

Characterization of Lactobacilli isolated from indigenous fermented foods and human gut with special reference to their Probiotic attributes

*A thesis
submitted in fulfillment of the requirement
for the award of the degree of*

**DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY**

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CERTIFICATE

Certified that the thesis "**Characterization of *Lactobacilli* isolated from indigenous fermented foods and human gut with special reference to their probiotic attributes**" which is submitted by **Mr. Mukesh Kumar**, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.



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I hereby declare that the work which is being presented in this thesis “**Characterization of lactobacilli isolated from indigenous fermented foods and human gut with special reference to their probiotic attributes**” submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. Abhijit Ganguli**, Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.


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In deed God and Guide work hand in hand”

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List of Abbreviations

ABTS	2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analysis of Variance
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
Bsh	Bile salt hydrolase
CFU	Colony Forming Units
CPC	Cetylpyridinium chloride
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
FAO	Food agricultural organization
GI	Gastrointestinal tract
GRAS	Generally recognised as safe
HPLC	High performance liquid chromatography
IEC	Intestinal epithelial cells
KOH	Potassium hydroxide
LAB	Lactic Acid Bacteria
Min	minutes
MRD	Maximum recovery diluents
MRS	De Mamm, Rogosa and Sharpe
NCBI	National centre for biotechnology information
OD	Optical Density
PCA	Plate count agar
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
ppm	Parts per million
QSR	Quarter-strength Ringer solution
rDNA	Ribosomal deoxyribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SCAN	Scientific Committee for Animal Nutrition
Sec	Seconds
SEM	Scanning electron micrography
SOD	Superoxide dismutase
TLC	Thin Layer Chromatography
Td	Degradation temperature
WTO	World Trade Organization

List of Symbols

%	Percentage
μ	Micron
μg	Microgram
μl	Microlitre
a _w	Water activity
bp	Base pair
C	Carbon
d	Days
Da	Dalton
g	Gram
H	Hydrogen
H ₂ O ₂	Hydrogen peroxide
hr	Hours
Hz	Hertz
kb	Kilo base
KDa	Kilo Dalton
kV	Kilo volt
L	Litre
M	Molar
mA	Milli ampere
mg	Milligram
Mg	Magnesium
Min	Minutes
mL	Milliliter
N	Nitrogen
ng	Nanogram
U	Unit
UV	Ultraviolet
V	Volt
v/v	volume by volume
w/v	Weight by volume
α	Alpha
β	Beta

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Chapter I
INTRODUCTION

I. Introduction

Lactic acid bacteria (LAB) including *Lactobacillus* spp. such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Bifidobacterium* spp. have been recognized to be important in the maintenance of the human intestinal microbial ecosystem. Member of LAB are widely-used probiotics in fermented foods and beverage industry and also contributes to the sensory qualities of the food and also in the prevention of spoilage. These organisms are present in large number in normal animal gastrointestinal flora (Sgouras *et al.*, 2004). Improvement in intestinal disorders and lactose intolerance, altered vitamin content of milk, antagonism against various pathogenic organisms and antimutagenic and anti-carcinogenic activities are some of the health benefits of LAB.

The beneficial properties associated with *lactobacilli* fermented food would improve health was postulated in the beginning of the 20th century by Metchnikoff (1908). He related the longevity of the Caucasians to the consumption of fermented milk products with *Lactobacillus bulgaricus*. Since then, these organisms have created a new generation of health foods in human nutrition. These health foods were termed as ‘*probiotic*’ by Fuller (1989), which means a live microbial feed supplement that beneficially affects the host by improving its intestinal microbial balance. The definition of probiotics has been modified and improved several times; a consensus definition had been stated by Schrenzenmeir and de Verse (2001). They defined probiotics as ‘a preparation or product containing viable, defined micro-organisms in sufficient numbers that alter the microflora in a compartment of the host and that exerts health effects in the host’.

Recent research work has credited several health benefits to probiotic organisms that are indigenous to the gastrointestinal tract, as well as those consumed through probiotic products. Probiotic organisms has been reported to overcome symptoms of lactose intolerance (deVerse *et al.*, 1992), improved immune function, cholesterol lowering potential (Noh *et al.*, 1997), antimutagenic activity (Lankaputhra and Shah, 1998) and treatment of diarrhea (Guandilini *et al.*, 2000). Therapeutic activity of probiotic bacteria may be due to competition with pathogens for nutrients and mucosal adherence, production of antimicrobial substances, and modulation of mucosal immune functions (O'sullivan *et al.*, 2005).

Gut microflora

Intestine, its microflora and the associated immune system are the 'natural' target of ingested probiotics (De Verse *et al.*, 2002). Therefore, investigations and clinical studies of non-intestinal infections are rather scarce. Human gastrointestinal tract constitutes a complex microbial ecosystem (Simon and Gorbach, 1986). More than 400 different species have been identified in feces of a single subject (Finegold *et al.*, 1977; Moore *et al.*, 1974). The equilibrium that exists in the large intestine is dynamic and is affected by age, diet composition and other environmental factors. Although these factors are significant, the continued maintenance of the intestinal microflora, which predominantly contains beneficial species such as probiotic bacteria, can improve our well being.

The normal intestinal flora and some pathogenic bacteria possess the ability to colonize the mucosal surface of gastrointestinal tract and carries out fermentation by consuming substrates and producing end products which significantly influence our health (Haenel, 1961). There is, however, host specificity in colonization by individual species; for example, *L. acidophilus*, *L. fermentum* and *L. plantarum* are commonly found in the feces of humans; whereas *L. delb. spp. bulgaricus*, which is used in combination with *Streptococcus*

thermophilus to make yoghurt, is unable to colonize the bowl and is not isolated in the feces (Finegold *et al.*, 1977).

The human gastrointestinal tract is known to possess active clearance mechanisms for microorganisms, and has proven difficult to introduce new bacterial strains into this ecosystem (Savage, 1979; Paul and Hoskins, 1972). However, in different circumstances it can be beneficial to alter the intestinal microflora by introducing *lactobacilli*. The nutritional and therapeutic benefits derived from this approach have been discussed by Gorbach (1990), and the claimed benefits of bacterial supplementation include increased nutrient utilization, alleviation of lactose intolerance, treatment of hepatic encephalopathy and intestinal infections, and inhibition of bacterially derived generation of carcinogens in the intestinal tract (Finegold *et al.*, 1977).

***Lactobacillus* spp.**

Lactic acid bacteria constitutes group of bacteria which cause fermentation and coagulation in milk and produce lactic acid from lactose. The family name Lactobacteriaceae was coined by Orla-Jensen (1919) for a physiological group of Gram-positive rods and cocci. They are anaerobic bacteria, non-sporulating, acid tolerant and produce mainly lactic acid as an end product of carbohydrate fermentation. On the basis of end product of their fermentation they can be divided into different genera which include homofermenters and heterofermenters. The homofermenters produces lactic acid as the major product of fermentation of glucose and include the genera *Lactococcus*, *Streptococcus* and *Pediococcus*. In contrast, the heterofermenters produce a number of by-products along with lactic acid, such as carbon dioxide, acetic acid, and ethanol from the fermentation of glucose and includes the genus *Leuconostoc* and a subgroup of the genus *Lactobacillus*, the Betabacteria (Kandler *et al.*, 1986; Schillinger and Lücke, 1987). Members of *Lactobacillus* spp. are

Gram-positive, facultatively anaerobic, catalase-negative, facultatively heterofermentative, non-spore-forming rods and are isolated from many habitats (e.g., meats, milks, dairy products, sour dough, silage, and sewage). Strains of *Lactobacillus* are important for many food fermentations and are normal constituents of intestinal microflora. Some *Lactobacillus* strains have desirable and functional characteristics (Saxelin *et al.*, 1996). The *Lactobacillus casei* group contains a number of well-known probiotics strains including *L. casei* shirota (Sugita and Togawa, 1994) and *L. rhamnosus* GG (Saxelin, 1997).

For both basic studies and applications in food industries, the identification of *Lactobacillus* strains within this group (*L. casei*, *L. paracasei*, *L. rhamnosus* and *L. zeae*) is very important. Most of these strains grow under similar environmental conditions and have very similar physiological properties and nutritional requirements because of which the traditional phenotypic tests for *Lactobacillus* identification can be difficult to interpret. These methods give ambiguous results and are also time consuming (Charteris *et al.*, 1997; Hammes *et al.*, 1992).

Although, this group of *Lactobacillus* can be readily distinguished from other members of the *Lactobacillus* genus by fermentation profiles (Hammes *et al.*, 1992), but it is not possible to unequivocally distinguish above mentioned four species on the basis of fermentation patterns. They form a closely related taxonomic group. In recent years, the taxonomy of this group has changed considerably with increasing knowledge of genomic structure and phylogenetic relationships between *Lactobacillus* species (Klein *et al.*, 1998; Tisala-Timisjarvi *et al.*, 1999). Identification of LAB mostly depends on traditional phenotypic analyses and molecular biology-based methods (Zoetendal *et al.*, 1998).

The core LAB genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* share a long history of safe usage in the processing of fermented foods.

Moreover, the antimicrobial effects and safety of LAB in food preservation is widely accepted (EFSA, 2005). Their preservative effect is mainly due to the production of lactic acid and other organic acids which results in lowering of pH (Daeschel, 1989). Preservation is enhanced by the production of other antimicrobial compounds, including hydrogen peroxide, CO₂, diacetyl, acetaldehyde, and bacteriocins (Klaenhammer, 1988; 1993). In addition to the antimicrobial effects, specific LAB also possesses health promoting properties. Evidence from *in vitro* systems, animal models and human clinical studies suggests that LAB function as immune-modulators and can enhance both specific and nonspecific immune responses (Ouwehand *et al.* 2002; O’Flaherty and Klaenhammer, 2009), justifying their use as health promoting supplements or probiotics both for humans and animals.

Antimicrobial peptides produced by *Lactobacillus* spp.

Lactic acid bacteria are capable of producing a wide range of ribosomally synthesized proteins and peptides which have antimicrobial activity to compete with other bacteria of the same species (narrow spectrum) or to counteract bacteria of other genera (broad spectrum) (Cotter *et al.*, 2005). The first description of bacteriocin-mediated growth inhibition was reported 85 years ago, when antagonism between strains of *Escherichia coli* was first discovered (Gartia, 1925). The inhibitory substances were called ‘colicins’, to reflect the producer organism, whereas gene-encoded antibacterial peptides produced by bacteria are now referred to as ‘bacteriocins’. In 2005, an estimated 1.8 million people died from diarrhoeal diseases, largely attributable to contaminated food and drinking water (Newell *et al.*, 2010). About 76 million cases of food-borne diseases, resulting in 325,000 hospitalizations and 5000 deaths, are estimated to occur each year in the United States of America (USA) alone (Newell *et al.*, 2010). In Finland, a total of 39,500 persons contracted

food-borne illness in outbreaks during years 1975-2006 (Niskanen *et al.*, 2007). Clearly, food and water safety need to be improved.

Microbes have an extraordinary array of defense systems. These include classical antibiotics, metabolic by-products such as lactic acid, numerous types of protein exotoxins, and antimicrobial peptides such as bacteriocins. The most promising antimicrobial peptides are those produced by LAB. Most LAB bacteriocins are non-toxic to eukaryotic cells and are generally recognized as safe substances, active in the nanomolar range (Cotter *et al.*, 2005; Peschel and Sahl, 2006). LAB antimicrobial peptides typically exhibit antibacterial activity against food-borne pathogens, as well as spoilage bacteria. Therefore, they have attracted the greatest attention as tools for food biopreservation (Collins *et al.* 1998; O'Sullivan *et al.*, 2002; Reid *et al.*, 2003). They either show bacteriostatic or bactericidal activity against targeted pathogens. Probiotics are now widely available commercially and marketed through food products such as yoghurt, fermented milks, fermented juices and freeze dried supplements. As commercially available probiotic strains are being produced and marketed for human consumption on a large scale, it is important to identify them at species and strain level.

In past 10 years, there has been an increase in the consumption of probiotics and functional foods in Western diets (O'sullivan, 1996). Probiotic bacteria are able to suppress potentially pathogenic microorganism in the gastrointestinal tract and enhance the population of beneficial microorganisms (Yaeshima *et al.*, 1997). The health benefits derived by the consumption of foods containing probiotic bacteria are well documented and more than 90 probiotic products are available worldwide. To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 CFU/g of a product (Shah, 2000). However, studies have shown low viability of probiotics in market preparations (Shah *et al.*, 1995). A number

of factors have been claimed to affect the viability of probiotic bacteria in fermented food including acid and hydrogen peroxide produced by bacteria, oxygen content in the product, and oxygen permeation through the package (Shah, 2000). Viability of probiotic bacteria declines over time in the fermented products because of the acidity of the product, storage temperature, storage time, and depletion of nutrients (Dave and Shah, 1997a). Due to this, these products have limited shelf life (Dave and Shah, 1996). However, in order to achieve maximum viability and maximum health benefits in a product, there is a need to have a better understanding of this organism as an emerging probiotic.

Thus host specificity, the generally regarded as safe (GRAS) status, colonization, antimicrobial activity, and desirable metabolic activity are reasonably agreed upon (Tannock, 1998), but issues such as the effect of living versus non living probiotics or even their survival in the intestinal tract remain open (Reid *et al.*, 2003). Criteria for quality, including the sensory characteristics of probiotic strains, is well established (O'Sullivan *et al.*, 2002; Reid *et al.*, 2003) as are those for technological suitability (Charteris *et al.*, 1998). In addition to *in vitro* experiments (Gibson and Fuller, 2000) and animal model studies (Borriello, 1990; Cross, 2002; Mallett *et al.*, 1986), GI tract simulation studies (Gmeiner *et al.*, 2000) have been employed for probiotic detection. However, the ultimate test for probiotic functionality is a double blind, placebo-controlled and randomized human study (Gibson *et al.*, 2000). In addition to this, for the demonstration of probiotic activity the given strain should also show resistance to technological processes, meaning viability and activity throughout processing phases (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, products and study populations in trials. It is mandatory that a potential probiotic strain must be accurately identified following their fulfillment of a set of

beneficial/probiotic characteristics. Currently, identification of LAB mostly depends on traditional phenotypic analyses and molecular biology based methods (Zoetendal *et al.*, 1998). From the above deliberations it is amply clear that lactic acid bacteria with unique as well diverse probiotic attributes may exist in fermented food and beverages (cereals, legumes, vegetables and fruits). It is also expected that the diversity in food culture existent in different geographical locations may yield equally prospective lactic acid bacteria from gut. Thus exploration of LAB with superior and strong probiotic attributes need to consider both fermented foods and human gut; some studies have actually exploited LAB with the above attributes from non dairy Indian fermented foods and this is equally true for LAB isolates from human gut.

