (1994). Purification of collagen binding proteins of *Lactobacillus reuteri* NCIB 11951. Curr Microbiol **28:** 231–236.

Allan, W.W. (2008). Mechanisms of action of probiotics. *Clin Infectious Diseases* **46:** 101-111.

Allison, C. and Hughes, C. (1991). Bacterial swarming: an example of prokaryotic differentiation and multicellular behaviour. *Sci Prog* **75(298 Pt 3-4):** 403–422.

Alm, L. (1982). Effect of fermentation on milk fat of Swedish fermented milk products. J Dairy Sci **65:** 521–532.

Alonso, L. and Fraga, M.J. (2001). Simple and rapid analysis for quantitation of most important volatile flavor compounds in yogurt by headspace GC-MS. *J Chromatographic Sci* **39(7):** 297-300.

Aneja, R.P. and Murthy, T.N. (1990). Conjugated linoleic acid contents of Indian curds and ghee. *Indian J Dairy Sci* **43:** 231–238.

Antunes, A., Rainey, F.A., Nobre, M.F., Schumann, P., Ferreira, A.M., Ramos, A., Santos, H. and de Costa, M.S. (2002). *Leuconostoc ficulneum sp. nov*., a novel lactic acid bacterium isolated from a ripe fig, and reclassification of *Lactobacillus fructosus* as *Leuconostoc fructosum comb. nov*. *Int J Sys Evolutionary Microbiol* **52:** 647–655.

Arunachalam, K., Gill, H.S. and Chandra, R.K. (2000). Enhancement of natural immune function by dietary consumption of *Bifidobacterium lactis* (HN019). *Eur J Clin Nutr* **54:** 1-5.

Ashar, M.A. and Prajapathi, J.B. (1998). Bile tolerance, bile deconjugation and cholesterol reducing properties of dietary *Lactobacilli*. *Ind J Microbiol* **38:** 145–148.

Aso, Y., Akaza, H., Kotake, T., Tsukamoto, T., Imai, K. and Naito, S. (1995). Preventive effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer in a double blind trial. The BLP Study Group. *Euro Urol* **27:** 104–109.

Astley, S.B., Elliott, R.M., Archer, D.B. and Southon, S. (2004). Evidence that dietary supplementation with carotenoids and carotenoid rich foods modulates the DNA damage: repair balance in human lymphocytes. *Brit J Nutr* **91:** 63-72.

Auge, B., Donnio, P.Y., Le Dleaut, P. and Avril, J. L. (1987). Influence de la vancomycine intraveineuse sur lesores alerobies salivaires et flecales. *Pathologie Biologie* **35:** 673– 675.

Bachhawat, N., Gowda, L.R. and Bhat, S.G. (1996). Single step method of preparation of detergent permeabilized *Kluyveromyces fragilis* for lactose hydrolysis. *Process Biochem* **31:** 21–25.

Bakalinsky, A.T., Nadathur, S.R., Carney, J.R. and Gould, S.J. (1996). Antimutogenicity of yoghurt. *Mut Res* **350(1):**199–200.

Barker, D.C. and Thorne, K.J. (1970). Spheroplasts of *Lactobacillus casei* and the cellular distribution of bactoprenol. *J Cell Sci* **7:** 755-785.

Barrangou, R., Sung-Sik, Y., Breidt Jr., F., Fleming, H. P. and Klaenhammer, T. R. (2002). Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial Sauerkraut fermentation. *Appl Environ Microbiol* **68:** 2877– 2884.

Barros, R.R, Carvalho, M.D.G.S., Peralta, J.M., Facklam, R.R. and Teixeira, L.M. (2001). Phenotypic and genotypic characterization of *Pediococcus* strains isolated from human sources. *J Clin Microbiol* **39–40:** 1241–1246.

Batra, N., Singh, J., Banerjee, U.C., Patnaik, P.R. and Sobti, R.C. (2002). Production and characterization of a thermostable *β*-galactosidase from *Bacillus coagulans* RCS3. *Biotechnol Appl Biochem* **36:** 1–6.

Bayless, T.M. and Rosensweig, N.S. (1966). A racial difference in incidence of lactase deficiency. A survey of milk intolerance and lactase deficiency in healthy adult males. *JAMA*. **197:** 968-972.

Beales, N. (2004). Adaptation of microorganisms to cold temperatures, weak acid preservatives, low ph, and osmotic stress: a review. *Comprehensive Rev Food Sci Food Safety* **3(1):** 1-20.

Bellengier, P., Richard, J. and Foucaud, C. (1997). Nutritional requirements of *Leuconostoc mesenteroides subsp. Mesenteroides* and *subsp. dextranicum* for growth in milk. *J Dairy Res* **64:** 95–103.

Benkerroum, N., Boughdadi, A., Bennani, N. and Hidane, K. (2003). Microbiological quality assessment of Moroccan camel's milk and identification of predominating lactic acid bacteria. *World J Microbiol Biotechnol* **19:** 645-648.

Bennenberg, H.J., Hock, C. and Van den, W. (1949). Estimation of milk protein by formal titration. *Dairy Sci Abstr* **45:** 103.

Berghofer, E., Grzeskowiak, B., Mundigler, N., Sentall, W.B. and Walcak, J.J. (1998). Antioxidative properties of faba bean, soybean and oat tempeh. *Int J Food Sci Nutr* **49:** 45–54.

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– 4135.

Bernet-Camard, M.F., Lievin, V., Brassart, D., Neeser, J.R., Servin, A.L. and Hudault, S. (1997). The human *Lactobacillus acidophilus* strain LA1 secretes a nonbacteriocin antibacterial substance(s) active *in vitro* and *in vivo*. *Appl Environ Microbiol* **63:** 2747– 2753.

Berrada, N., Lemeland, J.F., Laroch, G., Thouvenot, P. and Piaia, M. (1991). *Bifidobacterium* from fermented milks: survival during gastric transit. *J Dairy Sci* **74:** 409–413.

Bertazzoni, M.E., Benini, A., Marzotto, M., Hendriks, H., Sbarbati, A. and Dellaglio, F. (2001). Preliminary screening of health-promoting properties of new *Lactobacillus* strain: *in vitro and in vivo*. HEALFO abstracts, Italy.

Bertelsen, G., Christophersen, C., Nielsen, P.H., Madsen, H.L., Stadel, P. (1995). Chromatographic isolation of antioxidants guided by a methyl linoleate assay. *J Agric Food Chem* **43:** 1272-1275.

Beukes, E.M., Bester, B.H. and Mostert, J.F. (2001). The microbiology of South African traditional fermented milks. *Int J Food Microbiol* **63(**3): 189–197.

Beveridge, T.J. (2001). Use of the gram stain in microbiology. *Biotech Histochem* **76(3):** 111–118.

Bezares, B.R., Saenz, Y., Poeta, P., Zarazaga, M., Ruiz-Larrea, F. and Torres, C. (2006). Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. *Int J Food Microbiol* **111(3)**: 234-240.

Bhat, N., Naina, N.S., Gowda, L.K. and Bhat, S.G. (1993). Detergent permeabilized yeast cells as the source of intracellular enzymes for estimation of biomolecules. *Enzyme Microb Technol* **15:** 796–800.

Bhowmik, T. and Marth, E.H. (1989). Simple method to detect β-galactosidase. *Appl Environ Microbiol* **55**: 3240-3242.

Bibiloni, R., Zavaglia, A.G. and Antoni, G.d. (2001). Enzyme based most probable number method for the enumeration of *Bifidobacterium* in dairy products. *J Food Prot* **64(12):** 2001–2006.

Bjorkroth and Holzapfel. (2003). Genera *Leuconostoc, Oenococcus* and *Weissella*. In: M. Dworkin, et al. (Eds.), The prokaryotes: An evolving electronic resource for the microbiological community (3rd ed), release 3.12, March 28, 2003. Berlin: Springer, NY http : //link.springer-ng.com/link/service/books/10125/.

Bjorkroth, K.J., Geisen, R., Schillinger, U., Weiss, N., de Vos, P., Holzapfel, W.H., Korkeala, H.J. and Vandamme, P. (2000). Characterization of *Leuconostoc gasicomitatum sp. nov.*, associated with spoiled raw tomato marinated broiler meat strips packaged under modified atmosphere conditions. *Appl Environ Microbiol* **66:** 3764-3772.

Blackwood, A.C. and Blakley. E.R. (1960). Fermentation of glucose-c^{14} and xylose-c^{14} by various strains of *Leuconostoc mesenteroides*. *J Bacteriol* **79:** 411-416.

Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipids extraction and purification. *Can J Biochem Physiol* **37:** 911–917.

Blum, S., Haller, D., Pfeifer, A. and Schiffrin. E. J. (2002). Probiotics and immune response. *Clin Rev Allergy Immunol* **22:** 287–309.

Boirivant, M. and Strober, W. (2007). The mechanism of action of probiotics. *Curr Opin Gastroenterol* **23(6):** 679-692.

Boot, H.J., Kolen, C.P.A.M., Van Noort, J.M. and Pouwels, P.H. (1993). S-layer protein of *Lactobacillus acidophilus* ATCC 4356: Purification, expression in Escherichia coli, and nucleotide sequence of the corresponding gene. *J Bacteriol* **175(19):** 6089-6096.

Boris, S., Suarez, J.E., Vazquez, F. and Barbes. (1998). Adherence of human vaginal *Lactobacilli* to vaginal epithelial cells and interaction with uropathogens. *Infect Immun* **66:** 1985-1989.

Bottazzi, V. (1983). Other fermented dairy products. In *Biotechnology,* Vol. *5,* Food and Feed Production with Microorganisms ed. Reed, G. Weinheim: Verlag Chemie, pp. 315-363.

Boumerdassi, H., Monnet, C., Desmazeaud, M. and Corrieu, G. (1997) Effect of citrate on production of diacetyl and acetoin by Lactococcus Lactis ssp. Lactis CNRZ 483 cultivated in the presence of oxygen. *J Dairy Sci* **80:** 634–639.

Bourel, G., Henini, S., Krantar, K., Oraby, M., Divies, C. and Garmyn, D. (2001). Metabolisme sucrecitrate chez *Leuconostoc mesenteroides*. *Le Lait* **81:** 75–82.

Bourget, N.L., Philippe, H., Mangin, I. and Decaris, B. (1996). 16SrRNA and 16 to 23 internal transcribed spacer sequence analyses reveal inter and intraspecific *Bifidobacterium* phylogeny. *Int J Sys Bacteriol* **46:** 102–111.

Bozoglu, T.F., Ozilgen, M. and Bakir, U. (1987). Survival kinetics of lactic acid starter cultures during and after freeze drying. *Enzyme Microbiol Technol* **9:** 531–537.

Brand-Williams, W., Cuvelier, M.E. and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensm Wiss Technol* **26:** 25–30.

Brashears, M.M. and Durre, W.A. (1999). Antagonistic action of *Lactobacillus lactis* toward *Salmonella spp* and *Escherichia coli* 0151:H7 during growth and refrigerated storage. *J Fd Prot* **62:** 1336-1340.

Brashears, M.M., Jaroni, D. and Trimble, J. (2003). Isolation, selection, and characterization of lactic acid bacteria for a competitive exclusion product to reduce sheddingof E. coli O157:H7 in cattle. *J Food Prot* **66:** 355–363.

Brigidi, P., Vitali, B., Swennen, E., Bazzocchi, G. and Matteuzzi, D. (2001). Effects of probiotic administration upon the composition and enzymatic activity of human fecal microbiota in patients with irritable bowel syndrome or functional diarrhea. *Res Microbiol* **152:** 735-741.

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. *Cryobiol* **39:** 88–102.

Bronshtein,V. (2004). Preservation by foam formation. *Pharmaceutical Technol* **28(4):** 86–92.

Brown, J.L., Ross, T., McMeekin, T.A. and Nichols, P.D. (1997). Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. *Int J Food Microbiol* **37:** 163-173.

Bruno, E.A. and Shah, N.P. (2003) Viability of two freeze dried strains of *Bifidobacterium* and of commercial preparations at various temperatures during prolonged storage. *J Food Sci* **68(7)**: 2336-2339.

Buning, C., Genschel, J., Jurga, J., Fiedler, T., Voderholzer, W., Fiedler, E.M., Worm. M., Weltrich, R., Lochs, H., et al. (2005). Introducing genetic testing for adult type hypolactasia. *Digestion* **71:** 245-250.

Burgess, C., O'Connell-Motherway, M., Sybesma, W., Hugenholtz, J. and van Sinderen, D. (2004). RiboXavin production in *Lactococcus lactis*: potential for *in situ* production of vitamin-enriched foods. *Appl Environ Microbiol* **70:** 5769–5777.

Burns, A.J. and Rowland, I.R. (2000). Anti-Carcinogenicity of Probiotics and Prebiotics *Curr Issues Intest Microbiol* **1(1):** 13-24.

Bury, D., Jelen, P. and Kalab, M. (2001). Disruption of *Lactobacillus delbrueckii* ssp. *Bulgaricus* 11842 cells for lactose hydrolysis in dairy products: a comparison of sonication, high- pressure homogenization and bead milling. *Innovative Food Sci Emerging Technol* **2:** 23-29.

Busconi, L.and Denker, B.M. (1997). Analysis of the N-terminal binding domain of G_o *Biochem J* **328:** 23–31.

Cadieux, P., Wind, A., Sommer, P., Schaefer, L., Crowley, K., Britton, R.A. and Reid, G. (2008). Evaluation of reuterin production in urogenital probiotic *Lactobacillus reuteri* RC-14. *Appl Environ Microbiol* **6:** 12-19.

Calderon, M., Loiseau, G. and Guyot, J.P. (2001). Nutritional requirements and simplified cultivation medium to study growth and energetics of a sourdough lactic acid bacterium *Lactobacillus fermentum* Ogi E1 during heterolactic fermentation of starch. *J Appl Microbiol* **90(4):** 508-516.

Campieri, C., Campieri, M., Bertuzzi, V., Swennen, E., Matteuzzi, D., Stefoni, S., Pirovano, F., Centi, C., Ulisse, S., Famularo, G. and De Simone. C. (2001). Reduction of oxaluria after an oral course of lactic acid bacteria at high concentration. *Kid Int* **60:** 1097-1105.

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.

Canzi, E., Guglielmetti, S., Mora, D., Tamagnini, I. and Parini, C. (2005). Conditions affecting cell surface properties of human intestinal *Bifidobacteria*. *Antonie Van Leeuwenhoek* **88(3–4):** 207–219.

Canzi, E., Zanchi, R., Camaschella, P., Cresci, A., Greppi, G.F., Orpianesi, C., Serrantoni, M. and Ferrari, A. (2000). Modulation by lactic acid bacteria of the intestinal ecosystem and plasma cholesterol in rabbits fed a casein diet. *Nutr Res* **20:** 1329-1340.

Cao, G., Verdon, C.P., Wu, A.B.H., Wang, H. and Prior, R.L. (1995). Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. *Clin Chem* **41:** 1738–1744.

Caplice, E. and Fitzgerald, G.F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *Int J Food Microbiol* **50:** 131–149.

Carcoba, R. and Rodriguez, A. (2000). Influence of cryoprotectants on the viability and acidifying activity of frozen and freeze-dried cells of the novel starter strain *Lactococcus lactis ssp. lactis* CECT 5180. *Euro Food Res Technol* **211:** 433–437.

Carpenter, J. F., Arakawa, T. and Crowe**.** J. H. (1990). Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. *Dev Biol Stand* **74:**225–239.

Carr, F.J., Chill, D. and Maida, N. (2002). The lactic acid bacteria: A literature survey. *Crit Rev Microbiol* **28:** 281–370.

Carvalhoa, A.S., Silvaa, J., Hob, P., Teixeira, P., Malcataa, F.X. and Gibbsa, P. (2004). Relevant factors for the preparation of freeze-dried lactic acid bacteria. *Int Dairy J* **14:** 835–847.

Casas, I.A. and Dobrogosz, W.J. (2000). Validation of the probiotic concept: *Lactobacillus reuteri* confers broad spectrum protection against disease in humans and animals, *Microbial Ecology in Health and Disease* **12:** 247–285.

Cataldi, T.R.I., Bianco, G., Frommberger, M. and Schmitt-Kopplin. (2004). Direct analysis of selected *N*-acyl-L-homoserine lactones by gas chromatography/mass spectrometry. *Rapid Comm Mass Spectrometry* **18(12):** 1341 – 1344.

Challa, A., Rao, D.R., Chawan, C.B., and Shackelford, L. (1997). *Bifidobacterium longum* and lactulose suppress azoxymethane induced aberrant crypt foci in rats. *Carcinogenesis* **18:** 517-521.

Champagne, C.P., Mondou, F., Raymond, Y. and Roy, D. (1996). Effect of polymers and storage temperature on the stability of freeze dried lactic acid bacteria. *Food Res Int* **29:** 555–562.

Chancey, S. T., Wood, D.W. and Pierson, L.S. (1999). Two-component transcriptional regulation of *N*-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol* **65:** 2294-2299.

Charlton, T.S., de Nys, R., Netting, A., Kumar, N., Hentzer, M., Givskov, M. and Kjelleberg, S. (2000). A novel and sensitive method for the quantification of *N*-3-oxo- acyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. *Environ Microbiol* **2**: 530-541.

Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K. (2000). Effect of conjugated bile salts on antibiotic susceptibility of bile salt-tolerant *Lactobacillus* and *Bifidobacterium* isolates. *J Food Prot* **63(10):** 1369–1376.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. (1998). Ingredient selection criteria for probiotic microorganisms in functional dairy foods. *Int J Dairy Technol* **51(4):** 123–136.

Chateau, N., Deschamps, A. M. and Hadj Sassi, A. (1994). Heterogeneity of bile salts resistance in the *Lactobacillus* isolates of a probiotic consortium. *Lett Appl Microbiol* **18:** 42–44.

Chavarri, F.J., De Paz, M. and Nueez, M.M. (1988). Cryoprotective agents for frozen concentrated starters from non-bitter *Streptococcus lactis* strains. *Biotechnol Lett* **10:** 11–16.

Chen, T., Fowler, A. and Toner, M. (2000). Supplemented phase diagram of the trehalose–water binary mixture. *Cryobiol* **40:** 277–282.

Chen, X., Xu, J., Shuai, J., Chen, J., Zhang, Z. and Fang, W. (2007). The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157:H7 and *Salmonella typhimurium. Int J Food Microbiol* **115(3):** 307-312.

Chien, H.L. (2004). Change of isoflavones contents in cultured soymilk fermented with lactic acid bacteria and *Bifidobacteria*. M.S. Thesis, National Taiwan University, Taipei, Taiwan.

Childs, N.M. (1997). The functional food consumers: who are they and what do they want? Implications for product development and positioning. In: Yalpani M, ed. New technologies for healthy foods and nutraceuticals. Shrewsbury, MA: ATL Press, pp. 313–26.

Chin, S.F., Liu, W., Storkson, J.M., Ha, Y.L. and Pariza, M.W. (1992). Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. *J Food Composition Anal* **5:** 185–197.

Choi, S.S., Kang, B.Y., Chung, M.J., Kim, S.D., Park, S.H., Kim, J.S., Kang C.Y. and Ha, N.J. (2005). Safety assessment of potential lactic acid bacteria *Bifidobacterium longum* SPM1205 isolated from healthy Koreans. *J Microbiol* **43(6):** 493-498.