Therefore, the present study attempts to characterize *Lactobacilli* from human and indigenous (non dairy fermented) food of Indian origin and investigating their probiotic and technological properties. The following objectives were framed to achieve the above mentioned goal.

Objectives

Over the last decades, there has been a continuous increase in the consumption of functional foods and the market of these products is flourishing. There are already many studies about the effects of probiotics, prebiotics and synbiotics on health, but there are still a lot of unanswered questions. Not all probiotic bacteria act in the same way, their ability to inhibit pathogens are quite different and these differences are not related to the genus or the species but appears to be strain specific. Therefore, and because of safety reasons, for each single strain there should first be an intensive *in vitro* investigation of probiotic properties, which report a functional effect that justify further *in vivo* studies, which are associated with high costs and ethical requirements. The general objective of this work was to find new

probiotic *Lactobacillus* candidates, which can be used in functional foods and to characterize the strains *in vitro* as an initial part in their development, in order to elucidate their possible mechanisms of probiotic action. The procedure to achieve this goal was to isolate lactic acid bacteria from traditional fermented foods and children's faeces followed by selection of the strains which can survive under simulated gastrointestinal conditions and to accurately identify them by phenotypic and genotypic methods via a polyphasic taxonomic approach. The selected strains were screened for the technological and probiotic properties namely: antimicrobial activities against pathogens, presence of β -galactosidase and bile salt hydrolase, cholesterol removal, antibiotic resistance and surface properties including adhesion to the gut mucosa. Based on the evaluation of above screens, the next step was to recommend those selected strains for further probiotic development and to demonstrate functionality of bacteriocins in food matrix, as well as in selected processing and post processing studies with a view of commercial application.

The objectives of the present investigations are:

- Isolation and characterization of *lactobacilli* from indigenous fermented food with probiotic attributes
- Evaluation of bacteriocin as a function of pathogen exclusion by selected probiotic isolate and to elucidate their structure of action
- Evaluation of the function of bacteriocin in food matrix and post processing

Chapter II
REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) constitute a diverse group of Gram-positive bacteria, characterized by some common morphological, metabolic and physiological traits. They are anaerobic bacteria, non-sporulating, acid tolerant and produce mainly lactic acid as an end product of carbohydrate fermentation.

2.1.1 Historical background of lactic acid bacteria

Lactic acid produced by fermentation is a biological process which is known for centuries. Many different cultures in various parts of the world carry out fermentation to improve the storage qualities and nutritive value of perishable foods such as milk, vegetables, meat fish and cereals. LAB those produces such type of fermentation products plays an important role in preserving foods. In developed world, lactic acid bacteria are mainly associated with fermented dairy products such as cheese, buttermilk, and yogurt. The uses of dairy starter cultures gave rise to an industry during this century.

Lactic acid bacteria have also been associated with beneficial health effects since the days of Russian scientist Metchnikoff. Today, an increasing number of health food and so-called functional foods as well as pharmaceutical preparation are promoted with health claims based on the characteristics of certain strains of lactic acid bacteria. Most of these strains, however, have not been thoroughly studied, and consequently the claims are not well substantiated. Moreover, health benefits are judged mainly using subjective criteria. Additionally, the specific bacterial strains used in the studies are often poorly identified. Most information about the health effects of lactic acid bacteria is thus anecdotal. There is clear

need for critical study of the effect on health of strain selection and the quality of fermented foods and their ingredients.

The concept of the group name 'lactic acid bacteria' was created for bacteria causing fermentation and coagulation of milk, and defines as those which produce lactic acid from lactose. The family name Lactobacteriaceae was applied by Orla-Jensen (1919) to a physiological group of bacteria producing lactic acid alone or acetic and lactic acids, alcohol and carbon dioxide. LAB are a group of Gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. They are non-sporing, carbohydrate-fermenting lactic acid producers, acid tolerant of non-aerobic habitat and catalase negative. Typically, they are non-motile and do not reduce nitrite. Today, lactic acid bacteria are regarded as synonymous by and large with the family Lactobacteriaceae (Breed *et al.*, 1957). They are subdivided into four genera *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Lactobacillus*. Recent taxonomic revisions suggest that lactic acid bacteria group could be comprised of genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Vagococcus*. Originally, bifidobacteria were included in the genus *Lactobacillus* and the organism was referred to as *Lactobacillus bifidus*.

Although the classification of lactic acid bacteria into different genera is mainly based on the characteristics used by Orla-Jensen (1919). This work had a large impact on the systematic of lactic acid bacteria, and, although revised to some extent, it is still valid and the basis of classification remarkably unchanged. The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, and configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance. Some of the newly described genera of

lactic acid bacteria are classified with some additional characteristics such as fatty acid composition and motility. The term lactic acid bacterium was used synonymously with “milk souring organism.” Important progress in the classification of these bacteria was made when the similarity between milk-souring bacteria and other lactic-acid producing bacteria of other habitats was recognized (Axelsson, 1993). Lactic acid bacteria are generally associated with habitats rich in nutrients, such as various food products (milk, meat, vegetables), but some are also members of the normal flora of the mouth, intestine, and vagina of mammals. The genera that, in most respects, fit the general description of the typical lactic acid bacteria are (as they appear in the latest *Bergey's Manual* from 1986) *Aerococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. The genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus* have largely remained unchanged, but some rod-shaped lactic acid bacteria, previously included in *Lactobacillus*, is now forming the genus *Carnobacterium* (Collins *et al.*, 1987).

2.1.2 Classification of lactic acid bacteria at genus level

Lactic acid bacteria morphology is regarded as questionable as a key character in bacterial taxonomy (Woese, 1987), and is very important in the current descriptions of the lactic acid bacteria genera. Based on this, lactic acid bacteria can be divided into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera).

An important characteristic used in the differentiation of the lactic acid bacteria genera is the mode of glucose fermentation under standard conditions, i.e., nonlimiting concentrations of glucose, growth factors (amino acids, vitamins and nucleic acid precursors) and limited oxygen availability. Under these conditions, lactic acid bacteria can be divided into two groups: homofermentative, which convert glucose almost quantitatively to lactic acid and heterofermentative, which ferment glucose to lactic acid, ethanol/acetic acid, and CO₂ (Sharpe, 1979). In practice, a test for gas production from glucose will distinguish

between the groups (Sharpe, 1979). *Leuconostocs* and a subgroup of *Lactobacillus* are heterofermentative; all other lactic acid bacteria are homofermentative.

Growth at certain temperatures is mainly used to distinguish between some of the cocci. Enterococci grow at both 10°C and 45°C, lactococci and vagococci grow at 10°C, but not at 45°C. Streptococci do not grow at 10°C, while growth at 45°C is dependent on the species (Axelsson, 1993). Salt tolerance (6.5% NaCl) may also be used to distinguish among enterococci, lactococci/vagococci, and streptococci, although variable reactions can be found among streptococci (Mundt, 1986). Extreme salt tolerance (18% NaCl) is confined to genus *Tetragenococcus*. Tolerances to acid and/or alkaline conditions are also useful characteristics. Enterococci are characterized by growth at both high and low pH. The formation of the different isomeric forms of lactic acid during fermentation of glucose can be used to distinguish between *Leuconostoc* and most heterofermentative lactobacilli, as the former produce only D-lactic acid and the latter a racemate (DL-lactic acid).

2.2. *Lactobacillus* spp.

2.2.1 Historical background of *Lactobacillus*

The genus *Lactobacillus* is by far the largest of the genera included in lactic acid bacteria. It is heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. The heterogeneity is reflected by the range of mol% G+C of the DNA of species included in the genus. This range is 32-53%, which is twice the span usually accepted for a single genus (Schleifer and Stackebrandt, 1983). The heterogeneity and the large number of species are due to definition of the genus, which essentially are rod-shaped lactic acid bacteria. Such a definition is comparable to an arrangement where the entire coccoid lactic acid bacteria were included in one genus.

Table 2.1. Arrangement of the genus *Lactobacillus* (Kandler and Weiss, 1986) which summarizes the characters used to distinguish among the three groups and some of the more well-known species included in each group. The physiological basis for the division is (generally) the presence or absence of the key enzymes of homo- and heterofermentative sugar metabolism, fructose-1,6-diphosphate aldolase and phosphoketolase, respectively (Kandler, 1983, 1984; Kandler and Weiss, 1986).

	Group I:	Group II:	Group III:
Characteristic	Obligately	Facultatively	Obligately
	Homofermentative	Heterofermentative	Heterofermentative
Pantose fermentation	-	+	+
CO ₂ from glucose	-	-	+
CO ₂ from gluconate	-	+ ^a	+ ^a
FDP aldolase present	+	+	-
Phosphoketolase present	-	+ ^b	+
	<i>L. acidophilus</i>	<i>L. casei</i>	<i>L. brevis</i>
	<i>L. delbruckii</i>	<i>L. curvatus</i>	<i>L. buchneri</i>
	<i>L. helveticus</i>	<i>L. plantarum</i>	<i>L. fermentum</i>
	<i>L. salivarius</i>	<i>L. sake</i>	<i>L. reuteri</i>

a. when fermented, b. inducible by pentoses

Lactobacilli are widespread in nature, and many species have found applications in the food industry. They are Gram-positive, non-spore-forming, rods or coccobacilli with a G + C content of DNA usually <50 mol %. Lactic acid bacteria due to their higher molecular percentage of G + C contents in DNA are placed in the Actinomycete branch as presented, while other lactic acid bacteria are placed in the *Clostridium* branch.

Tables 1.2 compares the G + C contents in DNA of several genus of lactic acid bacteria and phylogenetic relationship of lactic acid bacteria according to G + C mol percent content in DNA, respectively.

Table 2.2. Phylogenetic relationship of lactic acid bacteria based on the mol percent of G + C content in DNA (Salminen and von Wright, 1998)

Mol % of G + C content in DNA	
Clostridium (<50)	Actinomycete (> 50)
<i>Lactobacillus</i>	<i>Bifidobacterium</i>
<i>Lactococcus</i>	<i>Propionibacterium</i>
<i>Enterococcus</i>	<i>Microbacterium</i>
<i>Leuconostoc</i>	<i>Corynebacterium</i>
<i>Pediococcus</i>	<i>Brevibacterium</i>
<i>Streptococcus</i>	<i>Atrophobium</i>
<i>Staphylococcus aureus</i>	
<i>Bacillus subtilis</i>	

Lactobacilli are strictly fermentative, aero-tolerant or anaerobic, aciduric or acidophilic and have complex nutritional requirements (e.g. for carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins) and do not synthesize porphyrinoids and thus, are devoid of hemedependent activities.

Lactobacilli are found in where rich, carbohydrates-containing substrate are available, and thus, in a variety of habitats such as mucosal membranes of humans and animals, (mainly in oral cavity, intestine, and vagina) and on plant material and fermenting food (Hammes *et al.*, 1991; Pot *et al.*, 1994).

2.2.2 Grouping of *Lactobacillus*

The primary interest of Orla-Jensen's (1919) early description of the lactic acid bacteria was directed to identify these bacteria useful in the dairy industry, with the particular interest in the study of those bacteria occurring in Danish 'dairy cheese'. Orla-Jensen recognized 10 species in his time. This number increased only slowly to 15 and 25 species, in the 7th and 8th editions of Bergey's Manual respectively. Finally 44 species have been recognized in the latest 9th edition of Bergey's Manual. The numbers of species are still

increasing due to emerging new taxonomic methods, which allow a more precise identification of strains isolated some time ago and, to some extent, from the continued investigation of habitats.

The latest grouping of lactobacilli by Kandler and Weiss (1986) relies on biochemical-physiological criteria and neglects classical criteria of Orla-Jensen such as morphology and growth temperature since many of recently described species did not fit into the traditional classification scheme. Unfortunately, the description of new species usually does not include the analysis of the end products derived from the fermentation of pentoses, and therefore, the enzymes of the pentose phosphate pathway may be present permitting a homofermentative metabolism of pentose in lactobacilli. Nevertheless, maintaining the traditional terms is justified with regards to hexose utilization. However, at low substrate concentration and under strictly anaerobic conditions, some facultatively heterofermentative species may produce acetate, ethanol and formate instead of lactate from pyruvate. Thus, the definitions have to be used in awareness of their limitations.

When glucose is used as a carbon source, lactobacilli could be homofermentative or heterofermentative. When homofermentative, they could produce more than 85% lactic acid, whereas, the heterofermentative strains produce lactic acid, carbon dioxide, ethanol or acetic acid. In the presence of oxygen or other oxidants, increased amounts of acetate may be produced at the expense of lactate or ethanol. A total of 56 species of lactobacilli have been divided into three metabolic groups (Hammes and Vogel, 1995).

Group A: *Obligatory homofermentative lactobacilli:* Hexoses are fermented to lactic acid by EMP (Embden-Meyerhof pathway) pathway. Pentose or gluconate are not fermented.

Group B: *Facultatively heterofermentative lactobacilli:* Hexoses are fermented to lactic acid by EMP (Embden-Meyerhof pathway) pathway. The organisms possess both

aldolase and phosphoketolase and therefore, not only ferment hexose but also pentoses (and often gluconate).

Group C: *Obligatory heterofermentative lactobacilli*: the phosphogluconate pathway ferments hexoses, yielding lactate, acetic acid (ethanol) and CO₂ in equimolar amounts. Pentose enters in this pathway and may be fermented.

Within these three groups the species are arranged according to their phylogenetic relationship. Thus, the combination of the letter “Aa” defines a species belonging to the obligatory homofermentative lactobacilli affiliated in the *L. delbrueckii* group, whereas “Cb” means that the species is obligatory heterofermentative phylogenetically belonging to the *L. casei-Pediococcus* group.

L. casei species belonging to the group facultatively heterofermentative organism comes under group B. Two species, *L. acetotolerans* and *L. hamsteri* constitute group “Ba”, means that phylogenetically these organisms fall into the *L. delbrueckii* group. The presence of the Lys-Dasp type peptidoglycan is consistent with this grouping.

Group “Bb” contains 15 species, 12 of which contain Lys-Dasp and three DAP in their peptidoglycan. In contrast to Kandler and Weiss (1986), Hammes and Vogel (1995) have included into group Bb *L. bif fermentans* since, in agreement with the group definition; this organism possesses key enzymes, aldolase and phosphoketolase. *L. bif fermentans* is characterized by fermenting glucose homofermentatively. However, dependent on the pH, lactate can be metabolized to ethanol, acetic acid and CO₂ and H₂. The utilization of lactate (and/or pyruvate) is rather common for group Bb-organisms.

2.2.3 Description of the species

All cells are Gram-positive and non-spore-forming, usually catalase-negative, non-motile, and facultatively anaerobic unless otherwise stated. The grouping of the species

together with the patterns of sugar fermentation and important physiological properties are given in Table 1.1

2.2.3.1 *Lactobacillus casei* belongs to group Bb. Cells are rods of 0.7 – 1.1 by 2.0 – 4.0 µm, often with square end and tending to form chains. Riboflavin, folic acid, calcium pantothenate and niacin are required for growth whereas pyridoxal or pyridoxamine is essential for stimulation. Thiamine, vitamin B12 and thymidine are not required. The strains were isolated from milk and cheese, dairy products and dairy environments, sour dough, cow dung, silage, human intestinal tract, mouth and vagina, sewage and the strain type is ATCC 393. (Orla-Jensen, 1919; Hansen and Lessel, 1971)

2.2.3.2 *Lactobacillus paracasei* belongs to group Bb. Cells are rod shaped, 0.8-1.0 by 2.0-4.0 µm, often with square ends, and occur singly or in chains. Although the favourable temperature for its growth is 10°C and 40°C but some strains can grow at 5 and 45°C. A few strains (formerly *Lb. casei* subsp. *pseudoplantarum*) produce inactive lactic acid due to the activity of L-lactic acid racemase. Two subspecies are validly published under *L. paracasei* (Collins *et al.*, 1989).

Lactobacillus paracasei subsp. *paracasei* and *Lactobacillus paracasei* subsp. *tolerans*. Strains were isolated from dairy products, sewage, silage, humans, and clinical sources (Collins *et al.*, 1989).

2.2.3.3 *Lactobacillus rhamnosus* belong to Group Bb. Cells are rod shaped, 0.8-1.0 by 2.0-4.0 µm, often with square ends, and occur singly or in chains. Some strains grow at 48° C. The strains were isolated from dairy products, sewage, humans, and clinical sources. The type strain is ATCC 7469 (Collins *et al.*, 1989).

2.2.3.4 *Lactobacillus helveticus* are placed in Group Bb (Orla- Jensen 1919), rod paired bacilli, No growth at 15° or below, Homofermentative. Ferments glucose, fructose, galactose,

mannose, maltose, lactose, dextrin. Does not ferment mannitol, sucrose, salicin, glycerol, inositol, rhamnose, arabinose, xylose, raffinose, inulin, sorbitol, amygdalin, cellobiose, melibiose, melezitose.

2.2.4 Taxonomic diversity of *Lactobacillus*

The human gastrointestinal tract contains hundreds of different bacterial species (Tannock, 1995). Members of the genus *Lactobacillus* are commonly present and have received considerable attention with respect to their putative health conferring properties as probiotics (Goldin and Gorbach, 1992). *Lactobacillus* has worldwide industrial use as starters in the manufacturing of fermented milk products. Moreover, some of *Lactobacillus* strains have probiotic characteristics and are therefore included in fresh fermented products or used in capsular health products, such as freeze-dried powder. The use of some *Lactobacillus* strains as probiotics is based on studies shows that these species belong to the normal intestinal flora and the strains have beneficial effects on human and animal health (Salminen *et al.*, 1996).

Major bacterial species isolated from human gastrointestinal tract fall generally into three distinct categories. These include 1) organisms almost always present in large number, and constituting the indigenous and resident flora, e.g. *Bacteroides*, *Bifidobacterium*; 2) organisms normally present in small or moderate numbers, and part of the resident flora, e.g. Enterobacteriaceae, *Streptococcus* and *Lactobacillus*; and 3) organisms present in small numbers, probably contaminants from other regions of the body e.g. *Staphylococcus*, *Haemophilus*, etc., or from the environment, e.g. *Bacillus*, *Corynebacterium*, which constitute transient flora.

More specifically, organisms of the human gastrointestinal tract include diverse bacterial genera or families, and are divided into the following three groups: 1) Lactic acid

bacteria in a broad sense, including *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* (including *Enterococcus*); 2) Anaerobic group, including *Bacteriodaceae*, *Eubacterium*, *Peptococcaceae*, *Veillonella*, *Megasphaera*, *Hemmiger*, *Clostridium* and *Treponema*; and 3) Aerobic group, including *Enterobacteriaceae*, *Staphylococcus*, *Bacillus*, *Corynebacterium*, *Pseudomonas* and yeasts.

2.2.5 Species-specific identification of *Lactobacillus* spp.

Traditionally, the identification of *Lactobacillus* has been based mainly on fermentation of carbohydrates, morphology, and Gram staining, and these methods are still used. The identification of *Lactobacillus* isolates by phenotypic methods is difficult because in several cases it requires the determination of bacterial properties beyond those of the common fermentation tests (for example, cell wall analysis and electrophoretic mobility of lactate dehydrogenase) (Kandler and Weiss, 1986). In general about 17 phenotypic tests are required to identify a *Lactobacillus* isolate accurately to the species level (Hammes and Vogel, 1995). In recent years, the taxonomy has changed considerably with the increasing knowledge of genomic structure and phylogenetic relationships between *Lactobacillus* spp. (Klein *et al.*, 1998).

Significant advances have been made in bacterial taxonomy of indigenous intestinal bacteria during the past 20 years. Newly developed research methods such as DNA-DNA homology, rRNA-DNA homology or the guanine-plus- cytosine (G+C) content of DNA have contributed a lot to the advances in bacterial taxonomy, and numerous new taxa of intestinal anaerobes were described. Results of DNA homology are used to indicate relationship among strains, establish genospecies and enable selection of those phenotypic tests that are the most useful for reliable identification of new isolates. Many different types of bacteria representing most bacterial groups have been isolated from the intestine. 30-40 species constitute

approximately 90% of the flora, but in order to be sure of identifying these species some hundred isolates from each sample should be examined. Such bacteria are generally identified on the base of their morphology as determined by Gram stain, fermentation reactions and metabolic tests. While several schemes have been developed for the identification of indigenous anaerobic bacteria, it is still difficult to identify many of these organisms by conventional tests at species level. Differentiation of major intestinal bacterial groups according to Gram-staining, aerobic growth, spore production and major fermentation products are presented below in Table 2.3.

The *Lactobacillus* commonly includes *Lactobacillus casei* and the taxonomically related species *L. paracasei* and *L. rhamnosus*. While this group of lactobacilli can be readily distinguished from other members of the *Lactobacillus* genus by fermentation profiles (Hammes *et al.*, 1992), it is not possible to unequivocally distinguish between these three species on the same basis.

The identification of *L. casei* by polymerase chain reaction (PCR) is important for basic studies and applications in food industries. *L. casei* along with *L. paracasei* and *L. rhamnosus* have very similar physiological properties, nutritional requirements and grow under similar environmental conditions (Mitsuoka, 1992).

Table 2.3. Differentiation of major intestinal bacterial groups (Mitsuoka, 1992).

Bacterial group	Gram-staining	Aerobic growth	Spore production	Major fermentation products
Lactic acid bacteria group				
<i>Lactobacillus</i>	+	+	-	Lactic acid
<i>Bifidobacterium</i>	+	-	-	Acetic acid+ lactic acid
<i>Streptococcus</i>	+	+	-	Lactic acid
Anaerobic group				
<i>Bacteroidaceae</i>	-	-	-	Various products
Anaerobic curved rods	-	-	-	Succinic acid, butyric acid
<i>Eubacterium</i>	+	-	-	Various products
<i>Peptococcaceae</i>	+	-	-	Various products
<i>Veillonella</i>	-	-	-	Acetic acid + propionic acid
<i>Megasphaera</i>	-	-	-	Caproic acid + butyric acid
<i>Gemmiger</i>	-	-	-	
<i>Clostridium</i>	+/-	-	+	Various products
<i>Treponema</i>	-	-	-	
Aerobic group				
<i>Enterobacteriaceae</i>	-	+	-	
<i>Staphylococcus</i>	+	+	-	
<i>Bacillus</i>	+	+	+	
<i>Corynebacterium</i>	+	+	-	
<i>Pseudomonas</i>	-	+	-	
<i>Yeasts</i>	+	+	-	

2.3 Probiotics

One manner in which modulation of the gut microbiota composition has been attempted is through the use of live microbial dietary additions, as probiotics. The word probiotic is translated from the Greek meaning ‘for life’. An early definition of probiotic was given by Parker (1974) as: ‘Organisms and substances which contribute to intestinal microbial balance.’ However, this was subsequently refined by Fuller (1989) as: ‘a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.’ This latter version is the most widely used definition and has

gained widespread scientific acceptability. A probiotic would therefore incorporate living microorganisms, seen as beneficial for gut health, into diet.

Probiotics has a long history. In fact, the first records of intake of bacterial drinks by humans are over 2000 years old. However, at the beginning of this century probiotics were first put onto a scientific basis by the work of Metchnikoff at the Pasteur Institute in Paris. Metchnikoff (1907) observed longevity in Bulgarian peasants and associated this with their elevated intake of soured milks. During these studies, he hypothesized that the normal gut microflora could exert adverse effects on the host and that consumption of certain bacteria could reverse this effect. Metchnikoff refined the treatment by using pure cultures of what is now called *Lactobacillus delbruckeii* subsp. *bulgaricus*, which, with *Streptococcus salivarius* subsp. *thermophilus*, is used to ferment milk in the production of traditional yoghurt.

Subsequent research has been directed towards the use of intestinal isolates of bacteria as probiotics (Fernandes *et al.*, 1987). Over the years many species of micro-organisms have been used. They mainly consist of lactic acid producing bacteria (lactobacilli, streptococci, enterococci, lactococci, bifidobacteria) but also *Bacillus* spp. and fungi such as *Saccharomyces* spp. and *Aspergillus* spp.

Despite the very widespread use of probiotics, the approach may have some difficulties. The bacteria used are usually anaerobic and do not relish extremes of temperature. To be effective, probiotic must be amenable to preparation in a viable form at a large scale. During use and under storage the probiotic should remain viable and stable, and be able to survive in the intestinal ecosystem, and the host animal should gain beneficially from harbouring the probiotic. It is therefore proposed that the exogenous bacteria reach the intestine in an intact and viable form, and establish there and exert their advantageous properties. In order to do so, microbes must overcome a number of physical and chemical

barriers in the gastrointestinal tract. These include gastric acidity and bile acid secretion. Moreover, on reaching the colon the probiotics may be in some sort of stressed state that would probably compromise chances of survival.

2.3.1 Quality parameters for probiotics for being effective in nutritional and therapeutic settings

A probiotic can be used exogenously or endogenously to enhance nutritional status and/or the health of the host. In the case of exogenous use, microorganisms are most commonly used to ferment various foods and by this process can preserve and make bioavailability of nutrients. In addition, microorganisms can metabolize sugars, such as lactose in yoghurt, making yoghurt more acceptable for consumption by individuals suffering from lactose intolerance. However, the most interesting properties that exogenously acting probiotics can have are the production of substances that may be antibiotics, anticarcinogens or have other pharmaceutical properties. The properties required for exogenously derived benefits from probiotics are the ability to grow in the food or the media in which the organism is placed, and the specific metabolic properties which result in the potential beneficial effects stated above. The selection of organisms that can be helpful therapeutically and nutritionally would be based on specific properties that are desired.

This can be achieved by either classical biological selection techniques or genetic engineering. Probiotics that are ingested by the host and exert their favourable properties by virtue of residing in the gastrointestinal tract need to have certain properties in order to exert an effect.

2.3.2 Requirements for probiotics

It is of great importance that the probiotic strain should survive in the location where it is presumed to be active. For a longer and perhaps higher activity, it is necessary that the

strain should proliferate and colonize on the specific location. Probably only host-specific microbial strains are able to compete with the indigenous micro flora and to colonize the niches. Besides, the probiotic strain must be tolerated by the immune system and not provoke the formation of antibodies against the probiotic strain. So, the host must be immuno-tolerant to the probiotic. On the other hand, the probiotic strain can act as an adjuvant and stimulate the immune system against pathogenic microorganisms. It goes without saying that a probiotic has to be harmless to the host: there must be no local or general pathogenic, allergic or mutagenic/carcinogenic reactions provoked by the microorganism itself, its fermentation products or its cell components after decrease of the bacteria.

For the maintenance of its favourable properties the strain must be genetically stable. For the production of probiotics it is important that the microorganisms multiply rapidly and densely on relatively cheap nutrients and remain viable during processing and storage. Besides the specific beneficial property, these general requirements must be considered in developing new probiotics, also for determining the scientific value of a claimed probiotic. A number of these requirements can be screened during *in vitro* experiments. It is advised for drawing up of a decision-tree for the minimal requirements which can be tested *in vitro*, such as culture conditions and viability of the probiotic strains during processing and storage; sensitivity to low pH values, gastric juice, bile, pancreas, intestinal juice and intestinal or respiratory mucus; adherence to isolated cells or cell cultures and interactions with other (pathogenic) microorganisms. If these *in vitro* experiments are successful, further research can be performed during *in vivo* experiments in animals or humans. Requirements of some basic quality parameters of probiotics for their use in humans are presented in Table 1.4.