Chou, L.S. and Weimer, B. (1999). Isolation and characterization of acid and bile tolerant isolates from strains of *Lactobacillus acidophilus*. *J Dairy Sci* **82:** 23–31.

Christensen, J.E., Dudley, E.G., Pederson, J.A. and Steele, J.L. (1999) Peptidases and amino acid catabolism in lactic acid bacteria. *Ant Leeuwenhoek* **76:** 217–246.

Christopher, N.L. and Bayless, T.M. (1971). Role of the small bowel and colon in lactose induced diarrhea. *Gastroenterol* **60:** 845-852.

Chugani, S.A., Whiteley, M., Lee, K.M., D'Argenio, D., Manoil, C. and Greenberg, E.P. (2001). QscR, a modulator of quorum sensing signal synthesis and virulence in *P. aeruginosa*. *Proc Natl Acad Sci USA* **98:** 2752-2757.

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 Microbiol* **47:** 25–32.

Chung, Y.C., Chang, C.T., Chao, W.W., Lin, C.F. and Chou, S.T. (2002). Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. *J Agric Food Chem* **50:** 2454–2458.

Cibik, R. and Chapot-Chartier, M. P. (2000). Autolysis of dairy *Leuconostocs* and detection of peptidoglycan hydrolases by renaturing SDS-PAGE . *J Appl Microbiol* **89:** 862–869.

Cibik, R., Lepage, E. and Tailliez, P. (2000). Molecular diversity of *Leuconostoc mesenteroides* and *Leuconostoc citreum* isolated from traditional French cheeses as revealed by RAPD fingerprinting, 16srDNA sequencingand 16srDNA fragment amplification. *Sys Appl Microbiol* **23:** 267–278.

Clark, P.A., Cotton, L.N. and Martin, J.H. (1993). Selection of *Bifidobacteria* for use as dietary adjuncts in cultured dairy foods: II. Tolerance to simulated pH of human stomach. *Cult Dairy Pro J* **28:** 11-14.

Clegg, J.S. (1986). The physical properties and metabolism status of Artemia cysts at low water contents. The 'water replacement hypothesis'. In Membranes, Metabolism and Dry Organisms ed. Leopold, A.C. Ithaca, NY: Cornell University Press, pp. 169-187.

Cocconcelli, P.S. Cattivelli, D. and Gazzola, S. (2003). Gene transfer of vancomycin and tetracycline resistances among *Enterococcus faecalis* during cheese and sausage fermentations. *Int J Food Microbiol* **88:** 315–323.

Cocconcelli, P.S., Parisi, M.G., Senini, L. And Bottazzi, V. (1997). Use of RAPD and 16S rDNA sequencing for the study of *Lactobacillus* population dynamics in natural whey culture. *Lett Appl Microbiol* **25**: 8-12.

Cogan, T.M. and Jordan, K.N. (1994). Metabolism of *Leuconostoc* bacteria. *J Dairy Sci* **77:** 2704–2717.

Collado, M.C., Gueimonde, M., Hernandez, M., Sanz, Y. and Salminen, S. (2005). Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and its role in enteropathogen exclusion. *J Food Prot* **68(12):** 2672–2678.

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

Collins, M.D. and Gibson, G.R. (1999). Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am J Clin Nutr* **69(5):**1052S-1057S.

Cook, M.E., Miller, C.C., Park, Y. and Pariza, M.W. (1993). Immune modulation by altered nutrient metabolism: Nutritional control of immune induced growth depression. *Poultry Sci* **72:** 1301–1305.

Coppola, R., Giagnacovo, B., Iorizzo, M. and Grazia, L. (1998). Characterization of *Lactobacilli* involved in the ripening of *Soppressata molisana*, a typical southern Italy fermented sausage. *Food Microbiol* **15:** 347–353.

Corcoran, B.M., Ross, R.P., Fitzgerald, G.F. and Stanton, C. (2004). Comparative survival of probiotic *Lactobacilli* spray-dried in the presence of prebiotic substances. *J Appl Microbiol* **96:** 1024–1039.

Corcoran, B.M., Ross, R.P., Fitzgerald, G.F., Dockery, P. and Stanton, C. (2006). Enhanced survival of GroESL-overproducing *Lactobacillus paracasei* NFBC 338 under stressful conditions induced by drying. *Appl Environ Microbiol* **72(7):** 5104–5107.

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

Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999). Bacterial biofilms: a common cause of persistent infections. *Sci* **284:** 1318-1322.

Cotter, A.A (2007). The health benefits of milk and dairy products. Society of Dairy Technology Bulletin no. 417. International Dairy Federation (IDF), Brussels, Belgium, pp. 213-214.

Cotter, P.D. and Hill, C. (2003). Surviving the acid test: responses of gram- positive bacteria to low pH. *Microbiol Mol Biol Rev* **67(3):** 429–453.

Cross, M.L. (2002). Microbes versus microbes: immune signals generated by probiotic *Lactobacilli* and their role in protection against microbial pathogens. *FEMS Immunol Med Microbiol* **34:** 245-253.

Crowe, J.H. and Crowe, L.M. (1986). Stabilization of membranes in an hydrobiotic organisms. In Membranes, Metabolism and Dry Organisms ed. Leopold, A.C. Ithaca, NY: Cornell University Press, pp. 188– 209.

Crowe, J.H., Carpenter, J.F. and Crowe, L.M. (1998). The role of vitrification in anhydrobiosis. *Ann Rev Physiol* **60:** 73–103.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Aurell Winstrom, C., Spargo, B.J. and Anchordoguy, T.J. (1988). Interaction of sugars with membranes. *Biochem Biophys Acta* **947:** 367–384.

Cvitkovitch, D.G., Li, Y.H. and Ellen, R.P. (2003). Quorum sensing and biofilm formation in streptococcal infections. *J Clin Investig* **112:** 1626-1632.

D'Aimmo, M.R., Modesto, M. and Biavati, B. (2007) International Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products. *J Food Microbiol* **115(1):** 35-42.

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:** 1361-1367.

Daeschel, M.A. (1989). Antimicrobial substances from acid lactic bacteria for use as food preservative. *Food Technol* **43:** 164-167.

dal Bello, F., Walter, J., Hammes, W. P. and Hertel, C. (2003). Increased complexity of the species composition of lactic acid bacteria in human feces revealed by alternative incubation condition. *Microbial Ecol* **45:** 455–463.

Daly, C. and Davis, R. (1998). The biotechnology of lactic acid bacteria with emphasis on applications in food safety and human health. *Agri Food Sci Finland* **7:** 251-264.

Dave, J.M. and Prajapathi, J.B.(1994). Lactic acid bacteria as antibacterials against food borne pathogens and food spoilage organisms. In: Microbes for better living Ed: Sankaran, R., Manja, K.S., Proceedings of MICON-INTL-94, Conference Secretariat, DFRL, Mysore. pp 361-367.

Dave, R.I. and Shah, N.P. (1998). Ingredient supplementation effects on viability of probiotic bacteria in yogurt. *J Dairy Sci* **81:** 2804–2816.

Davis, J.G., Ashton, T.R. and McCaskill, M. (1971). Enumeration and viability of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yoghurt. *Dairy Industries* **36:** 569–573.

De Angelis, M., Gallo, G., Corbo, M.R., McSweeney, P.L.H., Faccia, M., Giovine, M. and Gobbetti, M. (2003). Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. *Int J Food Microbiol* **87:** 259–270.

De Boever, P., Deplancke, B. and Verstraete, J. (2000). Fermentation by gut microbiota cultured in a simulator of the human intestinal microbiota ecosystem is improved by supplementing a soy germ powder. *J Nutr* **130:** 2599–2606.

De Giulio, B., Orlando, P., Barba, G., Coppola, R., De Rosa, M., Sada, A., De Prisco P.P. and Nazzaro, F. (2005). Use of alginate and cryoprotective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying. *World J Microbiol Biotechnol* **21:** 739–746.

de la Fuente, M.A., Montes, F., Guerrero, G.and Juarez, M. (2003). Total and soluble contents of calcium, magnesium, phosphorus and zinc in yoghurts. *Food Chem* **80(4):** 573-578.

De Smet, I., De Boever, P. and Verstraete, W. (1998). Cholesterol lowering in pigs through enhanced bacterial bile salt hydrolase activity. *Brit J Nutr* **79:** 185–194.

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 Valdez, G.F., de Giori, G.S., de Ruiz Holgado, A.P. and Oliver, G. (1983). Comparative study of the efficiency of some additives in protecting lactic acid bacteria against freeze-drying. *Cryobiol* **20:** 560–566.

De Vrese, M., Keller, B. and Barth, C.A. (1992). Enhancement of intestinal hydrolysis of lactose by microbial β-galactosidase (EC 3.2.1.23) of kefir. *Br J Nutr* **67:** 67-75.

De Vrese, M., Stegelmann, A., Richter, B., Fenselau, S., Laue, C., Schrezenmeir, J. (2001). Probiotics: compensation for lactase insufficiency. *Am J Clin Nutr* **73:** 421S-9S.

De Vries, R.P., van den Broek, H.C., Dekkers, E., Manzanares, P., de Graff, L.H. and Visser, J. (1999). Differential expression of three a-galactosidase genes and a single β-galactosidase gene from *Aspergillus niger*. *Appl Environ Microbiol* **65:** 2453– 2460.

Del Re, B., Sgorbati, B., Miglioli, M. and Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett Appl Microbiol* **31:** 438–442.

Delisle, J., Amiot, J. and Dore, F. (1995). Biological availability of calcium and magnesium from dairy products. *Int Dairy J* **5:** 87–96.

Denev, S.A. (2006). Role of *Lactobacilli* in gastrointestinal ecosystem. *Bulg J Agric Sci* **12:** 63-114.

Derre, I., Rapoport, G. and Msadek, T. (1999). CtsR, a novel regulator of stress and heat shock response, controls clp and molecular chaperone gene expression in Gram-positive bacteria. *Mol Microbiol* **31:** 117–131.

Desmond, C., Ross, R.P., O'Callaghan, E., Fitzgerald, G. and Stanton, C. (2002). Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. *J Appl Microbiol* **93:** 1003–1011.

Devery, R., Miller, A. and Stanton, C. (2001). Conjugated linoleic acid and oxidative behavior in cancer cells. *Biochem Society Trans* **9:** 341–344.

Devoyod, J.J. and Poullain, F. (1988). Les *leuconostocs*. Proprietes: Leur role en technologie laitiere. *Le Lait* **68:** 249–280.

Di Cagno, R., De Angelis, M., Auricchio, S., Greco, L., Clarke, C., De Vincenzi, M., Giovannini, C., D'Archivio, M., et al. (2004). Sourdough bread made from wheat and non toxic flours and started with selected lactobacilli is tolerated in celiac sprue patients. *Appl Environ Microbiol* **70:** 1088-1096.

Di Palma, J.A. and Collins M.S. (1989). Enzyme replacement for lactose malabsorption using a beta-d- galactosidase. *J Clin Gastroenterol* **11(3):** 290-293.

Di Stefano, M., Veneto, G., Malservisi, S., Cecchetti, L., Minguzzi, L., Strocchi, A. and Croazza, G.R. (2002). Lactose malabsorption and intolerance and peak bone mass. *Gastroenterol* **122:** 1793–1799.

Dicks, L.M.T., Fantuzzi, L., Gonzalez, F.C., Du Toit, M. and Dellaglio, F. (1993). *Leuconostoc argentinum sp. nov.* isolated from Argentine raw milk. *Int J Sys Bacteriol* **43:** 347–351.

Dogan, B. and Boor, K.J. (2003). Genetic diversity and spoilage potentials among *Pseudomonas* spp. Isolated from Fluid Milk Products and Dairy Processing Plants. *Appl Environ Microbiol* **69(1):** 130–138.

Doncheva, N.I., Antov, G.P., Softova, E.B. and Nyagolov, Y.P. (2002). Experimental and clinical study on the hypolipidemic and antisclerotic effect of *Lactobacillus bulgaricus strain* GB N 1 (48). *Nutr Res* **22:** 393–403.

Dong, Y.H., Gusti, A.R., Zhang, Q., Xu, J.L. and Zhang, L.H. (2002). Identification of quorum quenching N-acyl-homoserine lactonases from *Bacillus* Species. *Appl Environ Microbiol* **68(4):** 1754-1759.

Donnet-Hughes, A., Rochat, F., Serrant, J.M., Aeschlimann, J. and Schiffrin, E.J. (1999). Modulation of non-specific mechanisms of defense by lactic acid bacteria: Effective dose. *J Dairy Sci* **82:** 863–869.

Draksler, D., Gonzales, S. and Oliver, G. (2004). Preliminary assays for the development of a probiotic for goats. *Reprod Nutr Dev* **44:** 397-405.

Drici-Cachon, Z., Cavin, J.F. and Divies, C. (1996). Effect of pH and age of culture on cellular fatty acid composition of *Leuconostoc oenos*. *Lett Appl Microbiol* **22:** 331–334.

Du Toit, M., Franz, C.M.A.P., Dicks, L.M.T., Schillinger, U., Haberer, P., Warlies, B., Ahrens, F. and Holzapfel, W.H**. (**1998). Characterization and selection of probiotic *Lactobacilli* for a preliminary mini pig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content. *Int J Food Microbiol* **40**: 93-104.

Dubois, M., Gilles, K.A. Hamilton, J.K. Rebers, P.A. and Smith. F.(1956). Colorimetric method for determination of sugars and related substances. *Anal Chem* **28:**350.

Duc, L.H., Hong, H.A., Barbosa, T.M., Henriques, A.O. and Cutting, S.M. (2004). Characterization of *Bacillus* probiotics available for human use. *Appl Environ Microbiol* **70:** 2161–2171.

Duffy, L.C., Leavens, A., Griffiths, E. and Dryja, D. (1999). Perspectives on *Bifidobacteria* as biotherapeutic agents in gastrointestinal health. *Digestive Diseases and Sciences* **44(8):** 1499-1505.

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G.C., Shanahan, F. and Collins, K.C. (2001). *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Am J Clin Nutr* **73:** 386S-392S.

Dupont, H.L. (2005). *Shigella* species (Bacillary dysentery). In Principles and Practice of Infectious Diseases ed. Mandell, G.L., Bennett, J.E. and Dolin, R. Philadelphia, PA: Elsevier Inc, pp. 2655–2661.

Dwyer, J.H., Navab, M., Dwyer, K.M., Hassan, K., Sun, P., Shircore, A., HamaLavy, S., Hough, G., Wang, X., Drake, T., Merz, C.N. and Fogelman, A.M. (2001). Oxygenated carotenoid lutein and progression of early atherosclerosis: the Los Angeles atherosclerosis study. *Circulation* **103:** 2922-2927.

Eberl, L., Winson, M.K. Sternberg, C., Stewart, G.S.A.B., Christiansen, G., Chhabra, S.R., Daykin, M., Williams, P., Molin, S. and Givskov. M. (1996). Involvement of *N*-acyl-L-homoserine lactone autoinducers in control of multicellular behavior of *Serratia liquefaciens*. *Mol Microbiol* **20:** 127-136.

Eichholzer, M. and Stahelin, H. (1993). Is there a hypocholesterolemic factor in milk and milk products? *Int J Vit Nutr Res* **63:** 159-167.

El Zubeir, I.E.M., Abdalla, W.M. and El Owni, O.A.O. (2005). Chemical composition of fermented milk (roub and mish) in Sudan. *Food Control* **16:** 633–637.

Elmer, G.W., Martin, A.W., Horner, K.L., McFarland, L.V. and Levy, R.H. (1999). Survival of *Saccharomyces boulardii* in the rat gastrointestinal tract and effects of dietary fiber. *Microb Ecol Health Dis* **11:** 29–34.

Elmer, G.W., Surawicz, C.M. and McFarland. L.V. (1996). Biotherapeutic agents. A neglected modality for the treatment and prevention of selected intestinal and vaginal infections. *JAMA* **275:** 870–876.

Enig, M.G. (1998). Lauric oils a antimicrobial agents: theory of effect, scientific rationale and dietary application as adjuvant nutritional support for HIV-infected individuals. In: Watson R, ed. Nutrients and Foods in AIDS. Boca Raton, FL: CRC press, London, pp. 81-97.

Enig, M.G. (1999). Coconut: In Support of Good Health in the 21st Century, 36th session of Asian Pacific Coconut Community, Singapore, APCC, pp. 1-26.

Ennahar, S., Cai, Y. and Fujita, Y. (2003). Phylogenetic diversity of lactic acid bacteria associated with paddy rice silage as determined by 16S ribosomal DNA analysis. *Appl Environ Microbiol* **69:** 444–451.

Ennahar, S., Sonomoto, K., Ishizaki, A. (1999). Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. *J Biosci Bioeng* **87:** 705-716.

Ercolini, D., Moschetti, G., Blaiotta, G. and Coppola, S. (2001). Behavior of variable V3 region from 16S rDNA of important lactic acid bacteria in denaturing gradient gel electrophoresis. *Curr Microbiol* **42:** 199–202.

Erkkila, S. and Petaja, E. (2000). Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. *Meat Sci* **55:** 297-300.

Esaki, H., Onozaki, H. and Osawa, T. (1994). Antioxidative activity of fermented soybean products. In: Huang, M.T. (Ed.), Food Phytochemicals for Cancer Prevention I, Fruits and Vegetables. American Chemical Society, Washington, DC, pp. 353–360.

Esther, N., Julia, W., Sonia, G.M., Ligia, D., Javier, R. and Ascension, M. (2007). Immunomodulatory effects of probiotics in different stages of life. *Brit J Nutr* **98:** s90-95.

Fantes, P.A. and Roberts, C.F. (1973). *β*-Galactosidase activity and lactose utilization in *Aspergillus nidulans*. *J Gen Microbiol* **77:** 471– 486.

FAO/WHO (2002). Guidelines for the evaluation of probiotics in food. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food, London, Ontario, Canada, pp. 1-11.

Farmer, E.H., Bloomfield, G.F., Sundralingam, A. and Sutton, D.A. (1942). The course and mechanism of autoxidation reactions in olefinic and polyolefinic substances, including rubber. *Trans Faraday Soc* **38:** 348–356.

Farrow, J.A.E., Facklam, R.R. and Collins, M.D. (1989). Nucleic acid homologies of some vancomycin resistant *Leuconostocs* and description of *Leuconostoc citreum sp. nov.* and *Leuconostoc pseudomesenteroides sp. nov*. *Int J Sys Bacteriol* **39:** 279–283.

Fekete, E., Karaffa, L., Sandor, E., Seiboth, B., Biro, S. and Szentirmai, C. (2002). Regulation of formation of the intracellular *β*-galactosidase activity of *Aspergillus nidulans*. *Arch Microbiol* **179:** 7 – 14.

Felley, C.P. Corthesy-Theulaz, I., Blanco 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.

Femia, A.P., Luceri, C., Dolara, P., Giannini, A., Biggeri, A., Salvadori, M., *et al.* (2002). Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* on azoxymethane induced colon carcinogenesis in rats. *Carcinogenesis* **11:** 1953-1960.

Fernandes, C.F. and Shahani. K.M. (1990). Anticarcinogenic and immunological properties of dietary *Lactobacilli*. *J Food Prot* **53:** 704-710.