Table 2.4. Requirements of probiotics (Salminen and von Wright, 1998)

✓	Proliferation and/or colonisation on the location where it is active
✓	No immune reaction against the probiotic strain
✓	No pathogenic, toxic, allergic, mutagenic or carcinogenic reaction by the probiotic strain itself, its fermentation products or its cell components after decrease of the bacteria
✓	Genetically stable, no plasmid transfer
✓	Easy and reproducible production
✓	Viable during processing and storage
✓	Survival of the environmental conditions on the location where it must be active

2.3.2.1 Viability of probiotic organisms

Microorganisms introduced orally have to at least transiently survive in the stomach and small intestine. Although this appears to be a rather minimal requirement, many bacteria including the yoghurt-producing bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* often do not survive to reach the lower small intestine. The reason for this appears to be low pH of the stomach. In fasting individuals, the pH of the stomach is between 1.0 and 2.0 and most microorganisms, including lactobacilli, can only survive from 30 seconds to several minutes under these conditions. Therefore, in order for a probiotic to be effective, even the selection of strains that can survive in acid at pH 3.0 for sometime would have to be introduced in a buffered system such as milk, yoghurt or other food.

2.3.2.2 Acid and bile tolerance

One of the most important criteria for selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive in the bile concentrations encountered in the intestine. Lankaputhra and Shah (1995) showed that, among several strains of *L. acidophilus* and *Bifidobacterium* sp. studied, only a few strains

survived under the acidic conditions and bile concentrations normally encountered in fermented products and in the gastrointestinal tract, respectively. Therefore, it cannot be generalized that all probiotic strains are acid and bile tolerant. Lankaputhra and Shah (1995) showed that *Bifidobacterium longum* survives better in acidic conditions and is able to tolerate a bile concentration as high as 4%. Acid and bile tolerance is strain dependent, and care should be taken to select strains based on these attributes.

2.3.2.3 Adherence of probiotic bacteria

It is not clear if the adhesion to the intestinal epithelium is essential for the persistence of a probiotic in the human intestinal tract. However, adhesion seems to be a property that enhances long-term survival. The ability of microorganisms to adhere to epithelial cells is to a large extent species specific, although this may be relative. Screening of organisms for their ability to survive in the human gastrointestinal tract is not difficult. The selection of human bacterial isolates will enhance the possibility of finding organisms that will survive. The isolates can then be tested by administering orally between 10^9 and 10^{11} viable organism in a single dose with an appropriate buffering agent and the bacterial counts of the specific organism are then measured in the faeces over a several week period. This technique is most successful if the natural flora does not contain the organism being tested or only in small numbers. The first question of transient survival can be established in 48 to 96 h. The evaluation of the ability of the organism to permanently establish in the gastrointestinal tract, by proliferation, can be established by continuous appearance in the faeces over several weeks to several months. The faecal counts should exceed 10^6 /g of faeces. The application of this screen for selecting probiotics should be encouraged in the future. There are several tests for determining if a prospective probiotic can bind to intestinal epithelium. Radiolabelling the microorganisms with an amino acid and then counting for adhering radioactivity in either

ileal cells recovered from ileostroma effluent or from buccal cells obtained by gently scraping the inside of the cheek are effective methods. Good adhesion properties should enhance the possibility of long-term survival of the organism in the intestinal tract by countering the peristaltic action of the intestine. Blum *et al.*, 1999; Saarela *et al.*, 2000).

2.3.2.4 Specific Site of action of probiotics: the small intestine

The small intestine is a long tube of about 3-5 m in length; it begins with the duodenum, continues with the jejunum and ends with the ileum. Its main functions are digestion of food and absorption of water, electrolytes and nutrients necessary for cellular growth of the whole organism. In order to assure a maximal contact with the digested food in a limited place, the intestine presents different surface-increasing strategies: circular folds, intestinal villi and microvilli. This makes the gut mucosa (with a surface of approximately 200 m²) the largest area of the body in contact with the environment (Holzapfel *et al.*, 1998). The intestinal epithelium is a highly organized, single-cell layer covering the interface between tissues and the intestinal lumen. This monolayer is mainly constituted of enterocytes, which are the cells responsible for taking up nutrients, Paneth cells, which secrete the mucus bathing the epithelium, and intra-epithelial lymphocytes, which are part of the MALT. Yet, all epithelial cells arise from common non-differentiated precursors present in the epithelium (Brandtzaeg, 1995). This monolayer is constantly being renewed as epithelial cells undergo a lifecycle, which starts in the deep of the crypts, where they arise, continues with their differentiation and migration towards the tip of the villi and ends with apoptosis and exfoliation (Stadnyk, 1994; Turner, 2003; Dommett *et al.*, 2005). This cycle takes about 3 to 5 days in humans and allows epithelial self-renewal. Because of this turn over, the gut surface is covered by dead and exfoliating cells, which provide together with the mucus, bathing the

cells, and the nutrients passing through the lumen an excellent growth substrate for microorganisms (Stadnyk, 2002; Tlaskalova-Hogenova *et al.*, 2005).

So far, it was shown that the indigenous microflora is host-specific, location-specific, very complex in composition and it has beneficial properties to the host. However, it is not precisely known which species of microorganisms play the principal part in these beneficial properties. For man it is suggested that specific microbial strains could play an important role in;

- formation or reconstruction of a well-balanced indigenous intestinal and/or respiratory microflora, for example, in newborn children during admission to an ICU;
- after gastrointestinal decontamination by antibiotics in connection with bone-marrow transplantation;
- improving the colonization resistance of the indigenous microflora of the intestinal, respiratory and urogenital tracts;
- lowering the serum cholesterol level;
- inhibiting the mutagenicity of the intestinal contents and reducing the incidence of intestinal tumours; non-specific interactions with the immune system;
- metabolizing lactose and hence reducing lactose intolerance
- improving the absorption of calcium and hence inhibiting decalcification of the bones in elderly people;
- synthesis of vitamins and pre-digestion of proteins.

Probiotic foods are becoming increasingly popular. A number of health benefits have been claimed for *Bifidobacterium* sp. and therefore inclusion of these organisms in the diet is considered to be important in maintaining good health (Champagne *et al.*, 1996). Probiotics

have anticarcinogenic properties, a specific probiotic effect, which are of three types: (1) elimination of procarcinogens; (2) modulation of procarcinogenic enzymes; and (3) tumour suppression (Wollowski *et al.*, 2001). Furthermore, consumption of these organisms is an ideal method to re-establish the balance in the intestinal flora after antibiotic treatment (Gibson *et al.*, 1994). There is a growing agreement relating to the beneficial aspects of specific dairy products such as fermented milk, yoghurt and bacterial cultures that ferment the dairy products in human and animal nutrition. Experimental and epidemiological studies provide evidence that fermented milk and bacterial cultures that are routinely used to ferment the milk reduce the risk of certain types of cancer and inhibit the growth of certain tumours and tumour cells (Reddy and Rivenson, 1993).

Many health promoting effects have been attributed to certain *Bifidobacterium* sp. (Rolfe, 2000). These include reduction of ammonia levels, stimulation of the immune system, alleviation of lactose intolerance and prevention of gastrointestinal disorders (O'Sullivan, 1996). Several probiotic bacteria have been introduced in the market and the range of products in which probiotic bacteria are added is increasing. However, many of the prophylactic and therapeutic properties of these foods containing bifidobacteria are a matter of speculation because there are inherent difficulties in obtaining definitive evidence for proposed effects of ingesting bifidobacteria.

2.3.2.5 Colonization resistance

The indigenous microflora on body surface inhibits the colonization of non-indigenous microorganisms. Nevertheless, in some cases (potential) pathogenic microorganisms are able to penetrate and/or colonize these body surfaces, due to a massive attack of the pathogens or to a (temporarily) reduced colonization resistance. In different studies on humans and animals beneficial microorganisms are used to improve the

colonization resistance on body surfaces, such as gastrointestinal, the urogenital, and the respiratory tract.

2.3.2.6 Safety considerations

Even though *Lactobacillus* spp. belong to risk group 1 organisms (European Food Safety Authority, 2004), which include biological agents that are unlikely to cause human or animal disease, it is important to assess the safety of those microorganisms intended for use as food additives. Because of the serious concerns about the increasing level of resistance to antibiotics in regular use in human medicine, one of the aspects which need to be analyzed is antibiotic resistance. Probiotic strains as well as bacteria used in food fermentations may harbour resistant genes, which can be transferred to pathogenic bacteria (Havenaar *et al.*, 1992; Danielsen and Wind, 2003; Franz *et al.*, 2005). Therefore, probiotic strains intended for the market should be screened for transferable resistance genes. According to the report by the Scientific Committee for Animal Nutrition (SCAN) recommended to the European Union commission (Teuber, 1999), the absence of transferable resistance genes should be considered as an important pre-requisite for approval of probiotics.

2.3.2.7 Anticarcinogenic properties

In the last two decades, the number of people suffering from colon cancer has been gradually increasing, particularly in industrialized countries (Moore and Moore, 1995). Studies by Goldin and Gorbach (1981, 1984a) have indicated that diet and antibiotics can lower the generation of carcinogens in the colon and reduce chemically induced tumours. These effects appear to be mediated through the intestinal microflora. Additional studies have shown that the introduction of *L. acidophilus* into the diet lowered the incidence of chemically induced colon tumours in rats (Goldin and Gorbach, 1980). A possible mechanism for these anticancer effects relies on inhibiting intestinal bacterial enzymes that

convert procarcinogens to more proximal carcinogens. This technique can be expanded in the future by testing probiotics for their ability to inhibit the growth of organisms normally found in the flora that have high activities of enzymes such as β -glucuronidase (Reddy *et al.*, 1974), nitroreductase, azoreductase and β -glycosidase or the capability for nitrosation. The ability of probiotics to deactivate faecal mutagens can also be a marker used to introduce organisms that lower cancer risk.

2.3.2.8 Immunological enhancement

In recent years there have been several reports indicating that lactobacilli used in dairy products can enhance the immune response of the host. Organisms that have been identified as having this property are *Bifidobacterium longum*, *L. acidophilus*, *L. casei* subsp. *rhamnosum* and *L. helveticus* (Isolauri *et al.*, 2001b). In the future, prospective probiotics in the appropriate settings (anticancer or infection resistance) should be tested for enhancement of the immunological response. The measurements that should be considered are lymphocyte proliferation, interleukin 1, 2 and 6, tumour necrosis factor, prostaglandin E production and serum total protein, albumin, globulin and gamma interferon.

2.3.2.9 Cholesterol lowering

Experiments by Gilliland *et al.* (1985) have shown that dietary elevation of plasma cholesterol levels in pigs can be prevented by introduction of a *L. acidophilus* strain that is bile resistant and assimilates cholesterol. These findings were supported by research

conducted by Pereira and Gibson (2002) who demonstrated that probiotic strains were able to assimilate cholesterol in the presence of bile into their cellular membranes. Results however, were influenced greatly by the bacterial growth stage and inoculum used as resting cells did not interact with cholesterol as also shown by studies conducted by Dambekodi and Gilliland (1998). St-Onge *et al.* (2000) extensively reviewed the existing studies from animal

and human studies which detected moderate cholesterol-lowering was due to consumption of fermented products containing probiotic bacteria. Studies by Gopal *et al.* (1996) also showed cholesterol removal by *Bifidobacterium* spp. and *Lactobacillus acidophilus*.

2.3.2.10 Production of hormones and other agents

The possibility of genetically engineering strains of bacteria that can produce substances such as insulin, androgens, estrogens, growth hormone or cholesterol-lowering compounds, just to mention, a few is intriguing. The ability to produce *in situ* over a long period of time drugs or hormones that are constantly required by individuals suffering from various diseases (i.e. diabetes and hypercholesteremia) is of particular interest. These are problems to this approach, like control of production and contamination of normal individuals with the organism. Establishing the maximum achievable production level of the organism in the gut and thereby setting an upper limit on dose may solve the first problem. The contamination problem may be more difficult to solve, although antibiotic sensitivity can be introduced into the strains, so that the organism could be rapidly eliminated if a normal individual is infected with a specifically designed probiotic. This idea may have too many regulatory problems associated with it; however, it is still something that may have potential use in human disease regulation.

2.3.3 Claimed beneficial properties of probiotics

Probiotic strains have beneficial effects on the host by controlling undesirable micro-organisms and by modulating the immune system (Fuller and Perdigon, 2003). However, despite the efforts to elucidate their mechanism of action, it is still not well understood how probiotics work (Tannock, 2002). There are several postulated mechanisms through which probiotics exert their beneficial effects in the host (Fig. 1.1). Most of the mechanisms have been studied *in vitro* because of the complexity of the gut ecosystem and the numerous

interactions taking place in the gut (bacteria-bacteria, nutrients-bacteria, nutrients-epithelium, epithelium-bacteria, epithelium-immune system and bacteria-immune system). Some *in vitro* experiments can be extrapolated to the *in vivo* situation, as shown in several studies that compare *in vitro* results with *in vivo* studies (Jacobsen *et al.*, 1999; Cesena *et al.*, 2001). Animal models are also useful tools; nevertheless, they have substantial differences in the anatomy of the gastrointestinal tract and MALT (mucosa associated lymphoid tissue) and in the microbial composition of the gut microbiota, when compared with human beings. In addition, some strains show host-specificity for adherence and exerting their health effects (Morelli, 2000).

The ability to adhere to the intestinal epithelium is one of the main criteria for selecting new probiotic strains, as this property allows strains to remain, at least transiently, in the intestinal tract, and exert their probiotic effects such as excluding pathogenic bacteria by competing for adhesion sites (Alander *et al.*, 1999; Blum *et al.*, 1999; Saarela *et al.*, 2000). Some of the mechanisms through which probiotics may antagonize pathogens include production of antimicrobial compounds such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins (Alakomi *et al.*, 2000; Annuk *et al.*, 2003). Other functional properties to characterize probiotics are their ability to modulate immune responses and to adhere to gut tissues (Holzapfel and Schillinger, 2002) (Fig. 1.1).

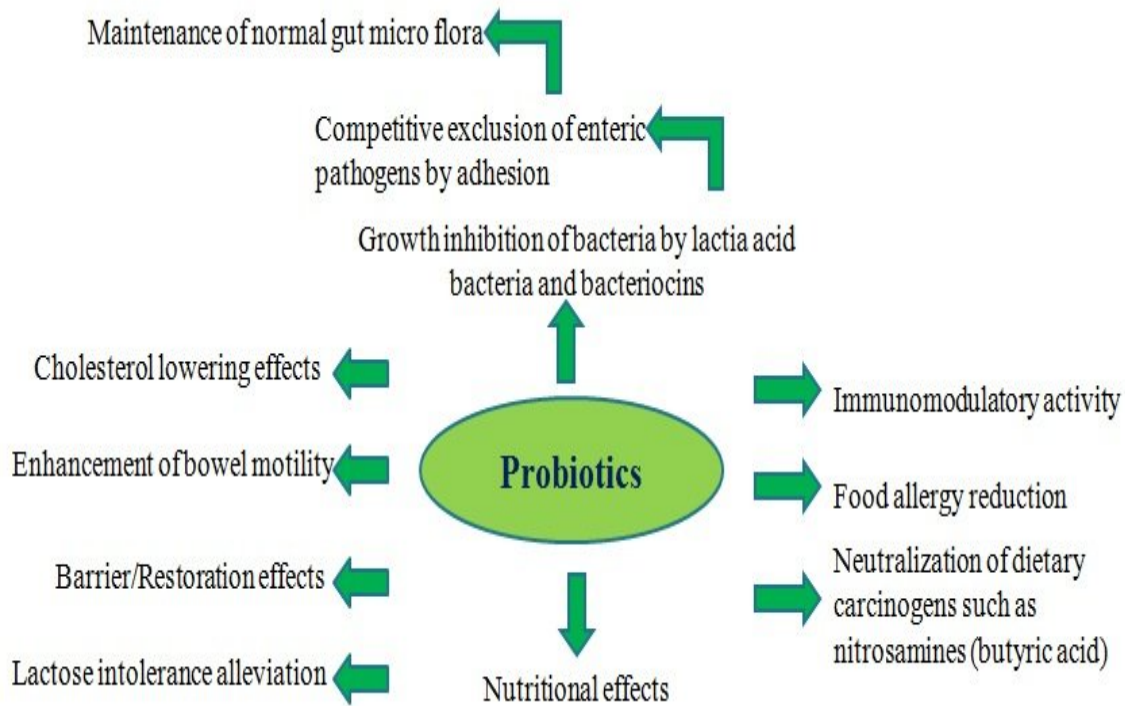


Fig. 2.1 Postulated mechanisms of action of probiotics

Lactic acid bacteria have been shown to reduce the symptoms of lactose intolerance. Lactose, a disaccharide composed of glucose and galactose, is the main sugar present in milk and needs to be cleaved to the monosaccharides in order to be absorbed in the small intestine. This enzymatic hydrolysis is catalysed by lactase (β -galactosidase), an enzyme present in the brush border of the enterocytes in childhood. Some adults still express this enzyme and can benefit from milk as a protein and calcium source, but the expression of this enzyme generally decreases with age and is, in some cases, completely lost (Szilagyi, 2002). In individuals with low lactase contents, lactose cannot be absorbed and it can be used as fermentable substrate by the intestinal microbiota. In addition, water accumulates in the intestinal lumen due to lactose osmotic properties. As a result, the patient may suffer from bloating, flatulence, pain, nausea and even diarrhoea (Hove *et al.*, 1999; de Vrese *et al.*,

2000). The consumption of yogurt and fermented milk containing lactic acid bacteria has been proven to alleviate lactose indigestion symptoms because of the lower lactose content, and its hydrolysis by microbial β -galactosidase (Gilliland and Kim, 1984; Mustapha *et al.*, 1997). This enzyme is sensitive to low pH and, therefore, bacteria surviving low pH protect the enzyme from activity loss until it reaches its site of action, which is the small intestine (Zarate *et al.*, 2000).

On the other hand, bacteria sensitive to bile salts present a membrane with increased permeability, which allows two events which may or may not be exclusive: one is the transport of the substrate at higher rates into the bacterial cell, and the other one is the increased release of the bacterial enzyme into the lumen. Probiotic bacteria, with higher resistance to bile salts, release less amounts of enzyme into the lumen. Therefore, yogurt starter cultures which do not survive the effect of bile, seem to be more effective in alleviation of lactose intolerance (de Vrese *et al.*, 2000).

Another desirable property of some probiotic strains is their ability to reduce cholesterol levels (De Smet *et al.*, 1998; du Toit *et al.*, 1998). This property has been partly related to bile salt hydrolase (BSH), which deconjugates bile salts by releasing the amino acid (taurine or glycine) bound to the side chain of the steroid core. Deconjugated bile salts are less soluble, and thus easily excreted via faeces, resulting in decreased reabsorption and recirculation of bile salts into the liver. Consequently, more cholesterol is needed for *de novo* synthesis of bile salts (Usman and Hosono, 1999). BSH activity has also been related to the ability of some bacteria to survive bile and colonize the intestine (De Smet *et al.*, 1995; Klaenhammer and Kullen, 1999), but this hypothesis is controversial because others have found that deconjugated salts are more toxic than their conjugated counterparts (Grill *et al.*,

2000). In humans, excess bile deconjugation may lead to adverse effects such as steatorrhea and formation of secondary bile salts, which are toxic and/or mutagenic (Marteau *et al.*, 1995).

Probiotics have also been shown to modulate the immune system at different levels. They may have anti- and/or pro-inflammatory properties. Some strains have shown to influence the adaptive immunity, whereas others affect the innate immunity (Fuller and Perdigon, 2000).

2.3.4 New probiotic strains and sources of isolation

There is still no consensus about the need for viability of probiotics to exert health effects (Gopal *et al.*, 2001). Some studies maintain that the viability of probiotic strains is necessary for stimulation of the gut associated immune system (Lammers *et al.*, 2002) or for anti-genotoxic effects (Galdeano and Perdigon, 2004; Ma *et al.*, 2004). Others have shown that heat-killed probiotics (Pool-Zobel *et al.*, 1996), or specific components derived from probiotic strains such as DNA (Nagy *et al.*, 2005) or bacterial cell wall (Lammers *et al.*, 2002; Jijon *et al.*, 2004) are responsible for some immunomodulatory effects. If this is the case, and live microorganisms are not necessary to obtain the desired effects, a revision of the definition of probiotics or the development of a new concept defining these probiotic-derived components will be needed.

A general agreement among those in favor of the classical definition of probiotics, refers to the need of live microorganisms exerting health-promoting effects, and thus, the need of survival in host conditions to reach the site of action. The general criteria for selection of strains to be used as probiotics include: safety and origin of the bacteria, their tolerance to the hostile conditions of the stomach and small intestine, and their ability to adhere gut epithelial tissue (Davidkova *et al.*, 1992; Tannock, 2002). The first step in the

choice of new microbial strains to be used as probiotics should be their safety and origin. GRAS (generally recognised as safe) microorganisms include *Lactobacillus* spp. and *Bifidobacterium* spp., which are bacteria with a long history of safe use, as they have been consumed by humans for centuries.

Selection of other microorganisms must include expensive and time consuming short- and long-term toxicological studies (Morelli, 2000; Chesson *et al.*, 2002). Havenaar *et al.* (1992) considered that the origin of the strain regarding host species and location specificity play an important role if colonization is essential for achieving the desired effect of the probiotic. In this study, strains from human origin and from traditional fermented food were compared in their *in vitro* probiotic potential. The bacterial isolates screened to be selected as potential probiotics in this study were isolated from Indian traditionally fermented food products and human meconium.

2.4 Antimicrobial properties

As indicated previously, the intestinal microflora is a complex ecosystem. Introducing new organisms into this highly competitive environment is difficult. Thus organisms which can produce such products which inhibit the growth or kill existing organisms in the intestinal milieu have a distinct advantage. The growth media filtrates and sonicates from the bacterial cells of prospective probiotics should be tested for bactericidal and bacteriostatic activity in well-plates against a wide variety of pathogens. The ability of probiotics to establish in the gastrointestinal tract will be enhanced by their ability to eliminate competitors.

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens (Klaenhammer Kullen 1999). Antimicrobial effects of lactic acid bacteria are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide,

diacetyl, low molecular weight antimicrobial substances and bacteriocins (Quwehand and Vesterlund 2004, Çakır 2003). They are active against other bacteria, either in the same species (narrow spectrum) or across genera (broad spectrum) (Klaenhammer, 1988). Producer organisms are immune to their own bacteriocin(s), a property that is mediated by specific immunity proteins (Cotter *et al.*, 2005). Both Gram-negative and Gram-positive bacteria produce small heat-stable bacteriocins, but so far they are found less frequently in Gram-negative bacteria (Diep and Nes, 2002), while within the Gram-positive bacteria group LAB seem to produce a large variety of these compounds (Dridger, Fimland, Hechard, McMullen and Prévost, 2006; Nes and Johnsborg, 2004). Most of the bacteriocin-producing LAB are isolated from endogenous fermented food. It appears that the preservative effect of many LAB is partly due to their bacteriocin production, which is considered to give the producers an advantage in competing with other bacteria sharing the same ecological niches (Diep *et al.*, 2002). Bacteriocins produced by LAB can be categorized into three different classes according to their biochemical and genetic properties (Table 2.1). The present review focuses on class II bacteriocins, since all bacteriocins produced by LAB isolated from wine are classified as class II.

Class II bacteriocins can be divided into three subclasses. Class IIa is the largest group. It has a conserved N-terminal amino acid sequence (YGNGVXC) and displays a high specific activity against the food pathogen *Listeria monocytogenes* (Hechard and Sahl, 2002).

A large variety of LAB belonging to the genera *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Carnobacterium*, and *Leuconostoc* is producing subclass IIa bacteriocins. Subclass IIb includes bacteriocins with two peptides which require the combined activity of both peptides and show very low, if any, bacteriocin activity when tested individually. Moreover, no sequence similarities appear between these complementary peptides. Subclass

IIc bacteriocins are grouped on the fact that their N- and C-termini are covalently linked, resulting in a cyclic structure (Maqueda *et al.*, 2004). Class II bacteriocins are small, heat-stable, cationic and hydrophobic peptides. They are generally very stable at acidic pH and as pH increases, their heat stability decreases. Moreover, bacteriocins are usually sensitive to proteolytic enzymes, such as trypsin, proteinase K and protease (Chen and Hoover, 2003).

Unlike lantibiotics, class II bacteriocins are not subject to extensive post-translational modification, but synthesized as precursor molecules mostly containing a leader peptide of the so called double glycine type (Cotter *et al.*, 2005; Håvarstein, Diep and Nes, 1995).

Table 2.5 Classification scheme of bacteriocins

Class	Characteristics	Sub class	Description	Example	Reference
Class I	Lantibiotics (containing lanthionine and β -lanthionine)	A (1)	Elongated, cationic, membrane active, slight + or - net charge	Bacteriocin Nisin A Nisin Z	Producer <i>Lactococcus lactis</i> Buchmann and Banerjee (1988)
		A (2)	Elongated, cationic, membrane active, highly net charge	Lacticin 481	<i>Lactococcus lactis</i> Piard et al. (1992)
		B	Globular, inhibit enzyme activity	Mersacidin	<i>Bacillus</i> spp. strain HILY -85, 547 2 8 Niu and Neu (1991)
Class II	Small (< 10 kDa), moderate (100 °C) to high (121°C) heat-stable, non-lanthionine-containing membrane active peptides	IIa	antilisterial bacteriocins	pediocin –like	<i>Pediococcus acidilactici</i> PAC 1.0 Marugg et al. (1992)
				Leucocin A	<i>Leuconostoc gelidum</i> UAL 187 Hastings et al. (1991)
				<i>plantarum</i> 423	<i>Lactobacillus</i> Plantaricin 423 Van Reenen et al. (1998)
		IIb	Two-peptide Bacteriocins	Plantaricin EF	<i>Lactobacillus plantarum</i> C11 Moll et al. (1999)
		IIc	Other peptide Bacteriocins	Lactococcin 972	<i>Lactococcus lactis</i> IPLA 972 Martinez et al. (1999)
Class III	Large (>30 kDa) heat-labile proteins			Helveticin J	<i>Lactobacillus helveticus</i> 481 Joerger and Klaenhammer (1990)

source: adapted from drier et al., 2006

The ability of numerous LAB to produce one or more bacteriocin displays an important skill sustained over many generations. Bacteriocin production is advantageous, since these peptides inhibit the growth of bacteria competing for the same ecological niche and the same resources. This is supported by the fact that their inhibition spectrum is mostly narrow and most likely to be effective against related bacteria competing for the same nutrients (Drider *et al.*, 2006). It appears that, by producing several bacteriocins belonging to different classes with different inhibitory spectra, LAB compensates their narrow spectrum. *Lactobacillus plantarum* C11 for example, produces two types of bacteriocins which have different target cell specificities (Anderssen, Diep, Nes, Eijsink and Nissen-Meyer, 1998). Moll *et al.* (1999) demonstrated that plantaricin EF shows high conductivity for monovalent cations, while plantaricin JK is more selective for anions. Consequently, having opposite ion selectivity, plantaricin EF forms pores with cation selectivity and plantaricin JK with anion selectivity. This may also help to overcome the development of resistance mechanisms in target organisms (Eijsink, Axelsson, Diep Dzung, Håvarstein, Holo and Nes, 2002).

Table 2.6 Presents some examples of antimicrobial-producing organisms. (Fuller, 1992)

Probiotic	Compound
<i>Lactobacillus</i> GG	Wide spectrum antibiotic
<i>L. acidophilus</i>	<i>Acidolin, Acidophilin, Lactocidin</i>
<i>L. delbrueckii</i> ssp. <i>bulgaricus</i>	<i>Bulgarican</i>
<i>L. plantarum</i>	<i>Lactolin</i>
<i>L. brevis</i>	<i>Lactobacillin, Lactobrevin</i>
<i>L. reuteri</i>	<i>Reuterin</i>

2.4.1 Mode of action

Although the mode of activity of bacteriocins can differ, the cell envelope is commonly their target. The majority is active by inducing membrane permeabilization. This is reflected by the fact that Class II bacteriocins have an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarization and death (Fig. 2.3), (Cotter *et al.*, 2005). To form the core of the pores, this structure is believed to face with the polar side towards the centre of the channel, while the non-polar side faces the hydrophobic phase of the phospholipid bilayer (Diep *et al.*, 2002).