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

Fernandez, J.C., Mas, R., Castano, G. *et al.* (2001). Comparison of the efficacy, safety and tolerability of policosanol versus fluvastatin in elderly hypercholesterolaemic women. *Clin Drug Investig* **21:** 103-113.

Fernandez-Murga, M.L., De Ruiz Holgado, A.P. and De Valdez, G.F. (1998). Survival rate and enzyme activities of *Lactobacillus acidophilus* following frozen storage. *Cryobiol* **36:** 315–319.

Fiedurek, J. and Szczodrak, J. (1994). Selection of strain, culture conditions and extraction procedures for optimum production of *β*-galactosidase from *Kluyveromyces fragilis*. *Acta Microbiol Pol* **43:** 57– 65.

Fiocchi, C. (2006). Probiotics in inflammatory bowel disease: yet another mechanism of action? *Gastroenterol* **131(6):** 2009-2012.

Fleming, H.P., Kyung, K.H. and Breidt, F. (1995). Vegetable fermentations, In G. Reed and T.W. Nagodawithana (ed.), Biotechnology, 2nd ed., Vol. 9. VCH Publishing Co., Weinheim, Germany, pp. 631-661.

Flores, M.V., Voget, C.E. and Ertola, R.J. (1994). Peneabilization of yeast cells (*Kluyveromyces lactis)* with organic solvents. *Enzyme Microb Technol* **16:** 340-346.

Fontaine, I.F., Aissi, E.A. and Bouquelet, S.J.L. (1994). *In vitro* binding of *Bifidobacterium bifidum* DSM 20028 to mucosal glycoproteins and hemagglutination activity. *Curr Microbiol* **28**: 325–330.

Food and Drug Administration. (2001). Guidance for industry involuntary labeling indicating whether foods have or have not been developed using bioengineering. Draft guidance. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, http://www.cfsan.fda.gov/~dms/biolabgu.html//

Fooks, L.J., Fuller, R. and Gibson, G.R. (1999). Prebiotics, probiotics and human gut microbiology. *Int Dairy J* **9**: 53–61.

Foucaud, C., Franaois, A. and Richard, J. (1997). Development of a chemically defined medium for the growth of *Leuconostoc mesenteroides*. *Appl Environ Microbiol* **63:** 301–304.

Frank, J.C., Chill, D. and Maida, N. (2002). The lactic acid bacteria: A literature survey. *Crit Rev Microbiol* **28:** 281-284.

Franz, C.M.A.P. and von Holy, A (1996). Bacterial populations associated with pasteurized vacuum-packed Vienna sausages. *Food Microbiol* **13(2):** 165-174.

Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H. and Holzapfel, W.H. (2003). Enterococci in foods: a conundrum for food safety. *Int J Food Microbiol* **88:** 105-122.

Franz, M. and Horl, W.H. (1999). Common errors in diagnosis and management of urinary tract infection. I: Pathophysiology and diagnostic techniques. *Nephrol Dial Transpl* **14:** 2754–2762.

Frece, J., Kos, B., Beganovic, J., Vukovic, S. and Suskovic, J. (2005). *In vivo* testing of functional properties of three selected probiotic strains. *World J Microbiol Biotechnol* **21:** 1401-1408.

Friend, B.A. and Shahani. K. (1984). Nutritional and therapeutic aspects of *Lactobacilli*. *J Appl Nutr* **36:** 126-36.

Frommberger, M., Schmitt-Kopplin, P., Ping, G., Frisch, H., Schmid, M., Zhang, Y., Hartmann, A. and Kettrup, A. (2004). A simple and robust set up for on column sample pre concentration nano-liquid chromatography electrospray ionization mass spectrometer for the analysis of N-acyl homoserine lactone. *Anal Bioanl Chem* **378:** 1014-1020.

Fuente, M.A., Fontecha, J. and Jufirez, M. (1993). Fatty acid composition of the triglyceride and free fatty acid fractions in different cows, ewes and goats milk cheeses. *Lebensm Unters Forsch* **196:** 155-158.

Fujisawa, T. and Mitsuoka. T. (1996). Homofermentative *Lactobacillus* species predominantly isolated from canine feces. *J Vet Med Sci* **58:** 591-593.

Fukushima, M., Yamada, A., Endo, T. and Nakano, M. (1999). Effects of a mixture of organisms, *Lactobacillus acidophilus* or *Streptococcus faecalis* on delta 6-desaturase activity in the livers of rats fed a fat and cholesterol enriched diet. *Nutr* **15:** 373-378.

Fuller, R. (1992). Probiotics: The Scientific Basis. Chapman & Hall, New York, pp. 377-386.

Fuller, R. (1999). Probiotics for farm animals. In: Tannock, G.W. (ed.): Probiotics: A General Review. Horizon Scientific Press, Wymondham, U.K.

Fuqua, C., Winans, S.C. and Greenberg**,** E.P. (1996). Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol* **50:** 727-751.

Fuqua, W.C., Winans, S.C. and Greenberg. E.P. (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176:** 269–275.

Gadaga, T.H., Mutukumira, A.N. and Narvhus, J.A. (2001). The growth and interaction of yeasts and lactic acid bacteria isolated from Zimbabwean naturally fermented milk in UHT milk. *Int J Food Microbiol* **68(**1–2): 21–32.

Gallardo-Escamilla, F.J., Kelly, A.L. and Delahunty, C.M. (2005). Influence of starter culture on flavor and headspace volatile profiles of fermented whey and whey produced from fermented milk. *Dairy Sci* **88:**3745-3753.

Gallo, G., De Angelis, M., McSweeney, P.L.H., Corbo, M.R. and Gobbetti, M. (2005). Partial purification and characterization of an X-prolyl dipeptidyl aminopeptidase from *Lactobacillus sanfranciscensis* CB1. *Food Chem* **91(3):** 535-544.

Gambelli, L., Manzi, P., Panli, G., Vivanti, V. and Pizzoferrato, L. (1999). Constituents of nutritional relevance in fermented milk products commercialized in Italy. *Food Chem* **66:** 353–358.

Garvie, E.I. (1986). Genus *Leuconostoc* van Tieghem 1878. In P. H. A. Sneath, N.S. Mair, M.E. Sharpe, and J. G. Holt (Eds.), Bergey's manual of systematic bacteriology. Baltimore: Williams and Wilkins, pp. 1071–1075.

Geddes, A. (2000). Infection in the twenty-first century: Predictions and postulates. *J Antimicrob Chemother* **46:** 873-878.

Gekas, V. and Lopez-Leiva, M. (1985). Hydrolysis of lactose: a literature review. *Process Biochem* **20**: 2–12.

Gennari, M. and Dragotto, F. (1992). A study of the incidence of different fluorescent *Pseudomonas* species and biovars in the microflora of fresh and spoiled meat and fish, raw milk, cheese, soil, and water. *J Appl Bact* **72:** 281-288.

Gevers, D., Huys, G. and Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett* **205:** 31–36.

Gherty, J. (1995). American dairy science association foundation, lecture. *J Dairy Sci* **78(7):** 1401.

Gibson, G.R. and Fuller, R. (2000). Aspects of *in vitro* and *in vivo* research approaches directed towards identifying probiotics and prebiotics for human use. *J Nutr* **130:** 391s–395s.

Gibson, G.R. and Wang, X. (1994). Bifidogenic properties of different types of fructooligosaccharides. *Food Microbiol* **11:** 491-598.

Gilbert, P., Maira-Litran, T., McBain, A.J., Rickard, A.H. and Whyte, F.W. (2002). The physiology and collective recalcitrance of microbial biofilm communities. *Adv Microb Physiol* **46:** 202–256.

Gill, H.S. and Rutherfurd, K.J. (2001). Immune enhancement conferred by oral delivery of *Lactobacillus rhamnosus* HN001 in different milk based substrates. *J Dairy Res* **68:** 611-616.

Gill, H.S., Rutherfurd, K.J., Prasad, J. and Gopal, P.K. (2000). Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *Brit J Nutr* **83:** 167–176.

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. and Speck, M.L. (1974). Relationship of cellular components to the stability of concentrated lactic *Streptococcus* cultures at -17^0C. *Appl Microbiol* **27**: 793- 796.

Gilliland, S.E. and Walker, D.K. (1990). Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *J Dairy Sci* **73:** 905-911.

Gilliland, S.E., Reilly, S.S., Kim, G.B. and Kim, H.S. (2002). Viability during storage of selected probiotic *Lactobacilli* and *Bifidobacteria* in a yogurt-like product. *J Food Sci* **67:** 3091–3095.

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

Gionchetti, P., Rizzello, F., Venturi, A., Brigidi, P., Matteuzzi, D., Bazzocchi, G., Poggioli, G., Miglioli, M. and Campieri, M. (2000). Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo controlled trial. *Gastroenterol* **119:** 305–309.

Givskov, M., de Nys, R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P.D. and Kjelleberg, S. (1996). Eukaryotic interference with homoserine lactone mediated prokaryotic signalling. *J Bacteriol* **178:** 6618-6622.

Godfrey, T. and West, S.I. (1996). Introduction to Industrial Enzymology. In : T. Godfrey Ed, Industrial Enzymology 2th Ed, MacMillan Trust, West. S. London, pp. 1-8.

Goldin, B.R. (1990). Intestinal microbiota: Metabolism of drugs and carcinogens. *Ann Med* **22:** 43-48.

Goldin, B.R., and Gorbach**,** S.L. (1992). Probiotics for humans, *In* R. Fuller (ed.), Probiotics, the scientific basis. Chapman & Hall, London, United Kingdom, pp. 355–376.

Gomez Zavaglia, A., Kociubinski, G., Perez, P. and De Antoni, G. (1998). Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. *J Food Prot* **61:** 865- 873.

Gonzalez-Siso, M.I. and Suarez-Doval, S. (1994). *Kluyveromyces lactis* immobilization on corn grits for milk whey lactose hydrolysis. *Enzyme Microb Technol* **16:** 303–310.

Goodenough, E.R. and Kleyn, D.H. (1976). Influence of viable yogurt microflora on digestion of lactose by the rat. *J Dairy Sci* **59(4):** 601-606.

Gorbach, S.L. (2000). Probiotics and gastrointestinal health. *Am J Gastroenterol* **95(suppl):** S2-S4.

Gould, T.A., Herman, J., Krank, J., Murphy, R.C. and Churchill, M.E.A. (2006). Specificity of acyl-homoserine lactone synthases examined by mass spectrometry. *J Bacteriol* **188:** 2773-783.

Gowda, L.R., Joshi, M.S. and Bhat, S.G. (1988). In situ assay of intracellular enzymes of yeast (*Kluyveromyces fragilis*) by digitonin permeabilization of cell membrane. *Anal Biochem* **175:** 531–536.

Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A., and Brassart, D. (1999). Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte like Caco-2 cells. *Appl Environ Microbiol* **65:** 1071–1077.

Grangette, C., Muller-Alouf, H. Goudercourt, D. Geoffroy, M.C. Turneer, M. Mercenier. A. (2001). Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant *Lactobacillus plantarum*. *Infect Immun* **69:** 1547-1553.

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

Greenberg, E.P. (2003). Bacterial communication and group behavior. *J Clin Invest* **112(9):** 1288-1290.

Greenberg, N.A. and Mahoney, R.R. (1982). Production and characterization of *β*- galactosidase from *Streptococcus thermophilus*. *J Food Sci* **37:** 248–254.

Gruber, K. and Sleytr, U.B. (1991). Influence of an S-layer on surface properties of *Bacillus stearothermophilus. Archives Microbiol* **156(3):** 181-185.

Guarino, A., Canani, R.B., Spagnuolo, M.I., Albano, F. and Benedetto, L.D. (1998). Oral bacterial therapy reduces the duration of symptoms and of viral excretion in children with mild diarrhea. *J Pediatr Gastroenterol Nutr* **25:** 516–519.

Guarner, F. (2005). Inulin and oligofructose: impact on intestinal diseases and disorders. *Brit J Nutr* **93(Suppl):** 61S-65S.

Guerzoni, M.E**.,** Lanciotti, R**.** and Cocconcelli**,** P.S**.** (2001). Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. *Microbiology* **147:** 2255-2264.

Gunn, J. S. (2000). Mechanisms of bacterial resistance and response to bile. *Microbes Infect* **2:** 907–913.

Gupta, P. K., Mital, B. K. and Garg, S. K. (1996). Characterization of *Lactobacillus acidophilus* strains for use as dietary adjunct. *Int J Food Microbiol* **29:** 105–109.

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

Hadani, A., Ratner, D. and Doron, O. (2002). Probactrix probiotic in the prevention of infectious bacterial diarrhoea of piglets. *Vet Med* **57(4):** 121-126.

Halliwell, B. and Cherico, S. (1993). Lipid peroxidation: its mechanism, measurement and significance. *Am J Clin Nutr* **57:** 715S-725S.

Halliwell, B. and Gutteridge, J.M.C. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* **219:** 1-4.

Halliwell, B., Murcia, H.A., Chirco, S. and Aruoma, O.I. (1995). Free radicals and antioxidants in food an *in vivo*: what they do and how they work. *CRC Crit Rev Food Sci Nutr* **35:** 7–20

Hamasaki, Y., Ayaki, M., Fuchu, H., Sugiyama, M. And Morita, H. (2003). Behavior of psychrotrophic lactic acid bacteria isolated from spoiling cooked meat products. *Appl Environ Microbiol* **69:** 3668–3671.

Hamdy, M.K. and Williams, F.S. (2001). Bacterial amelioration of bauxite residue water of industrial alumina plants. *J Ind Microbiol Biotechnol* **27:** 228–233.

Hammer, H.F., Petritsch, W., Pristautz, H., Krejs, G.J. (1996). Evaluation of the pathogenesis of flatulence and abdominal cramps in patients with lactose malabsorption. *Wien Klin Wochenschr* **108:**175-179.

Hammerman, C., Bin-Nun, A., Kaplan, M. (2006) Safety of probiotics: comparison of two popular strains. *BMJ* **333:** 1006-1008.

Hanafusa, J.V. (1985). The hydration of water and protein with cryoprotectant. In Fundamentals and Applications of Freeze-Drying to Biological Materials, Drugs and Food Stuffs. Paris: International Institute of Refrigeration, pp. 59–62

Hannes, A. N., Moes, C.J. and Dicks, M.T. (2000). Sluggish/ stuck malolactic fermentation in chardonnay: possible causes. Wynboer; Atechnical guide for wine producers.

Hart, C.A. and Winstanley, C. (2002). Persistent and aggressive bacteria in the lungs of children with cystic fibrosis. *Brit Med Bulletin* **61:** 81-96.

Hashimoto, H., Yamazaki, K., He, F., Kawase, M., Hosoda, M. and Hosono, A. (1999). Hypocholesterolemic effects of *Lactobacillus casei* subsp. *casei* TMV0409 strain observed in rats fed cholesterol contained diets. *Animal Sci J* **72:** 90–97.

Hassett, D.J., Ma, J.F., Elkins, J.G., McDermott, T.R., Ochsner, U.A., West, S.E., Huang, C.T., Fredericks, J., Burnett, S., Stewart, P.S., McFeters, G., Passador, L. and Iglewski, B.H. (1999). Quorum sensing in *Pseudomonas aeroginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Mol Microbiol* **34:** 1082–1093.

Hatakka, K., Savilahti, E., Ponka, A., Meurman, J.H., Poussa, T., Nase, L., Saxelin, M., Korpela, R. (2001). Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. *BMJ* **322:** 1327-1332.

Hatcher, G.E. and Lambrecht, R.S. (1993). Augmentation of macrophage phagocytic activity by cell-free extracts of selected lactic acid producing bacteria. *J Dairy Sci* **76:** 2485–2492.

Haudault, S., Lieven, V., Bernet-Camard, M.F. and Servin, A.L. (1997). Antagonistic activity exerted *in vitro* and *in vivo* by *L. casei* (strain *GG*) against *S. typhimurium* C5 infection. *Appl Environ Microbiol* **63:** 513-518.

Havenaar, R. and Huis in't Veld, J.H.J. (1992). Probiotics: a general view. The lactic acid bacteria. In *The Lactic Acid Bacteria in Health and Disease,* ed. B.J.B. Wood, Vol. 1, Elsevier Applied Science, London, pp. 151-71.

Hay, J.M., Yu, W.M. and Ashraf, T. (1999). Pharmacoeconomics of lipids lowering agents for primary and secondary prevention of coronary artery disease. *Pharmacoeconomics* **15:** 47- 74.

Hayatsu, H., Hamasaki, K., Togawa, K., Arimoto, S. and Negishi, T. (1983). Carcinogens and mutagens in the environment. In *Naturally Occurring Compounds*, vol. 2, Boca Raton, FL: CRC Press, pp. 91–99.

Hayatsu, H., Inoue. K., Ohta, H., Namba, T., Togawa, K., Hayatsu, T., Makita, M. and Wataya, Y. (1981). Inhibition of the mutagenicity of cooked-beef basic fraction by its acidic fraction. *Mut Res* **91:** 437–442.

Hechard, Y., Derijard, B., Letellier, F. and Cenatiempo, Y. (1992). Characterization and purification of mesentericin Y105, an anti- Listeria bacteriocin from *Leuconostoc mesenteroides*. J Gen Microbiol **138:** 2725–2731.

Hemme, D. and Foucaud-Scheunemann, C. (2004). *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. *Int Dairy J* **14:** 467–494.

Hemme, D., Nardi, M.B. and Jette, D. (1980). *β*-galactosidase and *β*-phospho–galactosidase in *Streptococcus thermophilus*. *Le Lait* **60**: 595-618.

Hendricks, C.L., Ross, J.R., Pichersky, E., Noel, J.P. and Zhou, Z.S. (2004). An enzyme-coupled colorimetric assay for S-adenosylmethionine-dependent methyltransferases. *Anal Biochem* **326:** 100–105.

Hensiek, R., Krupp, G. and Stackebrandt, E. (1992). Development of diagnostic oligonucleotide probes for four *Lactobacillus* species occurring in the intestinal tract. *Sys Appl Microbiol* **15:** 123-128.

Hentzer, M., Riedel, K., Rasmussen, T.B., Heydorn, A., Andersen, J.B., Parsek, M.R., Rice, S.A., Eberl, L., Molin, S., Hoiby, N., Kjelleberg, S. and Givskov, M. (2002). Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* **148**: 87–102.

Hentzer, M., Wu, H., Andersen, J.B., Riedel, K., Rasmussen, T.B., Bagge, N., Kumar, N., Schembri, M.A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J.W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., H¢iby, N. and Givskov, M. (2003). Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *The EMBO J* **22(15):** 3803-3815.

Herreros, M.A., Sandoval, H., Gonzalez, L., Castro, J.M., Fresno, J.M. and Tornadijo, M.E. (2005). Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). *Food Microbiol* **22(5):** 455-459.

Hertzler, S.R. and Savaiano, D.A. (1996). Colonic adaptation to daily lactose feeding in lactose maldigesters reduces lactose intolerance. *Am J Clin Nutr* **6(4):** 232-236.

Heyman, M. (2000). Effect of lactic acid bacteria on diarrheal diseases. *J Am College Nutr* **19(2):** 137S–146S.

Hickey, M.W., Hillier, A.J., Jago, G.R. (1986). Transport and metabolism of lactose, glucose, and galactose in homofermentative *Lactobacilli Appl Environ Microbiol* **51(4):** 825-831.