This creation of pores in the membrane of their target cells results in dissipation of the proton motive force, intracellular ATP depletion and leakage of nutrients and metabolites (Deegan *et al.*, 2006). Moreover, to form a pore, interactions with the cytoplasmic

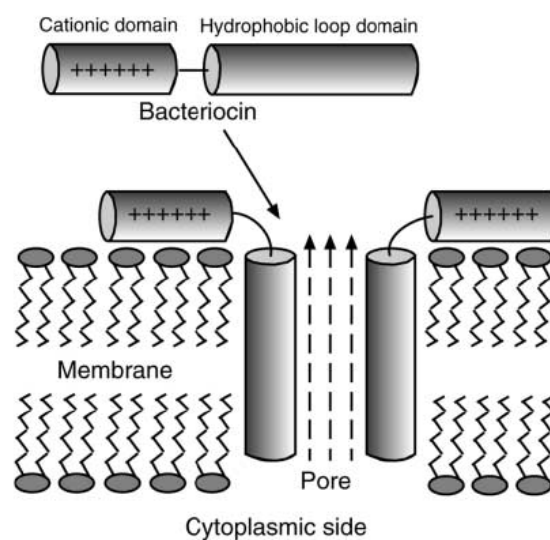


Fig: 2.2 Killing mechanism propose for bacteriocin

Source: Oscariz and Pisabarro, 2001

Initial electrostatic interactions between the positively charged peptide and anionic lipids, which are in large quantities present in the membranes of Gram-positive bacteria, play a role to some extent in this mode of action. Thus, the sensitivity to bacteriocins depends partly on the physiological state of the cell (Eijsink *et al.*, 2002). Up to this stage, it is not entirely clear whether bacteriocins act through receptors in the target cell membrane or if there is specificity in possible receptors.

2.4.2 Application of bacteriocins in food

Most bacteriocin-producing LAB are indigenous food isolates and due to their great potential in food preservation, bacteriocins has been subjected to extensive research during the last few years. The studies show their great potential in biopreservation, for example in dairy products, canned food and alcoholic beverages. Although numerous methods other than bacteriocins are available for the preservation of food and beverages, an increasingly health conscious public are looking for foods that have not undergone extensive processing and contain no chemical preservatives. Bacteriocins are often promoted as potential biopreservatives, but it is generally suggested that these antimicrobial peptides should not primarily be used to prevent the growth of spoilage microorganisms. They rather should be used in addition, to decrease the possibility of spoilage (Deegan *et al.*, 2006). Bacteriocins can be introduced into food to improve its safety in the following ways: i) in fermented food where bacteriocins can be produced in situ by bacterial cultures which can replace either all or part of a starter culture; (ii) purified or semi-purified bacteriocins can alternatively be added directly as an additive; or (iii) an additive based on a fermentate of a bacteriocin-producing strain (Cotter *et al.*, 2005). Incorporating purified bacteriocins might not always be attractive to the food and beverage industry, since in this form bacteriocins may have to be labelled as an additive like other preservatives and regulatory approval might be necessary (Deegan *et al.*, 2006). Several important factors must be considered when screening for a bacteriocin producing strain with potential in food application: the bacteriocin should have a broad spectrum of inhibition and be highly active; it should also be heat-stable, have no associated health risks and it should bring beneficial effects such as improved safety, quality and flavour (Cotter *et al.*, 2005). The physical and chemical properties of the food or beverage can also influence the efficiency and stability of a certain bacteriocin and have to be

considered (Deegan *et al.*, 2006). In case of purified bacteriocins, optimization of yield and kinetics during production must be taken into account in order to make commercial use of bacteriocins cost-effectively. The most broadly studied and commercially available bacteriocin is nisin. It was approved for use as an antimicrobial in food by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1969 which is an international scientific expert committee that is administratively joint by the Food and Agriculture Organisation of the United Nations-FAO and the World Health Organisation-WHO. Moreover, nisin has been given the food additive number E234 (EEC, 1983 EEC commission directive83/463/EEC) and is currently permitted for use in over 50 countries (Delves-Broughton, 2005).

Other bacteriocins, such as pediocins and lacticins, have found applications in various food systems which were reviewed recently by Chen *et al.* (2003) and Deegan *et al.* (2006). Bacteriocins have shown to be effective either added as an ingredient or produced by bacteriocin-producing bacteria strains in the food system (Deegan *et al.*, 2006). While nisin is mostly used in canned food or dairy products, pediocin has the ability to protect fresh and fermented meat. Lacticins have been tested as biopreservatives in natural yoghurt, cottage cheese and infant milk formula. The use of a plantaricin producing starter culture has been demonstrated for the fermentation and preservation of olives (Ruiz -Barba, Cathcart, Warner and Jimenez-Diaz, 1994).

2.5 Applications of probiotics

2.5.1 Importance of probiotic consumption in humans

The number of food and other dietary adjuncts products containing live *Bifidobacterium* and *Lactobacillus* bacteria have significantly increased over the last 20 years due to the beneficial

effects, these probiotic organisms are believed to provide (Laroia and Martin, 1990). Presented in Table 2.8, is a listing of bacterial species used as probiotic cultures in food products. Although research is ongoing, the available evidence indicates that ingestion of probiotic bacteria may promote desirable changes in the gastrointestinal tract of humans (Kaplan and Hutkins, 2000).

Table 2.7 Bacterial species primarily used as probiotic cultures (Krishnakumar and Gordon, 2001)

Species	Strains
<i>Lactobacillus acidophilus</i>	La2, La5 (also known as La1), Johnsonii (La1; also known as Lj1), NCFM, DDS-1, SBT-2062
<i>L. bulgaricus</i>	Lb12
<i>L. lactis</i>	La1
<i>L. plantarum</i>	299v, Lp01
<i>L. rhamnosus</i>	GG, GR-1, 271, LB21
<i>L. reuteri</i>	SD2112 (also known as MM2)
<i>L. casei</i>	Shirota, Immunitass, 744, 01
<i>L. fermentum</i>	RC-14
<i>Bifidobacterium longum</i>	BB536, SBT-2928
<i>B. breve</i>	Yakult
<i>B. bifidum</i>	Bb-12
<i>B. esselnsis</i>	Danone, (Bio Activia)
<i>B. lactis</i>	Bb-02
<i>B. infantis</i>	Shirota, Immunitass, 744, 01

Table 2.8 Organisms used as probiotics in the food and agricultural industry (Goldin and Gorbach, 1992)

Organism	Comment
<i>Lactobacillus acidophilus</i>	As a supplement in dairy products and used for fermentations; numerous health claims
<i>L. plantarum</i>	In dairy products, pickled vegetables and silage
<i>Lactobacillus GG</i>	In yoghurt and whey drink; numerous health claims
<i>L. casei</i> subsp. <i>rhamnosus</i>	In dairy products and silage
<i>L. brevis</i>	In dairy products and silage
<i>L. delbrueckii</i> spp. <i>bulgaricus</i>	Production of yoghurt; health claims have been made
<i>Bifidobacterium bifidum</i>	Component of new dairy products and in preparation for new born; health claims

LAB are capable of inhibiting various microorganisms in a food environment and display crucial antimicrobial properties with respect to food preservation and safety. In addition, it has been shown that some strains of LAB possess interesting health-promoting properties; one of the characteristics of these probiotics is the potential to combat gastrointestinal pathogenic bacteria such as *Helicobacter pylori*, *Escherichia coli* and *Salmonella* spp. An overview role of bacteriocin application as antibacterial peptides in both food safety and gastrointestinal health is depicted in Fig. 2.3.

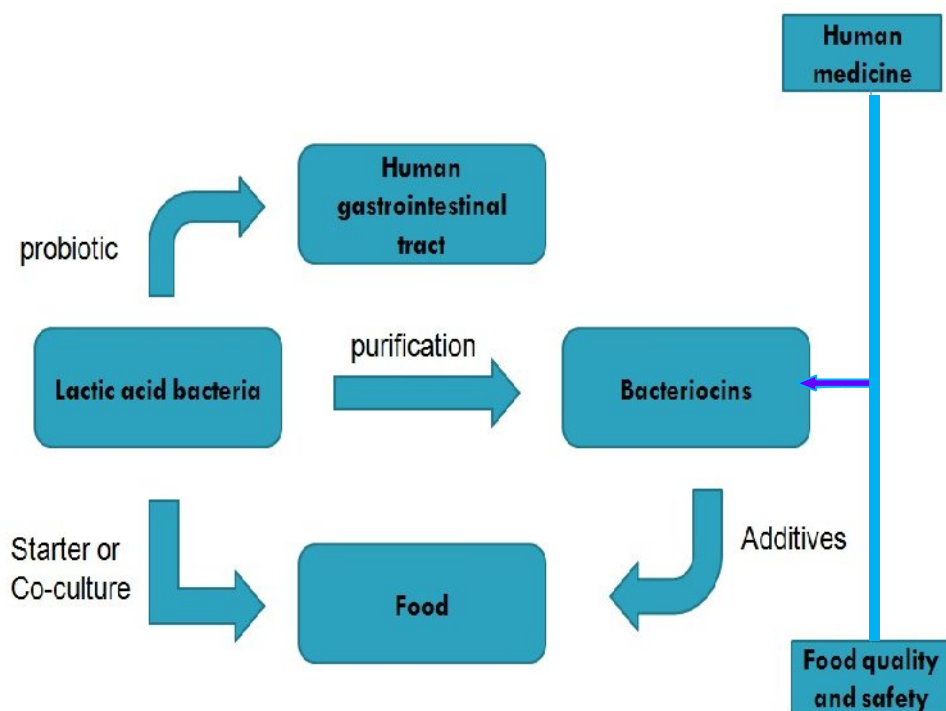


Fig. 2.3 Overview of the application potential of bacteriocin production by LAB in food quality and safety and in medicine, emphasizing their role as food ingredient and in the human gastrointestinal tract respectively.

Chapter III
MATERIALS & METHODS

III. Materials and Methods

3.1 Chemicals and Media

All chemicals and reagents used were of the highest analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified. Standard media components were purchased from Fisher Scientific (USA) or Sigma Aldrich (St. Louis, MO, USA) and Hi-Media (Mumbai, India). Chemicals of molecular biology grade were procured from New England Biolabs, UK.

3.2 Collection of samples

A total of four hundred and thirty six samples were selected, 100 each of fruits pickle mango and garlic–chilli samples were obtained from local market of Northern and North Eastern India. Samples of Bhaati jaanr (fermented rice liquor) and Mahula liquor (from fermented flowers of *Madhuca longifolia*) were obtained from local markets of Namchi; Sikkim, Cuttack; Orissa and Sitamadhi; Bihar. Thirty six meconium samples of new born babies were collected from Sehat Medicare, Patiala, Punjab (prior consent from parents was taken). All samples were collected in pre-sterile poly-bags and screw capped bottles, kept in an icebox and transported to the laboratory for analysis. Samples were processed within 6 h of receipt in local laboratories.

3.3 Isolation, selection and identification of *Lactobacillus* strains

3.3.1 Isolation of bacterial strains

One gram of each sample were weighed aseptically and homogenized for 2 min in stomacher lab-blender 400 (Seward, UK) in 9 ml Quarter-Strength Ringer's Solution (QRS) and diluted further in a ten-fold dilution series with the same. Freshly collected meconium (1 gm) from infants were suspended in 9 ml QRS and diluted further in a ten-fold dilution series with QRS. Aliquots (100 µl) of suitable ten-fold dilutions were plated onto MRS and Rogosa

agar to enumerate bacteria and predominant colonies were isolated. Plates were incubated anaerobically (Anaerobe jar, Hi-Media, India) for 48 h at 37°C. Several randomly selected colonies were picked from plates of highest dilution of each sample.

3.3.2 Gram staining

Bacterial smear from actively growing cells were spread on a glass slide and heat fixed. Smear was flooded with filtered crystal violet for 10 sec and then washed briefly in water to remove excess crystal violet. Later, it was flooded with Gram's iodine for ten sec and washed briefly in water. Smear was decolourised with acetone until the moving dye front had passed the lower edge of the section and washed immediately in tap water. Counterstaining was carried out with safranin for fifteen sec and washed with water to remove the excessive stain. Finally, samples were visualized under microscope at different magnification. The morphology of strains was studied upto 1000x magnification under microscope (Nikon E 200) and ninety six strains with rod-shaped morphology were further sub-cultured in MRS broth and streaked out three times to check for the purity. *Lactobacillus acidophilus* ATCC 4356 was taken as reference strain.

3.3.3 Catalase test

Microorganisms able to live in oxygenated environments produce catalase which neutralizes toxic forms of oxygen. Catalase breaks hydrogen peroxide (H_2O_2) into water and molecular oxygen. A small amount of freshly grown cultures were placed onto a clean grease free microscopic slide and few drops of H_2O_2 (3%) was added. A rapid evolution of O_2 as evidenced by bubbling indicates positive result. No bubbles or only a few scattered bubbles indicated the test as negative. Catalase test was performed by adding H_2O_2 to MRS broth inoculated with different isolates of *Lactobacilli*. Release of free oxygen gas bubbles indicate a positive catalase test. *Staphylococcus aureus* ATCC 9144 served as positive control. Stock cultures were kept at -80°C in MRS broth containing 20% (v/v) glycerol.

Table 3.1 Isolation of *Lactobacillus* from different sources

S. No.	Source name	Number of samples examined
1	Mango pickle	100
2	Garlic–chilli pickle	100
3	Chilli pickle	100
4	Fermented rice (Bhaati jaanr)	50
5	Fermented mustard (Kharoli)	50
6	New born babies meconium	36
	Total samples	436

3.3.4 Carbohydrate Fermentations

Isolates were characterized according to their fermentation ability to ferment eight different carbohydrates. All reactions were performed by using 96-well microtitre plates containing fermentation medium with different sugars, namely Glucose, Sorbitol, Mannitol, Lactose, Maltose, Mannose, Galactose and Arabinose. Active cells and sugar solutions were prepared separately. For preparation of active cells; isolates were activated in 10 ml MRS medium and incubated at 37°C for 24 h followed by centrifugation for 10 min at 10,000 rpm. Pellets obtained were washed twice with saline and resuspended in MRS broth without glucose, containing pH indicator bromothymol blue (0.01 g/l). The sugar solutions were prepared at a final concentration of 10% (w/v) after filter sterilization with sterile 0.22 µm membrane filters (MilliporeTM, India).

After preparative steps, following procedure was applied: 40 µl of sugar solutions were pipetted into each well followed with addition of 160 µl of suspended cells, giving the final sugar concentration of 2%. All the reactions were performed in triplicates. Positive and negative controls were used to indicate any contamination. Suspended cells (160 µl) and glucose (40 µl) solution were used as positive control while suspended cells (200 µl) alone were used as negative control. After overnight incubation at 37°C, the turbidity and the color

change from blue to yellow was recorded as positive fermentation results compared with the positive and negative controls. Results were also compared with the absorbance of samples read at 620 nm in an automated microplate reader (Bioscreen C, Oy Growth Curves Ab Ltd., Finland).

3.3.5 Strain selection and identification

Gram-positive, catalase-negative rods from traditional fermented nondairy products and infant gut were selected for further studies to determine, if they were able to survive the successive passage through solutions mimicking saliva, gastric juice and intestinal juice. Strains with high survival rates were further identified using phenotypic and genotypic characterization and identification techniques as described below.

3.3.5.1 *Survival after the successive passages through artificial saliva, gastric and intestinal juice*

To test survival under gastrointestinal conditions, the protective effect of milk and the effects of medium composition (lysozyme, pepsin, and pH of the medium) on bacterial viability were assessed *in vitro* using the method was adopted by (Ehrmann *et al.*, 2002; Suskovic *et al.*, 1997) in a model stomach/intestinal passage experiment in order to compare the survival of potential probiotic strains. Reconstituted skim milk (15% w/v, Merck) was inoculated with approximately 2×10^8 CFU/ml of an overnight culture of isolates. One ml aliquot was removed, serially diluted in QSR and plated onto MRS agar to determine the CFU/ml at 0 h.

To simulate the dilution and possible hydrolysis of bacteria in the human oral cavity, the suspension was diluted to 1:1 ratio in a sterile electrolyte solution containing 6.2 g/l NaCl, 2.2 g/l KCl, 0.22 g/l CaCl_2 and 1.2 g/l NaHCO_3 to which lysozyme was added to a final concentration of 100 ppm (parts per million), and incubated for 5 min at 37 °C. The sample was subsequently diluted in 3:5 ratio with an artificial gastric fluid, consisting of the

electrolyte solution mentioned above; containing 0.3% (w/v) pepsin and pH was adjusted to 2.5. If required, pH was readjusted to 2.5 with 5 M HCl. After 1 h of incubation at 37°C, another 1ml aliquot was removed, serially diluted in QSR and plated onto MRS agar. To simulate the dilution in the small intestine, the remaining volume was diluted 1:4 using an artificial duodenal secretion (pH 7.2). One ml aliquots were again removed after 3 h, serially diluted in QSR and spread-plated onto MRS agar to determine the CFU/ml in duplicate. Experiments were conducted in triplicate. Only those strains which survived the simulated gastrointestinal passage in detectable numbers were unequivocally identified and further investigated.

3.4 Phenotypic characterization

3.4.1 Growth at different temperatures

Overnight cultures were inoculated (1%) in MRS broth and incubated in water bath at 15°C and 45°C for 5 days. Turbidity and growth was measured at 600 nm using spectrophotometer (Hitachi U2900, Japan)

3.4.2 Production of gas from glucose

In order to determine the homofermentative and heterofermentative characterization of isolates, CO₂ production from glucose test was applied. Citrate lacking MRS broths and inverted Durham tubes were prepared and inoculated with 1% overnight fresh cultures. Then the test tubes were incubated at 37°C for 5 days. Gas production in Durham tubes was observed during 5th day which is the evidence for CO₂ production from glucose.

3.4.3 Hydrolysis of arginine

Overnight cultures were inoculated (1%) in MRS arginine broth at 37°C for 24-72 h. For detection of ammonia, 100 µl samples were spotted onto a white spotting tile and an equal volume of Nessler's reagent was added. Immediate appearance of a dark orange colour

indicates presence of ammonia. *Cronobacter sakazakei* MTCC 19111 was used as an arginine positive control strain.

3.4.4 Presence of *meso*-diaminopimelic acid (*mDAP*) in the cell walls

Overnight grown culture (1.5 ml) was centrifuged at 7,500 x g for 10 min in eppendorf tubes and then resuspended in 200 µl of 4 N hydrochloric acid (HCl) and hydrolysed at 100°C in a heating block overnight in tightly closed tubes. HCl was removed by gently streaming nitrogen at 40 to 50°C into the solution for 1 h. The dry residue was collected in a drop of water and spotted onto a thin layer chromatography (TLC) plate (pre-coated cellulose plastic sheets 20 cm x 20 cm, Merck, no. 5577). A *mDAP* standard (5 mg/ml) was also spotted as a positive control. The ascending one-dimensional chromatography was run in a solvent solution containing methanol: pyridine: 10 N HCl: water in the ratio of 32:4:1:7. After drying, the chromatograms were developed with an acidic ninhydrin spray (0.5 % w/v ninhydrin in 1-butanol:acetic acid [13:1] and heated at 100°C in oven for 5 min. *mDAP* was characterised by a low R_f (retention factor) and an olive green colour which changes to yellow with time and light exposure.

3.4.5 Determination of lactic acid enantiomers produced

Lactic acid enantiomers were determined using an enzymatic kit (API System; BioMerieux, Marcy l'Etoile, France) based on the oxidation of D-lactate or L-lactate into pyruvate in the presence of NAD^+ , which in turn reduces to NADH, by the corresponding enzymes, D-lactate-dehydrogenase or L-lactate-dehydrogenase.

The equilibrium of these reactions lies on the side of lactate. By trapping pyruvate in a subsequent reaction catalysed by glutamate pyruvate transaminase in the presence of L-glutamate, the equilibrium can be shifted in favour of pyruvate and NADH. The amount of NADH formed in the mentioned reactions is stoichiometric to the amounts of lactic acid oxidized by the lactate-dehydrogenases. The increase of NADH was determined

spectrophotometrically (spectrophotometer Hitachi U2900, Japan) by measuring the increase in light absorbance at 340 nm over time.

3.4.6 Sugar fermentation profiles

Overnight cultures grown in MRS broth were washed twice in QRS (7,500 x g, 10 min) and resuspended in basal medium for API 50 CH (API System; BioMerieux, Marcy l'Etoile, France). The API 50 CH strips consist of 50 microtubes containing dehydrated carbohydrates and its derivatives (heterosides, polyalcohols, uronic acids). The microtubes were inoculated with the bacterial suspension, which rehydrates the substrates. When fermentation occurred, it was revealed by a color change in the tube, caused by the anaerobic production of acid which was detected by the pH indicator present in the medium. The first tube, which did not contain any substrate, was used as negative control. Fermentation profiles were determined according to manufacturer's instructions.

3.4.7 Enzyme Activity

The enzyme profile of the isolates was carried out by using API ZYM kit according to manufacturer's instruction (API System; BioMerieux, Marcy l'Etoile, France). The isolates were grown overnight at 37°C on MRS agar. Pellets from centrifuged culture broth was used to prepare the suspension at 10^5 CFU/ml. After inoculation, cultures were incubated for 4 h at 37°C.

3.5 Genotypic characterization

3.5.1 Isolation of genomic DNA from bacteria

The total genomic DNA of selected *Lactobacillus* strains was isolated according to the guanidinium isothiocyanate method of Pitcher *et al.* (1989) as modified by Björkroth and Korkeala (1996) for Gram-positive bacteria. Briefly, 5 ml of overnight culture (grown in MRS) were centrifuged (17,860 x g, 10 min) and the pellet was resuspended in 1.5 ml 1x TE containing 0.5% NaCl and centrifuged again. The pellet was resuspended in 100 µl TERMLS

solution (see 2.2.1) and incubated at 37°C for 1 h. To degrade bacterial peptidoglycan, cells were lysed and protein denatured by adding 500 µl GES solution to the suspension and gently mixing by inversion. The preparation was incubated on ice for 5 min after which 250 µl of 7.5 M ammonium acetate was added and the tubes were gently inverted for 10 min before incubating and centrifuging at 15,000 x g for 10 min. The upper phase was carefully removed and placed in a new eppendorf tube. The DNA was precipitated by adding 460 µl ice cold 2-propanol and centrifugation at 15,000 x g for 10 min at 4°C. The supernatant was discarded and pellet was washed twice with 400 µl ethanol and was vacuum dried (Vacuum-drier, Eppendorf, Hamburg) at 45°C for about 3-5 min. The DNA was resuspended in 200 µl of 10 mM Tris-HCl (pH 8).

3.5.2 PCR amplification for 16S rRNA of *Lactobacilli* spp.

Almost complete 16S rDNA of selected strains was amplified by PCR using the primers 16Sseqfw (5'-AGAGTT TGATCM TGGC AG-3') and 16Sseqrev (5'-GGNTACCTTGTT ACGACT TC-3') corresponding to positions 8 to 27 and 1511 to 1491 of the 16S rDNA gene of *E. coli* (Brosius *et al.*, 1978), respectively. DNA amplification was performed with Genamp PCR system (Applied Biosystem, USA). DNA was amplified in 32 cycles (94°C - 1 min; 56°C - 1 min, 72°C - 2 min) in a 50 µl reaction volume containing 250 ng of genomic DNA as template, 10 per mol of each primer, 2.5 unit of Taq DNA polymerase (Bangalore Genei, India), 3 mM MgCl₂, 50 mM KCl, 20 mM Tris -HCL, 50 mM of each dNTP was added and final volume was adjusted to 50 µl with de-ionised water. The reaction was performed using the Gene Amp PCR system 2400 (Perkin Elmer, USA). The PCR products were cleaned using quantum prep PCR clean columns (BioRad, USA). The resin contained in the column was resuspended by vortexing for 5 sec. The column was placed in a 2 ml wash tube and spun for 1 min in a micro-centrifuge at 7350 x g. After this, the column was placed in a clean 1.5 ml collection tube and 25-100 µl samples was applied at the top

centre of the column without disturbing the resin. The tube was spun again at 735 x g for 2 min. The purified PCR product was recovered in the bottom of the collection tube. Determination of nucleotide sequence of the PCR purified fragments was performed using the ABI PRISM® BigDye™ Terminator cycle sequencing kit and the automatic DNA sequencer ABA PRISM at the DNA sequencing service of Bangalore Genei, India.

3.5.3 Analysis of sequence data

Sequences were analyzed by using CHECK-CHIMERA program (Maidak *et al.*, 2001), in order to detect the presence of possible chimeric artefacts generated by PCR. Similarities were calculated for nearly complete 16S rDNA sequences using only unambiguously determined nucleotide positions. The 16S rDNA gene sequences of isolates were compared with those available in GenBank/EMBL databases using BLAST program (Altschul *et al.*, 1997). The sequences of closely related strains aligned using multiple alignments CLUSTAL W program (Thompson *et al.*, 1997). The evolutionary distance was calculated by Kimura 2 parameter, phylogenetic dendrograms were constructed by neighbor-joining method by the use of MEGA 5 package (Tamura *et al.*, 2007). For analysis, 1500 bootstrap replicates were performed to assess the statistical support for the tree.

3.6 Storage of isolated culture

The isolates were inoculated in 5 ml of MRS broth in a test tube at 37°C for 24 ± 2 h in an anaerobic jar. The cultures were centrifuged and the pellets were washed twice with 0.85% saline, resuspended in fresh MRS and stored in cryo-vial containing 40% glycerol at -80°C. Working cultures were revived in MRS or M17 at least three times prior to any experiment.

3.7 Characterization of probiotic properties of *Lactobacillus* strains

3.7.1 Low pH and bile salt tolerance

The isolated *Lactobacillus* strains were tested for their ability to resist at low pH and bile salt. The pH value of gastric acid varies in the range of about 1.5-4.5 in a period of 2 h, depending on the entering time and the type of gastric contents. In the present study, pH 2 was used as a representative gastric pH value. 24 h old culture of each *Lactobacillus* (10^8 CFU/ml) was suspended in a MRS broth at pH 3 for 5 h at 37°C. The suspensions were then centrifuged at 3000 rpm for 10 min at 4°C twice and washed in sterile saline solution to remove the media. Cells were suspended in physiological solution and a series of 10-fold dilution (10^{-2} - 10^{-10}) was prepared. A given amount of each dilution (50 µl) was plated on to de Man Rogosa Sharpe (MRS) agar and incubated anaerobically at 37°C for 24 h. The percentage of the viable bacteria was calculated. Tolerance to bile salts was verified by inoculating 100 µl of bacterial suspension of each strain (10^8 CFU/ml) on to MRS agar containing bile salt at 0.3% concentration. Survival of the *Lactobacillus* strains was examined by counting the cells after 24 h of incubation at 37°C on to MRS agar.

3.7.2 Resistance to 0.4% phenol

Some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria, leading to the formation of phenols (Suskovic *et al.*, 1997). These compounds may exert a bacteriostatic effect against some *Lactobacillus* strains. Thus, testing for the resistance to phenol may generate further information on the potential for survival of *Lactobacilli* in gastrointestinal conditions (Xanthopoulos *et al.*, 2000). Therefore, the ability of *Lactobacillus* strains to grow in the presence of phenol by inoculating cultures (1% of an overnight culture) in MRS broth with and without 0.4% phenol was tested. Serial dilutions were spread plated (100 µl aliquots) onto MRS agar at

time 0 h and after 24 h of incubation at 37°C to enumerate surviving bacteria as described by Xanthopoulos *et al.* (2000).

3.7.3 Determination of antimicrobial potential of probiotic strains

3.7.3.1 Production of H₂O₂

Overnight grown cultures (10 µl) were spotted onto ABTS-agar plates (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) and incubated anaerobically at 37°C for 72 h. After anaerobic incubation, plates were exposed to the atmosphere. Oxidative coloration of ABTS by H₂O₂ was visible as a violet halo surrounding the colony of H₂O₂ producer *Lactobacillus* strains, indicating H₂O₂ production (Marshall, 1979).