Hilliam, M. (1998). The market for functional foods. *Int Dairy J* **8:** 349–353.

Hilton, E., Kolakowski, P., Singer, C. and Smith, M. (1997). Efficacy of *Lactobacillus GG* as a diarrheal preventive in travellers. *J Travel Med* **4:** 41–43.

Hirayama, K. and Rafter, J. (2000). The role of probiotic bacteria in cancer prevention. *Microbes and Infection* **2(6):** 681-686.

Hirschler, A., Rontani, J.F. and Raphel, D. et al. (1998). Anaerobic degradation of hexadecane-2-one by a microbial enrichment culture under sulfate reducing conditions. *Appl Environ Microbiol* **64:** 1576-1579.

Hobden, J.A. (2002). *Pseudomonas aeruginosa* Proteases and Corneal Virulence DNA and Cell Biology **21(5-6):** 391-396.

Hofi, M.A. (1990). Low-lactose labneh and zabady by ultrafiltration. *Scan Dairy Infor* **4:** 30–3.

Hogenauer, C., Hammer, H.F., Mellitzer, K., Renner, W., Krejs, G.J.K. and Toplak, H. (2005). Evaluation of a new DNA test compared with the lactose hydrogen breath test for the diagnosis of lactase nonpersistence. *Eur J Gastroenterol Hepatol* **17(3):** 371–376.

Holm, C., Jepsen, L., Larsen, M. and Jespersen, L. (2004). Predominant microflora of downgraded danish bulk tank milk. *J Dairy Sci* **87:** 1151-1157.

Holzapfel, W.H., Haberer, P., Geisen, R., Bjorkroth, J. and Schillinger, U. (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am J Clin Nutr* **73:** 365S–373S.

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U. and Huis Int Veld, J. H. J. (1998). Overview of gut flora and probiotics. *Int J Food Microbiol* **41:** 85– 101.

Holzapfel, W.H.N. and Wood, B.J. (1998). Genera of Lactic Acid Bacteria. Series: The Lactic Acid Bacteria, Vol. 2, Blackie Academic and Professional, Glasgow, pp. 307-420.

Hong, H.A., Le Hong Duc. and Cutting, S.M. (2005). The use of bacterial spore formers as probiotics. *FEMS Microbiol Rev* **29:** 813–835.

Hoolihan, L.E. Individualization of Nutrition Recommendations and Food Choices. Diet and Behavior. *Nutrition Today* **38(6):** 225-231.

Hoover, D.G. (1993). *Bifidobacteria*: activity and potential benefits. *Food Technol* **47(6):** 120-124.

Hourigan, J.A. (1984). Nutritional implication of lactose. *Aus J Dairy Tech* **39:** 114–120.

Houseknecht, K.L., Vanden-Heuvel, J.P., Moya-Camarena, S.Y., Portocarrero, C.P., Peck, L.W. and Nickel, K.P. (1998). Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty rat. *Biochem Biophy Res Comm* **244:** 678–682.

Howard, J.C., Heinemann, C., Thatcher, B.J., Martin, B., Siang Gan, B. and Reid, G. (2000). Identification of collagen-binding proteins in *Lactobacillus* spp. With surface enhanced laser desorption/ionization-time of flight protein chip technology. *Appl Environ Microbiol* **68:** 4396–4400.

Hsu, C.A., Yu , R.C., Lee, S.L. and Chou, C.C. (2007). Cultural condition affecting the growth and production of *β*-galactosidase by *Bifidobacterium longum* CCRC 15708 in a jar fermenter. *Int J Food Microbiol* **116:** 186–189.

Hsu, C.A., Yu, R.C. and Chou, C.C. (2005). Production of *β*-galactosidase by *Bifidobacteria* as influenced by various culture conditions. *Int J Food Microbiol* **104:** 197– 206.

Huang, J.S., Bousvaros, A., Lee, J.W., Diaz, A. and Davidson, E.J. (2002). Efficacy of probiotic use in acute diarrhea in children. *Dig Dis Sci* **47:** 2625-2634.

Huang, Y. and Adams, M.C. (2004). *In vitro* assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *Int J Food Microbiol* **91(3):** 253-260.

Hubalek, Z. (1996). Cryopreservation of microorganisms at ultra low temperature. Vyd. 1st Praha Academia, Czech Republic, pp. 70-72.

Hubalek, Z. (2003). Protectants used in the cryopreservation of microorganisms. *Cryobiol* **46**: 205-229.

Hugas, M. (1998). Bacteriocinogenic lactic acid bacteria for the biopreservation of meat and meat products. *Meat Sci* **49:** S139-S150.

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

Hughes, D.B. and Hoover, D.G. (1995). Viability and enzymatic activity of *Bifidobacteria* in milk. *J Dairy Sci* **78:** 268–276.

Huis in't Veld, J.H.J. (1996). Microbial and biochemical spoilage of foods: an overview. *Int J Food Microbiol* **33:** 1 – 18.

Hummel, A., Holzapfel, W.H. and Franz, C.M.A.P. (2007). Characterization and transfer of antibiotic resistance genes from enterococci isolated from food. *Syst Appl Microbiol* **19:** 1-7.

Hung, M.N. and Lee, B.H. (2002). Purification and characterization of a recombinant *β*- galactosidase with transgalactosylation activity from *Bifidobacterium infantis* HL96. *Appl Microbiol Biotechnol* **58:** 439–445.

Husni, R.N., Gordon, S.M., Washington, J.A., Longworth, D.L. (1997). *Lactobacillus* bacteremia and endocarditis: Review of 45 cases. *Clin Infect Dis* **25:**1048–1055.

Hynonen, V., Westerlund-Wikston, A.B. and Korhowen, T.K. (2002). Fibronectin-binding function in the SlpA surface protein of *Lactobacillus brevis*. *J Biotechnol* **174**: 3360–3362.

Ibrahim, S.A. and O'Sullivan D.J. (2000). Use of chemical mutagenesis for the isolation of food grade *β*-galactosidase overproducing mutants of *Bifidobacteria, Lactobacilli* and *Streptococcus thermophilus*. *J Dairy Sci* **83:** 923–930.

IDF (Intl Dairy Fed). (1992). General standard of identity for fermented milks. Vol. 163. Belgium: Intl Dairy Fed. Martinez-Villaluenga., Frias, J., Vidal-Valverde, C. and Gomez, R. (2005). Raffinose family oligosaccharides from lupin seeds as prebiotics: Application in Dairy Products. *J Food Prot* **68:** 1246–1252.

IDF- International standard (1996) Milk-Determination of fat content (Rose Gottlieb reference method). IDF, Brussels.

Inchaurrondo, V.A., Flores, M.V. and Voget, C.E. (1998). Growth and *β*-galactosidase synthesis in aerobic chemostat cultures of *Kluyveromyces lactis*. *J Ind Microbiol Biotech* **20:** 291– 298.

Ip, C., Chin, S.F., Scimeca, J.A. and Pariza, M.W. (1991). Mammary cancer prevention by conjugated dienoic derivatives of linoleic acid. *Cancer Res* **51:** 6118–6124.

Ishibashi, N. and Yamazaki, S. (2001). Probiotics and safety. *Am J Clin Nutr* **73(suppl):** 465S-470S.

Isolauri, E. (2004). Future aspects of probiotics in Health Probiotics: Immunomodulatory potential against allergic disease. *J Food Sci* **69(5):**11-15.

Isolauri, E., Arvola, T., Sutas, Y. and Salminen, S. (2000). Probiotics in the management of atopic eczema. *Clin Exp Allergy* **30:** 1605-1610.

Isolauri, E., Salminen, S. and Mattila-Sandholm. T. (1999). New functional foods in the treatment of food allergy. *Ann. Med*. **31:**299–302.

Isolauri, E., Suomalainen, H., Kaila, M., Jalonen, T., Soppl, E., Virtanen, E., and Arvilommi, H., (1992). Local immune response in patients with cow milk allergy follow up of patients retaining allergy or becoming tolerant. *J Pediatr* **120:** 9-15.

Itoh, T., Ohhashi, M., Toba, T. and Adachi, S. (1980). Purification and properties of *β*-galactosidase from *Lactobacillus bulgaricus*. *Milchwissenschaft* **35:** 593–597.

Jacob. M.B. (1958). The chemical analysis of foods and food products (3rd ed.), D. Van Nastrand Co Inc, Princeton, NJ, 393-394.

Jacobsen, C.N., Rosenfeldt, N.V., Hayford, A.E., Mille, P.L, Michaelsen, K.F., Paerregaard, A., Sandstrom, 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.

Jagerstad, M., Jastrebova, J. and Svensson, U. (2004). Folates in fermented vegetables: a pilot study. *Lebensmittel-Wissenschaft Und-Technologie* **37**: 603–611.

Jang, J., Kim, B., Lee, J. and Han, H. (2003). A rapid method for identification of typical *Leuconostoc* species by 16S rDNA PRCRFLP analysis. *J Microbiol Methods* **55:** 295–302.

Jaspe, A., Oviedo, P., Fernandez, L., Palacios, P. and SanJose, C. (1995). Cooling raw milk: change in the spoilage potential of contaminating *Pseudomonas*. *J Food Prot* **58:** 915-921.

Jay, J.M. (1992). Modern food microbiology. Van Nostrand-Reinhold, 4th Ed., Chapman and Hall, New. York.

Jay, J.M. (2000). Fermentation and fermented dairy products. In Modern Food Microbiology 6th edition. An Aspen Publication Aspen Publishers Inc. Gaithersburg USA, pp. 113- 130.

Jay, J.M., Vilai, J.P. and Hughes, M.E. (2003). Profile and activity of the bacterial biota of ground beef held from freshness to spoilage at 5–7 °C. *Int J Food Microbiol* **81**(2): 105–111.

Jennings, T.A. (1999). Lyophilization: introduction and basic principles. Interpharm Press, Englewood, Colorado, USA, pp. 165-252.

Jiang, T., Mustapha, A. and Savaiano, D.A. (1996). Improvement of lactose digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*. *J Dairy Sci* **79:** 750–757.

Jin, L.Z., Ho, Y.W., Abdullah, N. and Jalaludin, S. (1998). Growth performance, intestinal microbial populations and serum cholesterol of broilers fed diets containing *Lactobacillus* cultures. *Poult Sci* **77:** 1259–1265.

Joshi, M.S., Gowda, L.R., Katwa, L.C. and Bhat, S.G. (1989). Permeabilization of yeast cells (*Kluyveromyces fragilis*) to lactose by digitonin. *Enzyme Microbial Technol* **11:** 439–443.

Juhas, M., Eberl, L. and Tummler, B. (2005). Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ Microbiol* **7:** 459–471.

Juradol, E., Camacho, F., Luzon, G. and Vicaria, J.M. (2004). Kinetic models of activity for *β*-galactosidases: influence of pH, ionic concentration and temperature. *Enzyme Microb Technol* **34(1):** 33-40.

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

Kailasapathy, K and Rybka, S. (1997). *L. acidophilus* and *Bifidobacterium spp*.-their therapeutic potential and survival in yogurt. *Aust J Dairy Technol* **52(1):** 28–35.

Kalambaheti, T., Cooper, G. N. and Jackson, G. D. F. (1994). Role of bile in non-specific defence mechanisms of the gut. *Gut* **35:** 1047–1052.

Kalliomaki, M., Salminen, S., Kero, P., Arvilommi, H., Koskinen, P. and Isolauri, E. (2001). Probiotics in the primary prevention of atopic disease: a randomized placebo controlled trial. *Lancet* **357**: 1076-1079.

Kang, C.I., Kim, S.H., Kim, H.B., Park, S.W., Choe, Y.J., Oh, M.D., Kim, E.C. and Choe, K.W. (2003). *Pseudomonas aeruginosa* bacteremia: risk factors for mortality and influence of delayed receipt of effective antimicrobial therapy on clinical outcome. *Clin Infect Dis* **37:** 745-751.

Kannappan, S. and Manja. K.S. (2004). Antagonistic efficacy of lactic acid bacteria against seafood borne bacteria. *J Food Sci Technol Mysore* **41:**50-59.

Karasova, P., Spiwok, V., Mala, S., Kralova, B. and Russell, N.J. (2002). Beta-galactosidase activity in psychrotrophic microorganisms: Their potential use in food industry. *Czech J Food Sci* **20:** 41–42.

Kato, I., Endo, K. and Yokura, T. (1994). Effects of oral administration of *Lactobacillus casei* on antitumor responses induced by tumor resection in mice. *Int J Immunopharmacol* **16:** 29–36.

Kato, T., Haruki, M., Imanaka, T., Morikawa, M. and Kanaya, S. (2001). Isolation and characterization of long-chain-alkane degrading *Bacillus thermoleovorans* from deep subterranean petroleum reservoirs. *J Biosci Bioengineer* **91(1):** 64-70.

Kawata, T., Masuda, K., Yoshino, K., Fujimoto, M. (1974). Regular array in the cell wall of *Lactobacillus fermentum* as revealed by freeze-etching and negative staining. *J Microbiol* **18:** 469–476.

Khalil, R., Mahrous, H., El-Halafawy, K., Kamaly, K., Frank, J. and Soda, M.E. (2007). Evaluation of the probiotic potential of lactic acid bacteria isolated from faeces of breast fed infants in Egypt. *Afr J Biotechnol* **6(7):** 939-949.

Kim, B., Lee, J., Jang, J., Kim, J., & Han, H. (2003). *Leuconostoc inhae sp. nov*, a lactic acid bacterium isolated from kimchi. *Int J Sys Evolutionary Microbiol* **53:** 1123–1126.

Kim, D.S., Thomas, S. and Scott-Fogler, H. (2000). Effect of pH and trace minerals on long term starvation of *Leuconostoc mesenteroides*. *Appl Environ Microbiol* **66:** 976-981.

Kim, J.W. and Rajagopal, S.N. (2000). Isolation and characterization of β-galactosidase from *Lactobacillus crispatus*. *Folia Microbiol* **45:** 29–34.

Kimura, K., McCartney, A.L., McConnell, M.A. and Tannock G.W. (1997). Analysis of fecal populations of *Bifidobacteria* and *Lactobacilli* and investigation of the immunological responses of their human hosts to the predominant strains. *Appl Environ Microbiol* **63:** 3394–3398.

Kinsella, J.E. and Hwang, D.H. (1976). Biosynthesis of flavors by *Penicillium roqueforti*. *Biotechnol Bioeng* **18:** 927–938.

Kirjavainen, P.V, Salminen, S.J. and Isolauri, E. (2003). Probiotic bacteria in the management of atopic disease: underscoring the importance of viability. *J Pediatr Gastroenterol Nutr* **36(2):** 223-227.

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

Kishi, A., Uno, K. and Matsubara, Y. (1996). Effect of the oral administration of *Lactobacillus brevis* subsp. *coagulans* on interferon alpha producing capacity in humans. *J Am Coll Nutr* **15:** 408–412.

Kitchell, G. and Shaw, B.G. (1995). Lactic acid bacteria in fresh and cured meat. In: J.G. Carr, C.V. Cutting and G.C. Whiting, Editors, Lactic Acid Bacteria in Beverages and Foods, Academic Press, London pp. 209–222.

Klaenhammer, T.R. (1988). Bacteriocins of lactic acid bacteria. *Biochime* **70:** 337-349.

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 Environ Microbiol* **59**: 1120-1124.

Klijn, N., Weerkamp, A.H and de Vos, W.M. (1995). Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. *Appl Environ Microbiol* **61:** 2771-2774.

Klingberg, T.D., Axelsson, L., Naterstad, K., Elsser, D. and Budde, B.B. (2006). Identification of potential probiotic starter cultures for Scandinavian type fermented sausages. *Int JFood Microbiol* **105:** 419–431.

Knasmuller, S., Steinknellner, H., Hirschl, A.M., Rabot, S., Nobis, E.C. and Kassle, F. (2001). Impact of bacteria in dairy products and of intestinal microflora on the genotoxic and carcinogenic effects of heterocyclic aromatic amines. *Mut Res* **480:** 129–138.

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

Koch, A.K., Kappeli, O., Fiechter, A. and Reiser**,** J. (1989). Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *J Bacteriol* **173:** 4212– 4219.

Koch, B., Liljefors, T., Persson, T., Nielsen, J., Kjelleberg, S. and Givskov, M. (2005). The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. *Microbiology* **151:** 3589–3602

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

Konings, W.N. (2002). The cell membrane and the struggle for life of lactic acid bacteria. *Antonie van Leeuwenhoek* **82:** 3–27.

Konsoula, Z. and Kyriakides, M.L. (2007). Co-production of a-amylase and *β*-galactosidase by *Bacillus subtilis* in complex organic substrates *Biores Technol* **98:** 150–157.

Kontiokari, T., Sundqvist, K. and Nuutinen, M. (2001). Randomized trial of cranberry lingonberry juice and *Lactobacillus* GG drink for the prevention of urinary tract infections in women. *Brit Med J* **322:** 1571-1577.

Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J. and Matosic, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus M92*. *J Appl Microbiol* **94:**981–987.

Koval, S.F. (1988). Paracrystalline protein surface arrays on bacteria. *Can J Microbiol* **33:** 407–414.

Kowalska, A., Jachnowicz, A.Z. and Babuchowski, A. (2000). Yoghurt market in the United Kingdom. *Natural Sci* **6:** 131–141.

Kozlova, E.V., Pivovarenko, T.V., Malinovskaia, I.V., Aminov, R.I., Kovalenko, N.K. and Voronin, A.M. (1992). Antibiotic resistance of *Lactobacillus* strains. *Antibiot Khimioter* **37(6):** 12-15.

Kraft, A.A. (1992). Psychrotrophic Bacteria in Fooak Disease and Spoilage. CRC Press, Boca Raton, FL, pp. 99-112.

Krieg, N. (1984). Bergey's manual of systematic bacteriology, Vol-2, Baltirmore: Williams and Wilkins.

Kroger, M. and Weaver, J.C. (1983). Confusion about yoghurt; compositional and otherwise. *J Milk Food Technol* **36:** 383.

Kuipers, O.P., Buist, G. and Kok. J. (2000). Current strategies for improving food bacteria. *Res Microbiol* **151:** 815-822.

Kuisma, J., Mentula, S., Jarvinen, H., Kahri, A., Saxelin, M. and Farkkila, M. (2003). Effect of *Lactobacillus rhamnosus* GG on ileal pouch inflammation and microbial flora. *Alim Pharmacol Therap* **17:** 509–515.

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C. and Kilk, A. (2002). Two antioxidative *Lactobacilli* strains as promising probiotics. *Int J Food Microbiol* **72:** 215–224.

Kurman, A., Rasic J.L. and Kroger, M. (1992). Encyclopedia of Fermented Fresh Milk Products, Van Nostrand Reinhold, New York, pp. 309-316.

Kurmann, J.A. and Rasic, J.L. (1991). The health potential of products containing *Bifidobacteria*. In: Robinson RK, editor. Therapeutic properties of fermented milks. London: Elsevier. pp. 117–158.

Kurreck, J. (2003). Antisense technologies: Improvement through novel chemical modifications. *Eur J Biochem* **270:** 1628-1644.

Labadie, J. (1999). Consequences of packaging on bacterial growth. Meat is an ecological niche. *Meat Sci* **52:** 299-305.

Laemmli, U. K. **(**1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Lankaputhra, W.E.V. and Shah, N.P. (1998). Antimutagenic properties of probiotic bacteria and of organic acids. Mut Res **397:** 169–182.

Lara-Villoslada, F., Sierra, S., Martin, R., Delgado, S., Rodriguez, J.M., Olivares, M. and Xaus, J. (2007). Safety assessment of two probiotic strains, *Lactobacillus coryniformis* CECT5711 and *Lactobacillus gasseri* CECT5714. *J Appl Microbiol* **103(1):** 175-184.

Larena, I., Melgarejo, P. and De Cal, A. (2003). Drying of conidia of *Penicillium oxalicum*, a biological control agent against fusarium wilt of tomato. *J Pytopathol* **151:** 600–606.

Larsen, C.N., Nielson, S., Kaestel, P., Brockmann, E., Bennedsen, M., Christensen, H.R., Eskensen, D.C., Jocobsen, B.L., Michaelsen, K.E. (2006). Dose response study of probiotic bacteria *Bifidobacterium animalis* and *Lactobacillus paracasei* in healthy young adults. *Euro J Clin Nutr* **60:** 1284-1293.

Larsen, L.A., Raben, A., Haulrik, N., Hansen, A.S., Manders, M. and Astrop, A. (2000). Effect of 8 week intake of probiotic milk products on risk factors for cardiovascular diseases. *Eur J Clin Nutr* **54:** 288-297.

Launiala, K. (1968). The effect of unabsorbed sucrose and mannitol on the small intestinal flow rate and mean transit time. *Scand J Gastroenterol* **3:** 665-671.

Le Vay, D. (1988). 13 Digestion. In: *Human Anatomy Physiology* (3rd ed.), Hodder and Stoughton, Sevenoaks, Kent, pp. 231–233.

Lee, H.J., Park, S.Y. and Kim, J. (2000). Multiplex PCR-based detection and identification of *Leuconostoc* species. *FEMS Microbiol Lett* **15:** 243–247.

Lee, K.N., Kritchevsky, D. and Pariza, M.W. (1994). Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis* **108:** 19–25.

Lee, W.K., Lee, S.M., Bae, H.S.Y.J. and Baek, Y.J. **(**1999). Effect of *Bifidobacterium longum* HY8001 administration on human fecal bacterial enzyme and microflora. *Korean J Appl Microbiol Biotechnol* **27**: 267-272.

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

Lee, Y.K. and Wong, S.F. (1998). Stability of lactic acid bacteria in fermented milk. In: Salminen, S., von Wright, A., eds. Lactic acid bacteria, microbiology and functional aspects. New York: Marcel Dekker Inc, pp. 103–114.

Lee, Y.W. (1997). Effect of fermented milk on the blood cholesterol level of Korean. *J Food Safety* **12:** 83-95.

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

Leichter, J., Goda, T., Bhandari, S.D., Bustamante, S.and Koldovsky, O. (1984). Relation between dietary-induced increase of intestinal lactase activity and lactose digestion and absorption in adult rats. *Am J Physiol Gastrointest Liver Physiol* **247:** G729-G735.

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

Lesniewska, V., Rowland, I., Laerke, H.N., Grant, G. and Naughton, P.J. (2006). Relationship between dietary-induced changes in intestinal commensal microflora and duodenojejunal myoelectric activity monitored by radiotelemetry in the rat in-vivo. *Exp Physiol* **91:** 229-237.

Leverrier, P., Vissers, J.P., Rouault, A., Boyaval, P. and Jan, G. (2004). Mass spectrometry proteomic analysis of stress adaptation reveals both common and distinct response pathways in *Propionibacterium freudenreichii*. *Arch Microbiol* **181:** 215–230.

Lidbeck, A., Geltner Allinger, U., Orrhage, K.M., Ottova, L., Brismar, B., Gustafsson, J.A., Rafter, J.J., and Nord, C.E. (1991). Impact of *Lactobacillus acidophilus* supplements on the faecal microflora and soluble faecal bile acids in colon cancer patients. *Microbial Ecol Health Dis* **4:** 81-88.

Lidbeck, A., Nord, C.E., Gustafsson, J.A. and Rafter, J. (1992). *Lactobacilli*: anticarcinogenic activities and human intestinal microflora. *Eur J Cancer Prev* **1:** 341-353.

Lilley, D.M. and Stillwell, R.H. (1965). Probiotics, growth promoting factors produced by microorganisms. *Sci* **147:** 747–748.

Lim, H.J, Kim,S.Y. and Lee, W.K. (2004). Isolation of cholesterol-lowering lactic acid bacteria from human intestine for probiotic use. *J Vet Sci* **5(4):** 391–395.

Lin, M.Y. and Yen, C.L. (1999). Antioxidative ability of lactic acid bacteria. *J Agric Food Chem* **47(4):**1460–1466.

Lin, T.Y. (2000). Conjugated linoleic acid concentration as affected by lactic cultures and additives. *Food Chem* **69:** 27–31.

Linders, L.J.M., Meedink, G., Vant Riet, K. (1997). Effect of growth parameters on the residual activity of *L. plantarum* after drying. *J Appl Microbiol* **82:** 683–688.

Liong, M.T. and Shah, N.P. (2005) Optimization of cholesterol removal by probiotics in the presence of prebiotics by using a response surface method. *Appl Environ Microbiol* **71(4):** 1745–1753.

Litchfield, Jr. J.T. and Wilcox , F. (1949). A simplified method of evaluating dose effect experiments. *J Pharmacol Exp Ther* **96:** 99–113.

Littman. A., and Hammond, J.B. (1965). Diarrhea in adults caused by deficiency in intestinal disaccharidase. *Gastroenterol* **48:** 237-249.

Liu, M., and Griffiths, M.W. (2003). The role of quorum sensing in the regulation of protease by *Pseudomonas fluorescens* and its relation to food spoilage. In Proc. 2003 IFT Ann. Mtg. Chicago, IL. IFT, Chicago, IL, pp. 85 (29G–26)

Liu, S. Q., Holland, R. and Crow, V. L. (2003). The potential of dairy lactic acid bacteria to metabolise amino acids via non-transaminatingreactions and endogenous transamination. *Int J Food Microbiol* **86:** 257–269.

Logan, A.C., Venket, R.A. and Irani. D. (2003). Chronic fatigue syndrome: lactic acid bacteria may be of therapeutic value. *Med Hypoth* **60:** 915-923.

Logothetis, N. and Wynn, H.P. 1989. Quality through design. Clarendon Press, Oxford, New York, NY, pp. 464.

Lonvaud-Funel, A. and Desens, C. (1990). Constitution en acides gras des membranes des bacte´ries lactiques du vin. Incidences des conditions de culture. *Sci Aliments* **10:** 817– 829.

Lopez-Diaz, T.M., Alonso, C., Roman, C., Garcia Lopez, M.L., and Moreno, B. (2000). Lactic acid bacteria isolated from a hand made Blue cheese. *Food Microbiol* **17:** 23– 32.

Lorca, G., Torino, M.I., Font de Valdez, G. and Ljungh, A. (2002). *Lactobacilli* express cell surface proteins which mediate binding of immobilized collagen and fibronectin. *FEMS Microbiol Lett* **206:** 31–37.

Lorca, G.L. and Font de Valdez, G. (2001). A low-pH-inducible, stationary phase acid tolerance response in *Lactobacillus acidophilus* CRL 639. *Curr Microbiol* **42:** 21-25.

Lore, T.A., Mbugua, S.K. and Wangoh, J. (2005). Enumeration and identification of microflora in suusac, a Kenyan traditional fermented camel milk product, *Lebensm Wiss Technol* **38:** 125–130.

Lortal, S., van Heijenoort, J., Gruber, K. and Sleytr, U.B. (1992) S-layer of *L. helveticus* ATCC 12046: isolation, chemical characterization and re-formation after extraction with LiCl. *J Gen Microbiol* **138**: 611–618.

Lourens-Hattingh, A. and Viljoen, B.C. (2001). Yoghurt as probiotic carrier food. Int *Dairy J* **11(1/2):** 1–17.

Lovelace, H.Y. and Barr, S.I. (2005). Diagnosis, symptoms, and calcium intakes of individuals with self reported lactose intolerance. *J Am Coll Nutr* **24:** 51-57.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein estimation with the folin phenol (ciocalteau) reagent. *J Biol Chem* **193:** 265-271.

Lucke, F.K. and Earnshaw, R.G. (1990) Starter cultures. In *Food Preservatives* ed. Russell, N.J. and Gould, G.W. Glasgow and London: Blackie, pp. 215-234.

Macfarlane, G.T., Macfarlane, S. and Gibson, G. R. (1998). Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colonic microbiota. *Microb Ecol* **35:** 180–187.

Mack, D.R., Michail, S., Wei, S., McDougall, L. and Hollingsworth, M.A. (1999). Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. *Am J Physiol Gastrointest Liver Physiol* **276:** G941-G950.

Mackay, A.D., Taylor, M.B., Kibbler, C.C. and Hamilton-Miller, J.M.T. (1998). *Lactobacillus* endocarditis caused by a probiotic organism. *Clin Microbiol Infect* **5:** 290-292.

Madsen, K., Cornish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., Doyle, J., Jewell, L. and de Simone, C. (2001). Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterol* **121:** 580–591.

Mah, T.F.C. and O'Toole, G.A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9(1):** 34-39.

Mahoney, R.R. (1997). Lactose: enzymatic modification. In: P. F. Fox, *Advanced dairy chemistry* New York: Chapman & Hall, pp. 77-126.

Mahoney, R.R. (1998). Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. *Food Chem* **63(2):** 147–154.

Majamaa, H. and Isolauri, E. (1997). Probiotics: a novel approach in the management of food allergy. *J Allergy Clin Immunol* **99:**179–185.

Makras, L. and de Vyust, L. (2006). The *in vitro* inhibition of gram negative pathogenic bacteria by Bifidobacteria is caused by the production of organic acids. *Int Dairy J*, **16**: 1049-1057.

Manefield, M., Harris, L., Rice, S.A., de Nys, R. and Kjelleberg, S. (2000). Inhibition of luminescence and virulence in the black tiger prawn pathogen *Vibrio harveyi* by intercellular signal antagonists. *Appl Environ Microbiol* **66:** 2079-2084.

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

Margolles, A., Garcia, L., Sanchez, B., Gueimonde, M. and de los Reyes-Gavilan, C.G. (2003). Characterisation of a *Bifidobacterium* strain with acquired resistance to cholate: A preliminary study. *Int J Food Microbiol* **82(2):** 191-198.

Marotta,F., Naito,Y., Minelli,E., Tajiri,H., Bertuccelli,J., Wu,C.C., Min, C.H., Hotten, P. and Fesce, E. (2003). Chemopreventive effect of a probiotic preparation on the development of preneoplastic and neoplastic colonic lesions: an experimental study. *Hepatogastroenterology* **50(54):** 1914-1918.

Marteau, P., Minekus, M., Havenaar, R. and Huis Int Veld, J. H. J. (1997). Survival of lactic acid bacteria in a dynamic model of stomach and small intestine: validation and the effect of bile. *J Dairy Sci* **80:** 1031–1037.

Marteau, P., Pochart, P., Bouhnik, Y. and Rambaud, J.C. (1993). Fate and effects of transiting microorganisms in the human gastrointestinal tract. *World Rev Nutr Diet* **74:** 1-21.

Marteau, P., Rochart, P., Flourie, B., Pellier, P., Santos, L., Desjeux, J.F., and Rambaud, J.C. (1990). Effect of chronic ingestion of a fermented dairy product containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on metabolic activities of the colonic flora in humans. *Am J Clin Nutr* **52:** 685–688.

Marteau, P., Seksik, P. and Jian, R. (2002). Probiotics and intestinal health effects: a clinical perspective. *Brit J Nutr* **88:** S51–S57.

Martinez-Murcia, A.J. and Collins, M.D. (1991). A phylogenetic analysis of an atypical *Leuconostoc*: Description of *Leuconostoc fallax sp. nov*. *FEMS Microbiol Lett* **82:** 55–60.

Martley, F.G. and Crow, V.L. (1993). Interactions between non-starter microorganisms during cheese manufacture and ripening. *Int Dairy J* **3:** 461–483.

Masja, N., Groot, N. and de Bont, J.A.M. (1998). Conversion of Phenylalanine to Benzaldehyde Initiated by an Aminotransferase in *Lactobacillus plantarum. Appl Environ Microbiol* **64(8):** 3009–3013.

Masuda, K. (1992). Heterogeneity of S-layer proteins of *Lactobacillus acidophilus* strains. *Microbiol Immunol* **36:** 297–301.

Mathur, S. and Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria: a review. *Int J Food Microbiol* **105(3):** 281-295.

Matte-Tailliez, O., Quenee, P., Cibik, R., van Opstal, J., Dessevre, F., Firmesse, O. and Tailliez, P. (2001). Detection and identification of lactic acid bacteria in milk and industrial starter culture with fluorescently labeled rRNA-targeted peptide nucleic acid probes. *Le Lait* **81:** 237–248.

Mattila-Sandholm, T. (1999). The technology of probiotics. *Trends in Food Sci Technol* **10(12):** 387–392.

Maurad, K. and Meriem, K.H. (2008). Probiotic characteristics of *Lactobacillus plantarum* strains from traditional butter made from camel milk in arid regions (Sahara) of Algeria. *Grasas Y Aceites*, **59 (3):** 218-224.

Mauriello, G., Moio, L., Moschetti, G., Piombino, P., Addeo, F. and Coppola, S. (2001). Characterization of lactic acid bacteria strains on the basis of neutral volatile compounds produced in whey. *J Appl Microbiol* **90:** 928–942.

Mayra-Makinen, A., Manninen, M. and Gyllenberg, H. (1983). The adherence of lactic acid bacteria to the columnar epithelial cells of pigs and calves. *J Appl Bacteriol* **55:** 241-245.

McIntosh, G.H, Regester, G.O., LeLeu, R.K., Royle, P.J. and Smithers, G.W. (1995). Dairy proteins protect against dimethylhydrazine induced intestinal cancer in rats. *J Nutr* **125:** 809–816.

McKay, L.L., Miller, A., Sandine, W.E. and Elliker, P. R. (1970). Mechanisms of lactose utilization by lactic acid *Streptococci*: Enzymatic and genetic analyses. J Bacteriol **102:** 804-809.

McSweeney, P.L.H. and Sousa, M.J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening. A review. *Le Lait* **80:** 293–324.

Mercenier, A., Pavan, S. and Pot, B. (2002). Probiotics as Biotherapeutic Agents: Present Knowledge and Future Prospects. *Curr Pharmaceutical Design* **8:** 99-110.

Messner, P. and Sleytr, U.B. (1992). Crystalline bacterial cell-surface layers. *Adv Microbial Physiol* **33:** 213-275.

Metchnikoff, E. (1908). The Prolongation of Life, Putmans Sons, New York, NY.

Meydani, S.N. and Ha, W.K. (2000). Immunologic effects of yoghurt. *Am J Clin Nutr* **71:** 861–872.

Michaela, O., Hehn, R., Vogel, R.F. and Hammes, W.P. (1992). Lactose metabolism in *Lactobacillus curvatus* and *Lactobacillus sake*. *FEMS Microbiol Lett* **97:** 209-214.

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.

Miller, C.C., Park, Y., Pariza, M.W. and Cook, M.E. (1994). Feeding conjugated linoleic acid to animals partially overcomes catabolic responses due to endotoxin injection. *Biochem Biophy Res Comm* **198:** 1107–1112.

Miller, J.J. and Brand, J.C. (1980). Enzymatic lactose hydrolysis. *Fd Tech Aus* **32:** 144–147.

Miller, R.A. and Britigan, B.E. (1997). Role of oxidants in microbial pathophysiology. *Clin Microbiol Rev* **10:** 1-18.

Miller-Ihli, N.J. (1996). Trace element determinations in foods and biological samples using inductively coupled plasma atomic emission spectrometry and flame atomic absorption spectrometry. *J Agri Food Chem* **44:** 2675–2679.

Mishra, O.P. and Korachich, G.B. (1984). Inhibition of the autoxidation of ascorbate and norepinephrine by extracts of *Clostridium butyrich*, *Megasphaera elsdenii*, and *Escherichia coli*. *Life Sci* **35:** 849-854.

Mogensen, G., Salminen, S., O'Brien, J., Ouwehand, A., Holzapfel, W., Shortt, C., Fonden, R., Miller, G. D., Donohue, D., Playne, M., Crittenden, R., Salvadori, B. B. and Zink, R. (2002). Inventory of microorganisms with a documented history of use in food. *FIL/ IDF Bulletin* **377:** 10–19.

Montero, S., de Arriaga, D., Busto, F. and Soler, J. (1989). Induction of intracellular and extracellular beta-galactosidase activity in Phycomyces blakesleeanus. *Biochem Int* **18:** 637– 645.

Montes, R.G., and Perman. J.A. (1990). Disorders of carbohydrate absorption in clinical practice. *Maryland Med J* **39:** 383-388.

Montes, R.G., Bayless, T.M., Saavedra, J.M. and Perman, J.A. (1995). Effect of milks inoculated with *Lactobacillus acidophillus* or a yogurt starter culture in lactose- maldigesting children. *J Dairy Sci* **78:** 1657–1664.

Montgomery, R.K., Buller, H.A., Rings, E.H.H.M. and GRAND, R.J. (1991). Lactose intolerance and the genetic regulation of intestinal lactase-phlorizin hydrolase *FASEBJ* **5:** 2824-2832.

Morens, C., Bos, C., Pueyo, M.E., Benamouzig, R., Gausseres, N., Luengo, C., Tome, D. and Gaudichon, C. (2003). Increasing habitual protein intake accentuates differences in postprandial dietary nitrogen utilization between protein sources in humans. *J Nutr* **133:** 2733-2740.

Moskovitz, M., Curtis, G. and Gavaler, J. (1987). Does oral enzyme replacement therapy reverse intestinal lactose malabsorption. *Am J Gastroenterol* **82:** 632–635.

Mukai, T. and Arihara, K. (1994). Presence of intestinal lectin-binding glycoproteins on the cell surface of *Lactobacillus acidophilus*. *Biosci Biotechnol Biochem* **58:** 1851–1854.

Murakami, H., Asakawa, T., Terao, J. and Matsushita, S. (1984). Antioxidative stability of tempeh and liberation of isoflavones by fermentation. *Agric Biol Chem* **48:** 2971–2975.

Murch, S.H. (2001). Toll of allergy reduced by probiotics. *Lancet* **357:** 1057-1059.

Murti, T.W., Bouillanne, C., Landon, M. and Desmazeaud, M.J. (1993). Bacterial growth and volatile compounds in yoghurt type products from soymilk containing Bifidobacterium ssp. *J Food Sci* **58:** 153–157.

Mustapha, A., Jiang, T. and Savaiano, D.A. (1997). Improvement of lactose digestion by humans following ingestion of unfermented acidophilus milk: Influence of bile sensitivity, lactose transport, and acid tolerance of *Lactobacillus acidophilus*. *J Dairy Sci* **80:** 1537–1545.

Nader de Macias, M.E., Manca de Nadra, M.C., Strasses d Saad, A.M., Pesce de Ruiz Holgado, A.A. and Oliver, G. (1983). Isolation and purification of β-galactosidase of *Lactobacillus murinus* CNRZ 313. *Curr Microbiol* **9**: 99-104.

Nagy, Z., Keresztessy, Z., Szentirmai, A. and Biro, S. (2001). Carbon source regulation of β-galactosidase biosynthesis in *Penicillium chrysogenum*. *J Basic Microbiol* **41:** 351– 362.

Nakayama, J., Akkermans, A.D.L. and De Vos, W.M. (2003). Hight throughput PCR screening of genes for three component regulatory system putatively involved in quorum sensing from low G+C gram positive bacteria. *Biosci Biotectnol Biochem **67:*** 480-489.

Narvhus, J.A., Hulbaekdal, A. and Abrahamsen, R.K. (1992). Occurrence of *Lactobacilli* and *Leuconostoc* in Norwegian cheese of Gouda type and their possible influence on cheese ripening and quality. *International Dairy J,* **3:** 566.

Nase, L., Hatakka, K., Savilahti, E., Saxelin, M., Ponka, A., Poussa, T., Korpela, R. and Meurman, J.H. (2001). Effect of long term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. *Caries Res* **35:** 412-420.

Navarre, W.W. and Schneewind, O. (1999). Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* **63:** 174-229.

Nebedum, J.O. and Obiakor, T. (2007). The effects of different preservation methods on the quality of nunu, a locally fermented Nigerian dairy product. *African J Biotechnol* **6(4):** 454-458.

Nicolajsen, H. and Hvidt, A. (1994). Phase behavior of the system trehalose, NaCl, water. *Cryobiol* **31:** 199–205.

Nicolosi, R.J., Rogers, E.J., Kritchevsky, D., Scimeca, J.A. and Huth, P.J. (1997). Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. *Artery* **22:** 266–277.

Nikolaev, I.V. and Vinetski, Y.P. (1998). L-Arabinose induces synthesis of secreted *β*-galactosidase in the filamentous fungus *Penicillium canescens*. *Biochem (Moscow)* **63:** 1294– 1298.

Ning, W., Mackie, R.I. and Rex Gaskins, H. (1997). Biotype and ribotype diversity among *Lactobacillus* isolates from the mouse ileum. *Sys Appl Microbiol* **20:** 423-431.

Nomura, M., Nakajima, I., Fujita, Y., Kobayashi, M., Kimoto, H., Suzuki, I. and Aso, H. (1999). *Lactococcus lactis* contains only one glutamate decarboxylase gene. *Microbiol* **145:** 1375–1380.

O'Sullivan, D.J. (2001). Screening of intestinal microflora for effective probiotic bacteria. *J Agri Food Chem* **49:** 1755–1760.

O'Sullivan, M.G., Thornton, G., O'Sullivan, G.C., Collins, J.K. (1992). Probiotic bacteria: myth or reality? *Trends Food Sci Technol* **3:** 309–314.

Obermayer-Pietsch, B.M., Bonelli, C.M., Walter, D.E., Kuhn, R., Fahrleitner-Pammer, A., Berghold, A., Goessler, W., Stepan, V., Dobnig, H., Leb, G. and Renner, W. (2004). Genetic predisposition for adult lactose intolerance and relation to diet, bone density, and bone fractures. *J Bone Miner Res* **19(1):** 42–47.

Obodai, M.and Dodd, C.E.R. (2006). Characterization of dominant microbiota of a Ghanaian fermented milk product, Nyarmie, by culture and nonculture based methods. *J Appl Microbiol* **100:** 1355–1363.

Ochsner, U.A. **(**1993). Genetics and biochemistry of *Pseudomonas aeroginosa* rhamnolipid biosurfactant synthesis. Doctoral dissertation. *Swiss Federal Institute of Technology*, Zurich, Switzerland.

Ogier, J.C., Son, O., Gruss, A., Tailliez, P. and Delacroix-Buchet, A. (2002). Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Appl Environ Microbiol* **68:** 3691–3701.

Ohkuma, M. and Kudo, T. (1998). Phylogenetic analysis of the symbiotic intestinal microflora of the termite *Cryptotermes domesticus*. *FEMS Microbiol Lett* **164:** 389–395.

Okitsu-Negishi, S., Nakano, I., Suzuki, K., Hashira, S., Abe, T. and Yoshino, K. (1996). The induction of cardioangitis by *Lactobacillus casei* cell wall in mice. Clin Immunol Immunopathol **78:** 30–40.

Oksanen, P., Salminen, S., Saxelin, M., H.am.al.ainen, P., Ihantola-Vormisto, A., Muurasniemi-Isoviita, L., Nikkara, S., Oksanen, T., Porsti, T., Salminen, E., Siitonen, S., Stuckey, H., Toppila, A. and Vapaatalo, H. (1990). Prevention of traveler's diarrhea by *Lactobacillus GG*. *Annals Med* **22:** 53–56.

Oksanen, P., Vapaatalo, H. Salminen, S. (1990). Prevention of traveller's diarrhoea by *Lactobacillus GG. Ann Med* **22:** 53–56.

Olejnik, A., Lewandowska, M., Obarska, M. and Grajek, W. (2005). Tolerance of *Lactobacillus* and *Bifidobacterium* strains to low pH, bile salts and digestive enzymes. *Electronic Journal Of Polish Agricultural Universities* **8(1):** 5-10.

Onwulata, C.I., Rao, D.R. and Vankineni, P. (1989). Relative efficiency of yogurt, sweet acidophilus milk, hydrolyzed-lactose milk, and a commercial lactase tabled in alleviating lactose maldigestion. *Am J Clin Nutr* **49:** 1233–1237.

Ortensi, G., Gladstein, J., Vail, H. and Tesone, P.A. (1997). A comparativc study of policosanol vs. simvastatin in elderly patients with hypercholesterolemia. *Curr Ther Res* **58:** 390-401.

Ostlie, H.M., Treimo, J. and Narvhus, J.A. (2005). Effect of temperature on growth and metabolism of probiotic bacteria in milk. **15(10):** 989-997.

Ouwehand, A.C. (1998). Antimicrobial components from lactic acid bacteria. In Lactic Acid Bacteria, Microbiology and Functional Aspects, 2nd ed., editors, Salminen, S. and von Wright, A. New York: Marcel Dekker, pp. 139–159.

Ouwehand, A.C. and Salminen, S. (2003). *In vitro* adhesion assays for probiotics and their *in vivo* relevance: a review. *Microb Ecol Health Dis* **15:** 175–184.

Ouwehand, A.C., Salminen, S., Tolkko, S., Roberts, P., Ovaska, J. and Salminen, E. (2002). Resected human colonic tissue: new model for characterizing adhesion of lactic acid bacteria. *Clin Diag Lab Immuno* **9:** 184–186.

Oxman, T., Shapira, M., Diver, A., Klein, R., Avazov, N. and Rabinowitz, B. (2000). A new method of long-term preventive cardioprotection using *Lactobacillus*. *Am J Physiol Heart Circ Physiol* **278:** H1717–H1724.

Oyetayo, V.O., Adetuyi, F.C, and Akinyosoye, F.A. (2003). Safety and protective effect of *Lactobacillus acidophilus* and *Lactobacillus casei* used as probiotic agent *in vivo. Afr J Biotechnol* **2(11):** 448–452.

Palmfeldt, J. and Hahn-Hagerdal, B. (2000). Influence of culture pH on survival of *Lactobacillus reuteri* subjected to freeze-drying. *Int J Food Microbiol* **55:** 235–238.

Pariza, M.W., Park, Y. and Cook, M.E. (2001). The biologically active isomers of conjugated linoleic acid. *Prog Lipid Res* **40:** 283–298.

Park, B.T., Lee, S.J., Oh, T.K., Oh, J.W., Koo, B.T., Yum, D.Y. and Lee, J.K. (2003). AhlD, an N acyl homoserine lactonase in *Arthrobacter* sp and predicted homologous in other bacteria. *Microbiol* **149:** 1541-1550.

Park, S.Y., Bok, S.H., Jeon, S.M., Park, Y.B., Lee, S.J., Jeong, T.S. and Choi, M.S. (2002). Effect of rutin and tannic acid supplements on cholesterol metabolism in rats. *Nutr Res* **22:** 283-295.

Parker, R.B. (1974). Probiotics, the other half of the antibiotic story. *Ani Nutr Health* **29:** 4–8.

Parvez, S., Malik, K.A., Ah Kang, S. and Kim, H.Y. (2006). Probiotics and their fermented food products are beneficial for health. *J Appl Microbiol* **100:** 1171–1185.

Patrick T.C. van den Bogaard, Michiel Kleerebezem, Oscar P. Kuipers, and Willem M. de Vos. (2000). Control of lactose transport, *β*-galactosidase activity, and glycolysis by CcpA in *Streptococcus thermophilus*: evidence for carbon catabolite repression by a non-phosphoenolpyruvate-dependent phosphotransferase system sugar. *J Bacteriol* **182(21):** 5982-5989.

Pearson, J.P., Passador, L., Iglewski, B.H. and Greenberg**,** E.P. (1995). A second *N*-acyl homoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **92:**1490–1494.

Pearson, J.P., Pesci, E.C. and Iglewski, B.H. (1997). Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriology* **179(18):** 5756– 5767.

Pedone, C.A., Arnaud, C.C., Postaire, E.R., Bouley, C.F., Reinert, P., Cirde. (2000). Multicentric study of the effect of milk fermented by *Lactobacillus casei* on incidence of diarrhea. *Intl J Clin Pract* **54:** 568-571.

Pedone, C.A., Bernabeu, A.O., Postaire, E.R., Bouley, C.F., Reinert, P., Cirde. (1999). The effect of supplementation with milk fermented by *Lactobacillus casei* (Strain DN-114 001) on acute diarrhea in children attending day care centers. *Intl J Clin Pract* **53:** 179-184.

Pegues, D.A., Ohl, M.E. and Miller, S.I. (2005). *Salmonella* species, including *Salmonella typhi*. In Principles and Practice of Infectious Diseases ed. Mandell, G.L., Bennett, J.E. and Dolin, R. Philadelphia: Elsevier Inc, pp. 2636–2654.

Pelletier, C., Bouley, C., Bourliov, P. and Carbon, C. (1996). Evaluation of safety properties of *Lactobacillus* strains by using an experimental model of endocarditis in rabbit. Abstract, SOMED Meeting, Paris, France.

Pelletier, C., Bouley, C., Cayuela, C., Bouttier, S., Bourlioux, P., and Bellon-Fontaine, M.N. (1997). Cell surface characteristics of *Lactobacillus casei subsp. casei, Lactobacillus paracasei subsp. paracasei,* and *Lactobacillus rhamnosus* strains. *Appl Environ Microbiol* **63:** 1725–1731.

Pennacchia, C., Ercolini, D., Blaiotta, G., Pepe, O., Mauriello, G. and Villani, F. (2004). Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics. *Meat Sci* **67:** 309–317.

Penner, R., Fedorak, R.N. and Madsen, K.L. (2005). Probiotics and nutraceuticals: non-medicinal treatments of gastrointestinal diseases. *Curr Opin Pharmacol* **5(6):** 596-603.

Pereira, D.I.A. and Gibson, G.R. (2002). Effects of consumption of probiotics and prebiotics on serum lipid levels in humans. *Crit Rev Biochem Mol Biol* **37:** 259–281.

Perez, C., Pauli, M. and Bazevque, P. (1990). An antibiotic assay by the agar well diffusion method. *Acta Biologiae et Medicine Experimentalis* **15:** 113-115.

Perez, F.J. and Hanson, N.D. (2002). Detection of plasmid-mediated AmpC ß-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* **40:** 2153-2156.

Perez, G., Cardell, E. and Zarate, V. (2002). Random amplified polymorphic DNA analysis for differentiation of *Leuconostoc mesenteroides* subspecies isolated from Tenerife cheese. *Lett Appl Microbiol* **34:** 82–85.

Perez, P.F., Minnaard, Y., Disalvo, E.A. and De Antoni, G.L. (1998). Surface properties of *Bifidobacteria* strains of human origin. *Appl Environ Microbiol* **64:** 21–26.

Persky, V. and van Horn, L. (1995). Epidemiology of soy and cancer: perspectives and directions. *J Nutr* **125:** 709–712.

Pessi, G. and Haas, D. (2000). Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnabc* by the anaerobic regulator anr and the quorum-sensing regulators lasr and rhlr in *Pseudomonas aeroginosa. J Bacteriol* **182:** 6940-6949.

Petrof, E.O., Kojima, K., Ropeleski, M.J., Musch, M.W., Tao, Y., de Simone, C. and Chang, E.B. (2004). Probiotics inhibit nuclear factor B and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. *Gastroenterol* **127:** 1474-1487.

Piard, J.C. and Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria: Oxygen metabolites and catabolism end-products. *Lait* **71:** 525-541.

Picot, L., Mezghani-Abdelmoula, S., Chevalier, S., Merieau, A., Lesouhaitierm, O., Guerillon, J., Cazin, L., Orange, N., and Feuilloley. M.G.J. (2004). Regulation of the cytotoxic effects of *Pseudomonas fluorescens* by growth temperature. *Res Microbiol* **155:** 39–46.

Piotrowska, M. and Zakowska, Z. (2005). The elimination of Ochratoxin A by Lactic Acid Bacteria strains. *Polish J Microbiol* **54:** 279–286.

Pons, P., Rodriguez, M., Robaina, C., et al. (1994). Effects of successive dose increases of policosanol on lipid profile of patients with type-II hypercholesterolemia and tolerability to treatment. *J Clin Pharmacol Res* **14:** 27-33.

Pool-Zobel, B.L., Munzner, R., and Holzaapfel, H. (1993). Antigenotoxic properties of lactic acid bacteria in the *S. typhimurium* Mutagenicity assay. *Nutr Can*cer **20:** 261- 270.

Pouwels, P.H., Leer, R.J., Shaw, M., Heijne den Bak-Glashouwer, M.J., Tielen, F.D., Smit, E., Martinez, B., Jore, J. and Conway, P.L. (1998). Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. *Int J Food Microbiol* **41:** 155–167.

Prasad, J., Gill, H., Smart, J. and Gopal, P. K. (1998). Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. *Int Dairy J* **8:** 993– 1002.

Premi, L., Sandine, W.E. and Elliker, P.R. (1972). Lactose hydrolyzing enzymes of *Lactobacillus* species. *Appl Microbiol* **24:** 51-57.

Priest, f., Good, M. and Todd, C. (1988). Anumerical classification of the genus bacillus. *J Gen Microbiol* **134:** 184.

Pruitt, B.A., McManus, A.T. Kim, S.H. and Goodwin. C.W. (1998). Burn wound infections: current status. *World J Surg* **22:** 135-145.

Rabizadeh, E., Shaklai, M., Nudelman, A., Eisenbach, L. and Rephaali, A. (1993). Rapid alteration of c-myc and c-jun expression in leukemic cells induced to differentiate by a butyric acid. *FEBS Lett* **328:** 225–229.

Rachmilewitz, D., Katakura, K., Karmeli, F., Hayashi, T., Reinus, C., Rudensky, B., Akira, S., Takeda, K., Lee, J., Takabayashi, K. and Raz, E. (2004). Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterol **126:** 520–528.

Ram, C. and Chander, H. (2003).Optimization of culture conditions of probiotic *Bifidobacteria* for maximal adhesion to hexadecane. *World J Microbiol Biotechnol* **19:** 407–410.

Rashid, H., Togo, K., Ueda, M. and Miyamoto, T. (2007) Probiotic characteristics of lactic acid bacteria isolated from traditional fermented milk 'Dahi' in Bangladesh. *Pak J Nutr* **6 (6):** 647-652.

Rashid, M.H., Kato, F., Murata, A. and Kondo, W. (1993). Purification and characterization of the antioxidative substance produced by *Aspergillus sojae* K. *Biosci Biotech Biochem* **57:** 935-939.

Rasouli, I. and Kulkarni, P.R. (1994). Enhancement of *β*-galactosidase productivity of *Aspergillus niger* NCIM-616. *J Appl Bacteriol* **77:** 359–361.

Ratledge, C. and Wilkinson, S. G. (editors) (1989). *Microbial Lipids*, vol. 2. London: Academic Press. pp. 567-668.

Rautio, M., Jousimies-Somer, H., Kauma, H., Pietarinen, I., Saxelin, M., Tynkynen, S. and Koskela, M. (1999). Liver abscess due to a *Lactobacillus rhamnosus* indistinguishable from *L. rhamnosus* strain GG. *Clin Infect Dis* **28:** 1159–1160.

Ray, B., Jezeski, J.J. and Busta, F.F. (1971). Effect of rehydration on recovery, repair and growth of injured freeze-dried *Salmonella anatum*. *Appl Microbiol* **22 (2):** 184– 189.

Reale, A., Mannina, L., Tremonte, P., Sobolev, A.P., Succi, M., Sorrentino, E. and Coppola, R. (2004). Phytate degradation by lactic acid bacteria and yeasts during the whole meal dough fermentation: a 31P NMR study. *J Agri Food Chem* **52:** 6300–6305.

Reddy, B.S. (1998). Prevention of colon cancer by pre and probiotics: evidence from laboratory studies. *Brit J Nutr* **80:** S219-S223.

Reeson, A.F., Jankovic, T., Kasper, M.L., Rogers, S. and Austin, A.D. (2003). Application of 16S rDNA-DGGE to examine the microbial ecology associated with a social wasp *Vespula germanica*. *Insect Mol Biol* **12:** 85–91.

Reid, G. (2001). Probiotic agents to protect the urogenital tract against infection. *Am J Clin Nutr* **73:** 437S-443S.

Reid, G. (2008). Probiotic Lactobacilli for urogenital health in women. *J Clin Gastroenterol* **5:** 23-39.

Reid, G., Jass, J., Sebulsky, M.T. and McCormick, J.K. (2003). Potential uses of probiotics in clinical practice. *Clin Microbiol Rev* **16:** 658–672.

Reimmann, C., Ginet, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., Zala, M., Heurlier, K., Triandafillu, K., Harms, H., Defago, G. and Haas, D. (2002). Genetically programmed autoinducer destruction reduces virulence gene-expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiol* **148:** 923-932.

Rembacken, B.J., Snelling, A.M., Hawkey, P.M., Chalmers, D.M. and Axon, A.T. (1999). Non-pathogenic *Escherichia coli* versus mesalazine for the treatment of ulcerative colitis: a randomised trial. *Lancet* **354:** 635–639.

Rementzis, J. and Samelis, J. (1996). Rapid GC analysis of cellular fatty acids for characterizing *Lactobacillus sake* and *Lactobacillus curvatus* strains of meat origin. *Lett Appl Microbiol* **23(6):** 379-384.

Renault, P. (2002). Genetically modified lactic acid bacteria: applications to food or health and risk assessment. *Biochimie* **84:** 1073-1087.

Richelieu, M., Houlberg, U. and Nielsen, J.C. (1997). Determination of α- acetolactic acid and volatile compounds by head space gas chromatograph. *J Dairy Sci* **80:** 1918-1925.

Ridefelt, P. and Hakansson, L.D. (2005). Lactose intolerance: lactose tolerance test versus genotyping. *Scand J Gastroenterol* **40:** 822-826.

Ringo, E. and Gatesoupe, F. J. (1998). Lactic acid bacteria in fish: A review. *Aquaculture* **160:** 177–203.

Robinson, R.K., Tamime, A.Y. and Wszolek, M. (2002). Microbiology of fermented milks. In R. K. Robinson (Ed.), Dairy microbiology handbook: the microbiology of milk and milk products New York: Wiley, pp. 367–430.

Rojas, M., Ascencio, F. and Conway, P.L. (2002). Purification and characterization of a surface protein from *Lactobacillus fermentum* 104R that binds to porcine small intestinal mucus and gastric mucin. *Appl Environ Microbiol* **68:** 2330–2336.

Rolfe, R.D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *J Nutr* **130:** 396S-402S.

Roos, S. and Jonsson, H. (2002). A high-molecular mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiol* **148:** 433–442.

Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* **9:** 29–33.

Rowland, L.R. (1992). Metabolic interactions in the gut. In *Probiotics: The scientific basis,* (R Fuller, editor). Chapman and Hall, London, pp. 29-53.

Rudel, L.L., and Morris, M.D. (1973). Determination of cholesterol using o- phthalaldehyde. *J Lipid Res* **14:** 364-366.

Russell, N. J. and Fukanaga, M. (1990). A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. *FEMS Microbiol Rev* **75:** 171-182.

Rusynyk, R.A. and Still, C.D. (2001). Lactose intolerance. *JAOA* **101(4 suppl):** 10-12.

Ryan, M.P., Meaney, W.J., Ross, R.P. and Hill. C. (1998). Evaluation of lactacin 3147 and a teat seal containing this bacteriocin for inhibition of mastitis pathogens. *Appl Environ Microbiol* **64:** 2287-2292.

Saarela, M., Lahteenmaki, L., Crittenden, R., Salminen, S. and Mattila-Sandholm, T. (2002). Gut bacteria and health foods: the European perspective. *Int J Food Microbiol* **78:** 99–117.

Saavedra, J.M. (1995). Microbes to fight microbes: a not so novel approach to controlling diarrhoeal disease. *J Pediatric Gastroenterol Nutr* **21:** 125–129.

Saavedra, J.M. and Perman JA. (1989). Current concepts in lactose malabsorption and intolerance. *Annu Rev Nutr* **9:** 475-502.

Sadana, A. and Henley, J.P. (1986). Effect of chemical modification on enzymatic activities and stabilities. *Biotechnol Bioeng* **28:** 256–268.

Saha, B.K., Ali, M.Y., Chakraborty, M., Islam, Z. and Hira, A.K. (2003). Study on the preservation of raw milk with hydrogen peroxide for rural dairy farmers. *Pak J Nutr* **2(1):** 36-42.

Sahi, T. (2001). Hypolactasia and lactase persistence. Historical review and the terminology. *Scand J Gastroenterol* **l202(Supp)**: 1-6

Salminen, S. (1990). The role of intestinal microbiota in preserving intestinal integrity and health with special reference to lactic acid bacteria. *Ann Med* **22:** 35-42.

Salminen, S. (1996). Uniqueness of probiotic strains. Paper given at IDF Nutrition Week in Potsdam, Germany. *Newsletter of the International Dairy Federation* **145,** Brussels: IDF, pp. 18-19.

Salminen, S. and Marteau, P. (1997). Safety of probiotic lactic acid bacteria and other probiotics. Lactic 97 (Proceedings). *Adria Normandie Caen* 71–72.

Salotra, P., Singh, D.K., Seal, K.P., Krishna, N., Jaffe, H. and Bhatnagar, R. (1995). Expression of DnaK and GroEL homologs in *Leuconostoc mesenteroides* in response to heat shock, cold shock or chemical stress. *FEMS Microbiol Lett* **131:** 57–62.

Saltzman, J.R., Russell, R.M., Golner, B., Barakat, S., Dallal, G.E. and Goldin, B.R. (1999). A randomized trial of *Lactobacillus acidophilus* BG2FO4 to treat lactose intolerance. *Am J Clin Nutr* **69:** 140–146.

Samelis, J., Kakouri, A. and Rementzis, J. (2000). Selective effect of the product type and the packaging conditions on the species of lactic acid bacteria dominating the spoilage microbial association of cooked meats at 4°C. *Food Microbiol* **17(3):** 329-340.

Samona, A. and Robinson, R.K. (1991). Enumeration of *Bifidobacteria* in dairy products. *J Society Dairy Technol* **44:** 64–66.

Sanders, J. W., Venema, G. and Kok, J. (1999). Environmental stress responses in *Lactococcus lactis*. *FEMS Microbiol Rev* **23:** 483–501.

Sanders, M.E. (1998). Over view of functional foods: emphasis on probiotic bacteria. *Int Dairy J* **8:** 341-347.

Sanders, M.E. (2003). Probiotics: considerations for human health. *Nutr Rev* **61:** 91–99.

Sanders, M.E. and Huis in't Veld, J. (1999). Bringing a probiotic containing functional food to the market: microbiological, product, regulatory and labeling issues. *Antonie van Leeuwenhoek* **76:** 293–315.

Sanders, M.E., Morelli, L., Bush, S. (2001). *Lactobacillus sporogenes* is not a *Lactobacillus* probiotic. *ASM News* **67:** 385-386.

Sara, M. and Sleytr, U.B. (2000). S-layer proteins. *J Bacteriol* **182:** 859–868.

Sarkar, S. (2006). Potential of soyoghurt as a dietetic food. *Nutr Food Sci* **36 (1):** 43-49.

Sauer, U., Cameron, D.C. and Bailey, J.E. (1998). Metabolic capacity of *Bacillus subtilis* for the production of purine nucleosides, riboflavin, and folic acid. *Biotechnol Bioeng* **59:** 227–238.

Savadogo, A., Ouattara, C.A.T., Savadogo, P.W., Ouattara, A.S., Barro, N. and Traore, A.S. (2004). Microorganisms involved in fulani traditional fermented Milk in Burkina Faso. *Pak J Nutr* **3(2):** 134-139.

Savaiano, D.A. and Levitt, M.D. (1987). Milk intolerance and microbe containing dairy foods. *J Dairy Sci* **70:** 397–406.

Saxelin, M. (1996). Colonization of the human gastrointestinal tract by probiotic bacteria. *Nutr Today Suppl* **31:** 5S–8S.

Saxelin, M., Grenov, B., Svensson, U., Fonden, R., Reniero, R. and Mattila-Sandholm, T. (1999). The technology of probiotics. *Trends Food Sci Technol* **10(12):** 387–392.

Schaafsma, G. (1996). State of the art concerning probiotic strains in milk products. Paper given at IDF Nutrition Week in Potsdam, Germany. *Newsletter of the International Dairy Federation* **145,** Brussels: IDF, pp. 23-24.

Schaafsma, G., Meuling, W.J.A. van Dokkum, W. and Bouley. C. (1998). Effects of a milk product, fermented by *Lactobacillus acidophilus* and with fructooligosaccharides added, on blood lipids in male volunteers. *Eur J Clin Nutr* **52:** 436–440.

Schaefer, A.L., Val, D.L., Hanzelka, B.L., Cronan, J.E. and Greenberg, E.P. (1996). Generation of cell-to–cell signals in quorum sensing: acylhomoserine lactone synthase activity of a purified *V. fischeri* LuxI protein. *Proc Natl Acad Sci USA* **93:** 9505-9509.

Schiffrin, E.J., Brassart, D., Servin, A., Rochat, F. and Donnet-Hughes, A. (1997). Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. *Am J Clin Nutr* **66:** 515S-520S.

Schiffrin, E.J., Rochat, F., Link-Amster, H., Aeschlimann, J.M., Donnet- Hughes, A. (1995). Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *J Dairy* ***Sci*** **78:** 491–497.

Schillinger, U. and Lucke, F.K. (1989). Antibacterial activity of *Lactobacillus* sake isolated from meat. *Appl Environ Microbiol* **55(8):**1901–1906.

Schillinger, U., Guigas, C., Holzapfel, W.H. (2005). *In vitro* adherence and other properties of *Lactobacilli* used in probiotic yoghurt-like products. *Int Dairy J* **15:** 1289–1297.

Schleifer, K.H., Ehrmann, M., Krusch, U. and Neve, H. (1991). Revival of the species *Streptococcus thermophilus* (ex Orla-Jensen, 1919) nom. rev. *Sys Appl Microbiol* **14:** 386-388.

Schneitz, C., Nuotio, L. and Lounatma, K. (1993). Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer). *J Appl Bacteriol* **74:** 290–294.

Schnurer, J. and Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci Technol* **16(1-3):** 70-78.

Schonhuber, W., Le Bourhis, G., Tremblay, J., Amann, R. and Kulakauskas, S. (2001). Utilization of tmRNA sequences for bacterial identification. *BMC Microbiol* **1:** 20-27.

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

Scott, R. (1981). Cheesemaking Practice. Applied Science Publishers, London.

Scrimshaw, N.S. and SanGiovanni, J.P. (1997). Synergism of nutrition, infection and immunity: an overview. *Am J Clin Nutr* **66:** 464S–477S.

Selmer-Olsen, E., Birkeland, S.E. and Sorhaug, T. (1999). Effect of protective solutes on leakage from and survival of immobilized *Lactobacillus* subjected to drying, storage and rehydration. *J Appl Microbiol* **87:** 429–437.

Semenza, G. and Auricchio, S. (1995). Small – intestinal disaccharides. In: Scriver, C.R. and Beaudet, W.S. (Eds), Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, London, pp. 4451–79.

Sen, S., Mullan, M.M., Parker, T.J., Woolner, J.T., Tarry, S.A. and Hunter, J.O. (2002). Effects of *Lactobacillus plantarum* 299v on colonic fermentation and symptoms of irritable bowel syndrome. *Dig Dis Sci* **47:** 2615– 2620.

Shah, N.P. (2000). Probiotic bacteria: Selective enumeration and survival in dairy foods. *J Dairy Sci* **83(4):** 894–907.

Shah, N.P. (2001). Functional foods from probiotics and prebiotics. *Food Technol* **55(11):** 46–53.

Shah, N.P. and Jelen, P. (1990). Survival of lactic acid bacteria and their lactases under acidic conditions. *J Food Sci* **55:** 506–509.

Shah, N.P. and Lankaputhra, E.V. (1997). Improving viability of *Lactobacillus acidophilus* and *Bifidobacterium spp*. in yogurt. *Int Dairy J* **7:** 349–356.

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.

Shahani, K.M., Vakil, J.R. and Kilara, B.A. (1976). Natural antibiotic activity of *Lactobacillus acidophilus* and *bulgaricus*. Isolation of acidophilin from *L. acidophilin*. *Cult Dairy Prod J* **12(2):** 8-11.

Shaikh, S.A., Khire, J.M. and Khan, M.I. (1997). Production of *β*-galactosidase from thermophilic fungus *Rhizomucor sp*. *J Ind Microbiol Biotech* **19:** 239– 245.

Shanahan, F. (2004). Probiotics in inflammatory bowel disease: therapeutic rationale and role. *Adv Drug Deliv Rev* **56:** 809–818.

Shaw, B.G. and Harding, C.D. (1989). *Leuconostoc gelidum* sp. *nov*. and *Leuconostoc carnosum sp. nov*. from chill stored meats. *Int J Sys Bacteriol* **39:** 217–223.

Shaw, P.D., Ping, G., Daly, S., Cronan, J.E., Rhinehart, K. and Farrand, S.K. (1997). Detecting and characterizing acyl-homoserine lactone signal molecules by thin layer chromatography. *Proceedings of National Academy of Sciences of United States of America* **94:** 6036-6041.

Sheih, I.C., Wu, H.Y., Lai, Y.J. and Lin, C.F. (2000). Preparation of high free radical scavenging tempeh by a newly isolated Phizopus sp. R- 96 from Indonesia. *Food Sci Agric Chem* **2:** 35–40.

Shermark, 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 lactose-malabsorbing children. *Am J Clin Nutr* **62:** 1003-1006.

Sieber, R., Stransky, M. and deVrese, M. (1997). Lactose intolerance on consumption of milk and milk products. *Z Emahrungswiss* **36(4)**: 375-393.

Simakachorn, N., Pichaipat, V., Rithpornpaisam, P., Kongkaew, C., Tongpradit, P. and Varavithya, W. (2000). Clinical evaluation of the addition of lyophilized, heat-killed *Lactobacillus acidophilus LB* to oral rehydration therapy in the treatment of acute diarrhea in children. *J Pediatr Gastroenterol Nutr* **30:** 68–72.

Sindhu, S.C. and Khetarpaul, N. (2003). Effect of feeding probiotic fermented indigenous food mixture on serum cholesterol levels in mice. *Nutr Res* **23(8):** 1071-1080.

Siso, M.I.G., Cerdan, E., Freire, M.A., Ramil, E, Belmonte, E.R. and Torres, A.M.R. (1992). Permeabilization of *Kluyveromyces lactis* cells for milk whey saccharification: a comparison of different treatments. *Biotechnol Techn* **6:** 289–292.

Sivak, C. (2000). Growth culture. *Dairy Field* **183:** 1-33.

Sleytr, U.B. and Messner, P. (1988). Crystalline surface layers in procaryotes. *J Bacteriol* **170(7):** 2891-2897.

Smart, J. B., Pillidge, C. J. and Garman, J. H. (1993). Growth of lactic acid bacteria and *Bifidobacteria* on lactose and lactose related mono-, di- and trisaccharides and correlation with distribution of *β*-galactosidase and phospho-*β*-galactosidase. *JDairy Res* **60:** 557-568.

Smibert, R.M. and Krieg, N.R. (1981). General characterization. Manual of methods for general bacteriology eds Gerhardt, Murray, R. G. E, Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N.R and Briggs phillips, G. Washington DC: ASM press, pp 409-443.

Smit, E., Jager, D., Martinez, B., Tielen, F.J. and Pouwels, P.H. (2002). Structural and functional analysis of the S-layer protein crystallization domain of *Lactobacillus acidophilus* ATCC 4356: evidence for protein–protein interaction of two subdomains. *J Mol Biol* **324:** 953–964.

Smit, G., Smit B.A., Wim J.M. Engels. (2005). Flavor formation by lactic acid bacteria and biochemical flavor profiling of cheese products. *FEMS Microbiol Rev* **29:** 591–610.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. (1986). Bergey's Manual of Systematic Bacteriology. Vol 2, Baltimore, Williams and Wilkins, London, pp. 1104-1139.

Solis Pereyra, B. and Lemonnier, D. (1993). Induction of human cytokines by bacteria used in dairy foods. *Nutr Res* 13: 1127–1140.

Solomons, N.W., Guerrero, A.M. and Torun, B. (1985). Dietary manipulation of postprandial colonic lactose fermentation: II. Addition of exogenous microbial beta-galactosidases at mealtime. *Am J Clin Nutr* **41:** 209–221.

Somkuti, G.A. and Steinberg, D.H. (1994). Permeabilization of *Streptococcus thermophilus* and the expression of beta-galactosidase. *Enzyme Microb Technol* **16:** 573-576.

Soomro, A.H., Masud, T. and Anwaar, K. (2002). Role of lactic acid bacteria (LAB) in food preservation and Human health. *Pak J Nutr* **1:** 20-24.

Sorhaug, T. and Stepaniak**,** L. (1997). Psychrotrophs and their enzymes in milk and dairy products: quality aspects. *Trends Food Sci Technol* **8:** 35–40.

Spanhaak, S., Havenaar, R. and Schaafsma, G. (1998). The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. *Eur J Clin Nutr* **52:** 899-907.

Sperti, G.S. (1971). Probiotics. West Point, CT: Avi Publishing. Surawicz, C.M., Elmer, L.W., Speelman, P., McFarland, L.V., Chinn, J. and van Belle, G. (1989). Prevention of antibiotic associated diarrhea by *Saccharomyces boulardii:* a prospective study. *Gastroenterol* **96:** 981–988.

Standiford, T.J., Arenberg, D.A. Danforth, J.M., Kunkel, S.L., VanOtteren, G.M. and Strieter, R.M. (1994). Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. *Infect Immun* **62:** 119-125.

Stanley, G. (1998). Cheeses. In: Microbiology of fermented foods. Eds. Brian JB Wood, Vol. 1(2), Blackie Academic and Professional, New York, pp 263-307.

Stanton, C., Gardiner, G., Meehan, H., Collins, K., Fitzgerald, G., Lynch, P.B., Ross, R.P. (2001). Market potential for probiotics. *Am J Clin Nutr* **73(2 Suppl):** 476S–83S.

Stewart, P.S. and Costerton, J.W. (2001). Antibiotic resistance of bacteria in biofilms. *The Lancet* **358:**135-138.

Stiles, M.E. and Holzapfel, W.H. (1997). Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol* **36:** 1–29.

Stoner, G.D. and Mukhtar, H. (1995). Polyphenols as cancer chemopreventive agents. *J Cell Biochem* **22:** 169–180.

Streeter, J.G. (2003). Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. *J Appl Microbiol* **95:** 484–491.

Strompfova, V. and Laukova, A. (2004). Antibiotic resistance of lactic acid bacteria from canine faeces. *Bull Vet Inst Pulawy* **48:** 215-218.

Strus, M., Marewicz, E., Kukla, G., Ruranska-Smutnicka, D., Przondo-Mordarska, A. and Heczko, P.B. (2001). Surface properties of *Lactobacillus* strains of human origin. *Microb Ecol Health Dis* **13:** 240–245.

Suarez, F.L., Savaiano, D.A. and Levitt, M.D. (1995). A comparison of symptoms after the consumption of milk or lactose hydrolyzed milk by people with self-reported severe lactose intolerance. *New England J Med* **333:** 1–4.

Sutas, Y., Hurme, M. and Isolauri, E. (1996). Down-regulation of anti-CD3 antibody induced IL-4 production by bovine caseins hydrolysed with Lactobacillus GG derived enzymes. *Scand J Immunol* **43:** 687–689.

Swagerty, D.L., Walling, A.D. and Klein, R.M. (2002). Lactose intolerance. *Amer Fan Physician* **65(9):** 1845-1850.

Swaminathan, M. (1998). Essentials of Food Nutrition, Vol. 1, Bangalore Printing Pub. Co. Ltd, Bangalorep. pp. 488-490.

Swan, T.M. and Watson, K. (1997). Membrane fatty acid composition and membrane fluidity as parameters of stress tolerance in yeast. *Can J Microbiol* **43:** 70–77.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M. H. and Hugenholtz, J. (2003). Effects of cultivation conditions on folate production by lactic acid bacteria. *Appl Environ Microbiol* **69:** 4542–4548.

Tadesse, G., Ephraim, E. and Ashenafi, M. (2005). Assessment of the antimicrobial activity of lactic acid bacteria isolated from Borde and Shamita, traditional Ethiopian fermented beverages, on some food borne pathogens and effect of growth medium on the inhibitory activity. *Internet J Food Safety* **5:** 13-20.

Tagg, J.R., Dajani, A.S. and Wannamaker, L.W. (1976). Bacteriocins of gram-positive bacteria. *Bacteriological Reviews* **40:** 722–756.

Takahashi, M., Okada, S., Uchimura, T. And Kozaki, M. (1992). *Leuconostoc amelibiosum* is a later subjective synonym of *Leuconostoc citreum. Int J Sys Bacteriol* **42:** 649–651.

Takeyama, K., Kunishima, Y., Matsukawa, M., Takahashi, S., Hirose, T., Kobayashi, N., Kobayashi, I. and Tsukamoto, T. (2002). Multidrug-resistant *Pseudomonas aeroginosa* isolated from the urine of patients with urinary tract infection. *J Infect Chemother* **8:** 59-63.

Tannock, G.W. (1995). Normal Microflora : An introdction to microbes inhabiting the human body. Chapman and Hall, London, UK, pp. 480-495.

Tannock, G.W. (1998). Studies of the intestinal microflora: a prerequisite for the development of probiotics. *Int Dairy J* **8:** 527–533.

Tannock, G.W. (1999). A fresh look at the intestinal microflora. In Probiotics. A Critical Review ed. Tannock, G.W. Wymondham: Horizon Scientific Press, Norfolk, England, pp. 5–14.

Tapiero, H., Townsend, D.M. and Tew, K.D. (2004). The role of carotenoids in the prevention of human pathologies. *Biomed Pharmacother* **58:**100–110.

Taranto, M.P. Medici, M. Perdigon, G. Ruiz Holgado, A.P. and Valdez, G.F. (1998). Evidence for hypocholesterolemic effect of *Lactobacillus reuteri* in hypercholesterolemic mice. *J Dairy Sci* **81(9):** 2336-2340.

Tavaria, F.K., Dahl, S., Carballo, F.J. and Malcata, F.X. (2002). Amino acid catabolism and generation of volatiles by lactic acid bacteria. *J Dairy Sci* **85:** 2462– 2470.

Tayag, E. and Dayrit, C.S. (2000). Results analysis of clinical HIV trial with mono glycerides. The 37th Cocotech Meeting, Chennai, India.

Teixeira, P.C., Castro, M.H., Malcata, F.X. and Kirby, R.M. (1995). Survival of *Lactobacillus delbruckii ssp. bulgaricus* following spray drying. *J Dairy Sci* **78:** 1025– 1031.

Terahara, M., Kurama, S. and Takemoto, N. (2001). Prevention by lactic acid bacteria of the oxidation of human LDL. *Biosci Biotechnol Biochem* **65:** 1864–1868.

Ternstrom, A., Lindberg, A.M. and Molin, B. (1993). Classification of the spoilage flora of raw and pasteurized bovine milk, with special reference to *Pseudomonas* and *Bacillus*. *J Appl Bact* **75:** 25-34.

Terzaghi, B.E. and Sandine, W.E. (1975). Improved medium for lactic streptococci and their bacteriophages. *Appl Environ Microbiol* **29:** 807– 813.

Teuber, M., Meile, L. and Schwarz, F. (1999). Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek* **76(1):** 115-137.

Thapa, T. (2000). Small-scale milk processing technologies: other milk products. In: Report of the FAO E-mail conference on small-scale milk collection and processing in developing countries, Food and Agriculture Organization of the United Nations, Rome, Italy..

Theunissen, J.J.H., Stolz, E. and Michel, M.F. (1993). The effects of medium and rate of freezing on the survival of *Chlamydias* after lyophilization. *J Appl Bacteriol* **75:** 473–477.

Thibodeaux, B., Caballero, A., Dajcs, J., Marquart, M., Engel, L. and O'Callaghan, R. (2005). *Pseudomonas aeruginosa* Protease IV: A Corneal Virulence Factor of Low Immunogenicity. *Ocular Immunology and Inflammation* **13(2-3):** 169.

Thierry, A., Maillard, M.B. and Yvon, M. (2002). Conversion of L-leucine to isovaleric acid by *Propionibacterium freudenreichii* TL 34 and ITGP23. *Appl Environ Microbiol* **68:** 608–615.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:** 4673-4680.

Timisjarvi, T. A. and Alatossava, T. (1997). Development of oligonucleotide primers from the 16S–23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. *Int J Food Microbiol* **35:** 49-56.

Tissier, H. (1906). Recherches sur la flore intestinale des nourissons. University of Paris. G Carre and C Nord, editors. Paris, France.

Torres, A.G., Tutt, C.B., Duval, L., Papov, V., Ben Nasr, A., Michalski, J. and Scaletsky, I.C.A. (2007). Bile salts induce expression of the afimbrial LDA adhesin of atypical enteropathogenic E. coli. *Cell Microbiol* **9(4):** 1039-1049.

Tryfinopoulou, P., Tsakalidou, E. and Nychas, G.J. E. (2002). Characterization of *Pseudomonas* spp. associated with spoilage of gilt head sea bream stored under various conditions. *Appl Environ Microbiol* **68:** 65-72.

Tserovska, L., Stefanova, S and Yordanova, T. (2002). Identification of lactic acid bacteria isolated from katyk, goat's milk and cheese *J Culture Collections* **3:** 48-52.

Tuomola, E., Crittenden, R., Playne, M., Isolauri, E. and Salminen, S. (2001). Quality assurance criteria for probiotic bacteria. *Am J Clin Nutr* **73:** 393S–398S.

Tuomola, E.M.,Ouwehand, A.C. and Salminen, S. (2000). Chemical, physical and enzymatic pretreatments of probiotic *Lactobacilli* alter their adhesion to human intestinal mucus glycoproteins. *Int J Food Microbiol* **60:** 75–81.

Tyssandier, V., Cardinault, N., Caris-Veyrat, C., Amiot, M.J., Grolier, P., Bouteloup, C., Azais-Braesco, V. and Borel, P. (2002). Vegetable borne lutein, lycopene, and betacarotene compete for incorporation into chylomicrons, with no adverse effect on the medium-term (3-week) plasma status of carotenoids in humans. *Am J Clin Nutr* **75:** 526-534.

Tzanetaki, E.L. and Mastrojiannaki, A.V. (2006) Diacetyl and acetaldehyde concentrations during ripening of kefalotyri cheese. *J. Food Sci,* **53(2):** 663-664.

Usman. and Hosono. A. (2001). Hypocholesterolemic effect of *Lactobacillus gasseri* SBT0270 in rats fed a cholesterol enriched diet. *J Dairy Res* **68:** 617–624.

Uzunova-Doneva, T. and Donev, T.(2000). Influence of the freezing rate on the survival of strains *Saccharomyces cerevisiae* after cryogenic preservation. *J Culture Collections* **3:** 78–83.

Van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Stanislav, D., Ehrlich. and Maguin, E. (2002). Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek* **82:** 187–216.

Van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G. and Zehnder. A.J.B. (1987). The role of bacterial cell wall hydrophobicity in adhesion. *Appl Environ Microbiol* **53:**1893–1897.

Van Schaik, W., Gahan, C.G. and Hill. C. (1999). Acid-adapted *Listeria monocytogenes* displays enhanced tolerance against the lantibiotics nisin and lacticin 3147. *J Food Prot* **62:** 536–539.

Vanderhoof, J.A., Whitney, D.B., Antonson, D.L., Hanner, T.L., Lupo, J.V. and Young, R.J. (1999). *Lactobacillus* GG in the prevention of antibiotic-associated diarrhea in children. *J Pediatr* **135:** 564–568.

Vannini, A., Volpari, C., Gargioli, C., Muraglia, E., Cortese, R., De Francesco, R., neddermann, P. And Marco, S.D. (2002). The crystal structure of the quorum sensing protein Tra R bound to its autoinducer and target DNA. *EMBO J* **21:** 4393-4401.

Vedamuthu, E.R. (1994). The dairy *Leuconostoc*: Use in dairy products. *J Dairy Sci* **77:** 2725–2737.

Vergin, F. (1954). Anti-und Probiotika (Anti- and probiotics). *Hippokrates* **25(4):** 116–119.

Vesa, T.H., Marteau, P., Zidi, S., Pochart, P. and Rambaud, J.C. (1996). Digestion and tolerance of lactose from yogurt and different semi-solid fermented dairy products containing *Lactobacillus acidophilus* and *Bifidobacteria* in lactose maldigesters. Is bacterial lactase important?. *Eur J Clin Nutr* **50:** 730–733.

Vescovo, M., Torriani, S., Orsi, C., Macchiarolo, F. and Scolari, G. (1996). Application of antimicrobial-producing lactic acid bacteria to control pathogens in ready-to-use vegetables. *J Appl Microbiol* **81:** 113-119.

Villani, F., Moschetti, G., Blaiotta, G. and Coppola, S. (1997).Characterization of strains of *Leuconostoc mesenteroides* by analysis of soluble whole-cell protein pattern, DNA fingerprinting and restriction of ribosomal DNA. *J Appl Microbiol* **82:** 578–588.

Vinderola, C.G., Bailo, N. and Reinheimer, J.A. (2000). Survival of probiotic microflora in Argentinian yoghurts during refrigerated storage. *Food Res Int* **33(2):** 97–102.

Vinderola, C.G., Medici, M. and Perdigon, G. (2004). Relationship between interaction sites in the gut hydrophobicity, mucosal immunomodulating capacities and cell wall protein profiles in indigenous and exogenous bacteria. *J Appl Microbiol* **96:** 230–243.

Virtanen, T., Pihlanto, A., Akkanen, S. and Korhonen, H. (2007). Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria. *J Appl Microbiol* **102:** 106–115.

Visick, J. and Clark, S. (1995). Repair, refold, recycle: how bacteria can deal with spontaneous and environmental damage to proteins. *Mol Microbiol* **16:** 835–845.

Von Wright, A., Vilpponen-Salmela, T., Pages Llopis, M., Collins, K., Kiely, B., Shanahan, F. and Dunne, C. (2002). The survival and colonic adhesion of *Bifidobacterium infantis* in patients with ulcerative colitis. *Int Dairy J* **12:** 197-200.

Vorobjeva, L.I. and Abilev, S.K. (2002) Antimutagenic Properties of Bacteria: Review. *Appl Biochem Microbiol* **38(2):** 112-122.

Wadstrom, T., Andersson, K., Sydow, M., Axelsson, L., Lindgren, S. and Gullmar, B. (1987). Surface properties of *Lactobacilli* isolated from the small intestine of pigs. *J Appl Bacteriol* **62:** 513–520.

Wallenfels, K. and Weil, R. (1972). *β*-Galactosidase. In: Boyer, P.D. (Ed.), The Enzymes, vol. 7. Academic Press, New York, pp. 617-663.

Walsh, C. (2003). Antibiotic resistance by replacement or modification of the antibiotic target. In: Antibiotics: actions, origins, resistance. ASM Press, Hong Kong, pp 143–154.

Wanasundrara, U., Amarowicz, R., Shahidi, F. (1994). Isolation and identification of an antioxidative component in canola meal. *J Agric Food Chem* **420:** 1285-1290.

Wang, X. and Gibson, G.R. (1993). Effects of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in human large intestine. *J Appl Bacteriol* **75:** 373-380.

Wang, Y.C., Yu, R.C. and Chou, C.C. (2004). Viability of lactic acid bacteria and *Bifidobacteria* in fermented soymilk after drying, subsequent rehydration and storage. *Int J Food Microbiol* **93:** 209–217.

Wang, Y.C., Yu, R.C. and Chou, C.C. (2006). Antioxidative activities of soymilk fermented with lactic acid bacteria and *Bifidobacteria*. *Food Microbiol* **23:** 128–135.

Weizman, Z., Asli, G. and Alsheikh, A. (2005). Effect of a probiotic infant formula on infections in child care centers: Comparison of two probiotic agents. *Pediatrics* **115:** 5–9.

Wiemken, A. (1990). Trehalose in yeast, stress protectant rather than reserve carbohydrate. *Antonie van Leeuwenhoek Int J Gen Mol Microbiol* **58:** 209-217.

Wilderdyke, M.R., Smith, D.A. and Brashears, M.M. (2004). Isolation, identification and selection of lactic acid bacteria from alfalfa sprouts for competitive inhibition of food borne pathogens. *J Food Prot* **67:** 947-951.

Winson, M.K., Camara, M., Lati, A., Foglino, M., Chhabra, S.R., Daykin, M., Bally, M., Chapon, V., Salmond, G.P.C., Bycroft, B.W., Lazdunski, A., Stewart, G. and Williams, P. (1995). Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences of the United States of America **92:** 9427-9431.

Winzer, B., Middleton, Pritchard, D.I. and Bycroft**,** B.W. (2000). Quorum sensing and the population dependent control of virulence. *Philos Trans R Soc Lond B* **355:** 667–680.

Wojnicz, D. and Jankowski, S. (2007). Effects of subinhibitory concentrations of amikacin and ciprofloxacin on the hydrophobicity and adherence to epithelial cells of uropathogenic *Escherichia coli* strains. *Int J Antimicro Agents* **29(6):** 700-704.

Wouters, J.A., Rombouts, F.M., de Vos, W.M., Kuipers, O.P. and Abee, T. (1999). Cold shock proteins and low-temperature response of *Streptococcus thermophilus* CNRZ302. *Appl Environ Microbiol* **65:** 4436–4442.

Yaeshima, T., Takahashi, S., Ishibashi, N. And Shimamura, S. (1996). Identification of *Bifidobacteria* from dairy products and evaluation of a microplate hybridization method. *Int J Food Microbiol* **30:** 303–313.

Yamauchi, R., Goto, Y., Kato, K. and Ueno. Y. (1984). Prooxidant effect on dihydroxyacetone and reducing sugars on the autoxidation of methyl emulsions. *Agric Biol Chem* **48:** 843- 848.

Yan, F. and Polk, D.B. (2002). Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. *J Biol Chem* **277:** 50959–50965.

Yan, T., Lin, Y. and Lin, C. (1998). Purification and characterization of an extracellular *β*-glucosidase II with high hydrolysis and transglucosylation activities from *Aspergillus niger*. *J Agri Food Chem* **46:** 431–437.

Yarwood, J.M. and Schlievert, P.M. (2003). Quorum sensing in *Staphylococcus* infections. *J Clin Invest* **112(11):** 1620–1625.

Young, J. (1996). Functional foods: strategies for successful product development. FT management report. Pearson Professional Publishers, London.

Yu, W.C., Lee, J.C., Choi, Y.G. and Yang, H.C. (1986). Studies on production of *β*-galactosidase by a mutant of *Lactobacillus sporogenes*. *Kor J Appl Microbiol Bioeng* **14:** 75–84.

Zammaretti, S.P. and Ubbink, J. (2003). The cell wall of lactic acid bacteria: surface constituents and macromolecular conformations. *Biophy J* **85:** 4076–4092.

Zarate, G., Chaia, A.P., Gonzalez, S. and Oliver, G. (2000). Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. *J Food Prot* **63:**1214-1221.

Zareba, T. W., Pascu, C., Hryniewicz, W. and Waldstrom, T. (1997). Binding of extracellular matrix proteins by *Enterococci*. *Curr Microbiol* **34(1):** 6–11.

Zhang, R., Pappas, T.J., Miller, P.C., Oulmassov, T., Molyneaux, J.M., Anderson, J.C., Bashkin, J.K., Winans, S.C. and Joachimiak, A. (2002). Structure of a bacterial quorum sensing transcription factor complexed with pheromone and DNA. *Nature* **417:** 971-974.

Zhao, G. and Zhang, G. (2005). Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. *J Appl Microbiol* **99:** 333–338.

Zhou, J.S. Shu, Q., Rutherfurd, K.J., Prasad, J., Gopal, P.K. and Gill, H.S. (2000). Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria. *Food Chem Toxicol* **38(2-3):** 153-161.

Zhou, J.S., Pillidge, C.J., Gopal, P.K. and Gill, H.S. (2005). Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *Int J Food Microbiol* **98**: 211–217.

Zhou, X., Yuan-jiang, P., Yan-bo, W. and Li Wei-fen. (2007) *In vitro* assessment of gastrointestinal viability of two photosynthetic bacteria, *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides. J Zhejiang Univ* **8(9):** 686-692.

Zoetendal, E.G., Akkermans, A.D. and. De Vos, W.M. (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol* **64:** 3854–3859.

Zommara, M., Tachibana, N., Sakomo, M., Suzuki, Y., Oda, T., Hashida, H. and Imaizumi, K. (1996). Whey from cultured skim milk decreases serum cholesterol and increases antioxidant enzymes in liver and red blood cells in rats. *Nutr Res* **16:** 293–302.

Outcome of the present work

OUTCOME OF THE PRESENT WORK

Publications

1) Strain improvement by mutagenesis and optimum condition for culture parameter by response surface methodology for lactose tolerance in a novel native culture isolate *Leuconostoc mesenteroides subsp dextranicum*. Shobha Rani. P, Ramesh BS and Renu Agrawal, Research Journal of Biotechnology, 1(2), 5-11, 2006.
2) Studies on the stability and viability of a local probiotic isolate *Pediococcus pentosaceus* MTCC 5151 under induced gastrointestinal tract conditions in MRS broth medium. Sudha N, Shobha Rani. P and Renu Agrawal, Journal Food Science and Technology, 43(6), 677-678, 2006.
3) Volatile compounds of therapeutic importance produced by probiotic *Leuconostoc paramesenteroides* a native laboratory isolate. Shobha Rani. P and Renu Agrawal, Turkish Journal of Biology, 31, 35-40, 2007
4) Effect on cellular membrane fatty acids in the stressed cells of *Leuconostoc mesenteroides subsp dextranicum*: a native probiotic lactic acid bacteria. Shobha Rani. P and Renu Agrawal, Food Biotechnology, 22(1), 1-17, 2008.

PAPER PRESENTATIONS IN SYMPOSIA

1) Isolation and characterization of probiotic lactic acid bacteria from cheddar cheese. 45th Annual Conference, 2004, Association of Microbiologists of India, NDRI, Karnal- Hariyana
2) A potent probiotic isolate Lsh 1 to be used as a potent antimicrobial agent against diseases and toxic food pathogens. 45th Annual Conference, 2004, Association of Microbiologists of India, NDRI, Karnal- Hariyana

3) Flavor profile of a potent probiotic culture (*Leuconostoc paramesenteroides*) and their therapeutic importance. 16th Indian Convention of Food Scientists and Technologists, ICFOST- 2004, Mysore
4) Effect of adjuvant supplementation on different parameters of fermented milk produced from *Leuconostoc mesenteroides subsp dextranicum.* ICFOST, Bangalore 2005.
5) Quorum sensing in bacteria in relation to milk spoilage. Association of Microbiologists of India, Bhopal 2006.
6) *Leuconostoc mesenteroides subsp dextranicum* as an alternative sources for β-galactosidase enzyme to treat lactose intolerance. Society of Biological Chemists, New Delhi 2006.
7) Enhancement of cell stability and viability of probiotic *Leuconostoc mesenteroides* MTCC 5209 on freeze drying to be used in food formulation. Intl Conference on 'New Horizon in Biotechnology'. Trivandrum, 29 Nov 2007.
8) Interception of quorum sensing signal molecule by furanone to enhance shelf life of fermented milk. Intl conference on 'Applied Bioengineering' iCAB-07. Chennai, 5-7 Dec 2007 (Best poster award).

Process, products and patents

1) Bile resistant and antimicrobial lactic acid bacteria from whey. (303/DEL/2006)
2) A process for the preparation of nutritive probiotic fermented milk beverage with enhanced stability during storage with adjuvant supplementation (658/DEL/07).
3) A process for detection of hexanoyl homoserine lactone as quorum signal molecule in spoilt milk by GC and GCMS method (159/NF/06).
4) *Leuconostoc mesenteroides* as an alternative source for β-galactosidase to treat lactose intolerance (submitted to PMC).