3.7.3.2 Screening for antagonistic activity

The agar spot test as described by Schillinger and Lucke (1987) was used for screening the antagonistic activity of the selected *Lactobacillus* strains. 10 µl of overnight *Lactobacilli* culture were spotted onto modified MRS agar (2 g/l glucose and 13 g/l agar) and incubated at 37°C for 24 h. These plates were over-layered with MRS soft agar (7.5 g/l agar) inoculated with ca. 1×10^8 CFU/ml of indicator strains. The agar spot test method of Uhlman (1992) was used to test whether the inhibition zones observed in the screening for antagonistic activity were due to the bacteriocin production or as a result of acid inhibition. Briefly, cell-free neutralized supernatants were obtained from overnight producer cultures grown in MRS broth at 37°C. After centrifuging the culture at 7,200 x g for 10 min, the supernatants were neutralized with sterile 5 M NaOH and then boiled for 5 min to inactivate residual viable cells. The supernatants were tested against the same indicator strains as mentioned above.

3.7.3.3 Bile salt hydrolase activity

Bile salt hydrolase (Bsh) activity of the cultures was detected using the procedure described by Du Toit *et al.* (1998). Briefly, 10 µl of overnight cultures were spotted onto MRS agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt and 0.37 g/l of CaCl_2 . The plates were incubated anaerobically for 72 h. The strains with a white precipitation zone surrounding the colony were considered as positive (du Toit *et al.*, 1998; Franz *et al.*, 2001).

3.7.3.4 *In vitro* cholesterol assimilation

Water-soluble cholesterol was dissolved in 50% ethanol (5 mg/ml), filter sterilized and added to MRS broth supplemented with 0.3% ox-bile at a final concentration of 70 mg/ml. Medium was inoculated with each test culture *L. casei* LAM-1, LAM-2, *L. helveticus* LKH-5, *L. fermentum* Lamec-29 and *L. delbrueckii* LKH-2, LKH-3 (10^7 CFU/ml) and incubated anaerobically at 37°C for 24 h. After the incubation period, cells were centrifuged ($10\,000 \times g$ at 48°C for 10 min), and the remaining cholesterol concentration in the broth was determined using a colorimetric method as described by Rudel and Morris, 1973. Then 1ml of the aliquot was added with 1 ml of KOH (33%, w/v) and 2 ml of absolute ethanol, vortexed for 1 min and left at 37°C for 15 min. After cooling, 2 ml of distilled water and 3 ml of hexane were added followed by vortexing for 1 min. Then 1ml of the hexane layer was transferred into a glass tube and evaporated. The residue was immediately dissolved in 2 ml of *o*-phthalaldehyde reagent. After complete mixing, 0.5 ml of concentrated H_2SO_4 was added, and the mixture was again vortexed for 1 min. Finally, the absorbance was read at 550 nm after 10 min. The amount of cholesterol removed from broth was determined by subtracting the amount in each broth sample (mg/ml) from the amount present in the uninoculated control.

3.7.3.5 Production of β -galactosidase

The *o*-nitrophenyl- β -D-galactopyranoside (ONPG) substrate was used to determine β -galactosidase activity as described by Zárate *et al.* (2000), with modifications as described below. The strains which were able to grow in M17 medium (Merck) (which contains lactose as only carbon source), were harvested by centrifugation and washed twice in phosphate buffered saline (PBS) at pH 7.4. Strains growing on M17 were thought to have β -galactosidase activity, which enables utilization of lactose and hence growth on this type of medium. The samples were adjusted to an $A_{580\text{ nm}}$ of 1.0. Aliquots (100 μ l) of each of the bacterial suspensions were incubated in the presence of 2 mM ONPG for 40 min in a water bath at 37°C. The reaction was terminated by addition of 1 ml 0.25 M Na_2CO_3 . The samples were centrifuged at $7,200 \times g$ for 10 min at 4°C and the supernatants were recovered to measure the absorbance at 420 nm. A standard curve was obtained with *o*-nitro-phenol (ONP, Sigma) (concentrations of 0.05 to 0.5 μ mol/ml in 0.05 μ mol/ml increments). In order to compare the activity of those strains able to hydrolyze ONPG, cell-free extracts were prepared by disruption using a French pressure cell (SLM Aminco, SLM Instruments Inc., Lorch, Germany). Briefly, 10 ml of overnight cultures grown in M17 broth were harvested by centrifugation ($7,200 \times g$ at 4°C for 10 min) washed twice with buffer $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (50 mM, pH 7.25) and resuspended in the same buffer.

The cell suspension was passed through the French pressure cell at 1,000 psi for at least two times. Cell debris was separated by centrifugation at $10,000 \times g$ at 4°C for 10 min. Cell extracts were kept on ice until incubation with the substrate as previously described. Protein contents were determined by the method of Bradford (1976), using bovine serum albumin (Roche, Mannheim) as standard. One enzymatic unit was defined as the micromoles of ONP liberated from ONPG per milligram of protein and per min.

3.7.3.6 Safety considerations: antibiotic resistance profiles of *Lactobacillus* strains

The selected strains were investigated for their antibiotic resistance profile using the E-test (Viva Diagnostika, Cologne, Germany) using MRS agar and anaerobic incubation conditions following the manufacturer's instructions. The minimum inhibitory concentration (MIC) values were used to determine whether strains were susceptible or resistant were those as suggested by the scientific Commission on Animal Nutrition (SCAN, Europe) for *Lactobacillus* spp. (Chesson *et al.*, 2002).

3.8 Adhesion properties of selected *Lactobacillus* strains

3.8.1 Microbial adhesion to solvents

Microbial adhesion to solvents was measured using the methods described by Rosenberg *et al.* (1980) and Bellon-Fontaine *et al.* (1996) with slight modifications. Overnight cultures of *Lactobacilli* were harvested by centrifugation at 5,000 x g for 5 min, washed twice in PBS (pH 7.4), resuspended and diluted in PBS to reach an absorbance (A_0) of approx. 0.5 at 580 nm. Test solvent (2 ml) were added to 2 ml of the suspension and mixed by vortexing for 1 min. The three test solvents used were: n-hexadecane (Sigma, USA), which is an apolar solvent, chloroform (Merck, USA), which is a monopolar and acid solvent and ethyl acetate (Merck), which is a monopolar and basic solvent. The aqueous phase was taken after 20 min incubation at RT and its absorbance at 580 nm was measured (A_1). The percentage of bacterial adhesion to solvent was calculated as $(1 - A_1/A_0) \times 100$.

3.8.2 Aggregation

The auto-aggregation assay was performed as described by Del Re *et al.* (2000) with modifications. Overnight cultures were washed as described above and the cells were suspended in PBS pH 7.4, PBS pH 4.0, and supernatant of bacteria grown overnight in MRS or in neutralized supernatant (neutralized with 1 N HCl). Each of these suspensions (2 ml)

were vortexed for 10 sec and auto-aggregation was determined after 2 h at RT. For determination of auto-aggregation, 100 µl samples was removed from the top of the suspension and was transferred to a cuvette containing 900 µl PBS pH 7.4. The absorbance (A_1) was measured at 580 nm. The auto-aggregation percentage was expressed as: $(1 - A_1/A_0) \times 100$, where, A_0 represents the absorbance at zero time.

3.8.3. Coaggregation

Overnight cultures of *Lactobacilli* and pathogenic strains (Table 2.1) were washed twice with PBS pH 7.4. To each well of a 24-well microtray (Costar, VWR Int.), 500 µl of the *Lactobacillus* suspension (10^9 CFU/ml) and 500 µl of the pathogen suspension (10^9 CFU/ml) were added. After mixing, the trays were incubated at 37°C with shaking at 100 rpm for 2 h. The scoring system of 0 to 4 (0 for no coaggregation, 1 for small and dispersed clumps, 2 for medium-sized and dispersed clumps, 3 for abundant and medium sized clumps and 4 for very big clumps and clear supernatant) as described by Reid *et al.* (1988) was used. Each suspension was examined for aggregation microscopically at 200 x and 400 x magnification with an inverted microscope (CKX41, Olympus), though clumping at score levels of 3 and 4 could be seen macroscopically.

3.8.4 Caco₂ Cells Adhesion Assay

Adhesion of isolates was assayed as per the method described by Jacobsen and coworkers. Initially, 10^5 Caco₂ cells were seeded in each well of six-well tissue culture plates. The Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% (v/v) heat-inactivated (30 min, 56°C) fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin was used for culturing. The medium was changed with fresh medium every alternate day. Adhesion assay was done after 20 days of post confluency. The cells were then washed twice with 3 ml phosphate-buffered saline (pH 7.4). Two ml of DMEM without serum and antibiotics was added to each well and incubated at 37°C for 30 min.

Approximately 10^9 CFU/ml bacterial culture was suspended in one ml DMEM medium (without serum and antibiotics) and added to different wells. The plate was incubated at 37°C for 2 h in the presence of 5% CO₂ in incubator. The monolayer was washed with sterile PBS and the cells were detached by trypsinization. one ml of 0.25% Trypsin-EDTA solution (Sigma. USA) was added to each well of six-well plate which was then incubated for 15 min at room temperature. The cell suspension was plated on MRS agar by serial dilution for determining the adherent bacterial cells. The plate was incubated for 24-48 h at 37°C and colonies were counted. Bacterial cells initially added to each well of six-well plates were also counted by serial dilution and plating on MRS agar. The results of the adhesion assay were expressed as adhesion percentage, the ratio between adherent bacteria and added bacteria per well. Three independent experiments (n = 3) with two replicates in each experiment with Caco₂ cells of same passage were carried out.

3.9 Screening of *Lactobacillus* spp. for Bacteriocin Production

Cell-free supernatants (CFS) of *Lactobacillus* were obtained by centrifuging the cultures at 12000 x g for 10 min, collecting the supernatants, which were adjusted to pH 6.5 with 1 M NaOH, and filtered through a 0.22 µm filter (Millipore). Inhibitory activity from hydrogen peroxide was avoided by the addition of catalase (5 mg/ml). Bacteriocin production activity was determined using the well diffusion method (Motta and Brandelli, 2002) by using *Aeromonas hydrophila* ATCC 7966, *Yersinia enterocolitica* MTCC 840, *Staphylococcus aureus* ATCC 9144, *Enterobacter (Chronobacter) sakazakii* MTCC 659, *Shigella flexneri* 2a and *Listeria monocytogenes* ATCC 19111 as the indicator bacteria. Aliquots (20 µl) of culture supernatants were applied to well (5 mm in diameter) on agar plates previously inoculated with a cell suspension of indicator bacteria, which corresponded to a 0.5 McFarland turbidity standard solution.

Plates were incubated at 37°C for 24 h and the antimicrobial activity titer was determined by serial two-fold dilution method previously described (Kimura *et al.*, 1998). Bacteriocin activity was expressed as AU/ml and defined as the reciprocal of the highest dilution showing a distinct zone of inhibition. To evaluate whether the antimicrobial activity was due to peptides, culture supernatants were treated with 2 mg/ml pronase E (Sigma, USA) for 30 min at 37°C before being tested for antimicrobial activity.

3.9.1 Batch fermentation for bacteriocin production by selected isolates

The isolated strains *Lactobacilli casei* LAM-1 strain was used for the production of bacteriocin. Batch fermentation experiments using MRS broth (100 ml) were performed under static conditions. Incubation time was optimized by performing the fermentation experiment for 48 h at 37°C with 1% inoculum and the samples were collected upto 36 h of incubation. Analysis of growth (CFU/ml) and bacteriocin activity (AU/ml) were performed at each step.

3.9.2 Optimization of physical and chemical parameters for bacteriocin production by selected *Lactobacillus* spp.

Parameters such as incubation time, media pH, incubation temperature, inoculum size and media composition were optimized. Further the sets of experiments were performed at different temperatures 25, 30, 35, 37, 40 and 45 °C. The flasks were incubated for 36 hrs and samples were collected and analyzed. Optimization of media pH was done with MRS broth adjusted to pH 4, 5.5, 6, 6.5, 7.0, 7.5 8, 9 and 10, inoculated with 1% of bacteriocin producing culture grown overnight at 37°C. Inoculum size was optimized for 1, 2.5 and 5.0% of inoculum at 37°C and constant pH 7.0.

3.9.3 Partial purification of bacteriocins

Bacteriocin was isolated from culture of the *L. casei* LAM-1 grown in MRS broth medium at 37°C for 16 and 24 h. The pH of culture supernatant was adjusted to 6.5 by the

addition of 0.1 N NaOH. The cells were removed by centrifugation (5500 rpm, 4°C, 20 min). The cell-free culture supernatant was brought to a final ammonium sulphate concentration of 20 and 40% saturation by slow addition of the salt, and was stirred overnight at 4°C. Then, the mixture was centrifuged (5500 x g, 4°C, 30 min) and the surface pellicles and bottom pellets were harvested and resuspended in 10 ml of sodium phosphate buffer (pH 6.5). To one volume of the resuspended product, 10 volumes of a methanol-chloroform mixture (1: 2, v/v) were added, and the mixture was extracted at 4°C for 1 h. The sample was centrifuged (5500 rpm, 4°C, 30 min), supernatant fraction decanted and the pellet was air-dried. The pellet was resuspended in 2 ml of milli-Q water. The partially purified bacteriocin was stored at 20°C. Protein concentrations were determined by a modification of the method of Bradford (1976) using a Protein Assay Kit from BioRad Laboratories (California, USA). Bovine serum albumin was used as a standard.

3.10 Characterization of partially purified bacteriocin

3.10.1 Effect of enzymes and detergents

Sensitivity of the bacteriocin to enzymes: proteinase K, (1000 units mg/ml), α -amylase (724 units mg/ml), DNase and RNase (470 units mg/ml), and pepsin (290 units mg/ml) (Sigma, MO, USA) was tested using partially purified bacteriocin samples. Each enzyme was dissolved in 10 mM sodium phosphate buffer (pH 7.0), and the solutions were added to the bacteriocin solution for a final concentration of 1 mg/ml. Following incubation at 37°C for 2 h, the mixture was heated at 100°C for 10 min to denature the enzymes. The residual bacteriocin activity (AU/ml) was measured by bioassay, using *Listeria monocytogenes* ATCC 19111 as an indicator strain. The effect of detergents on bacteriocin activity was also determined by adding sodium dodecyl sulfate (SDS), Tween 80, Tween 20, EDTA and urea at final concentration 0.1, 1, 2 or 5% to the cell-free supernatants in a separate experiment. All detergent-treated supernatants were incubated at 37°C for 1 h, and

tested for residual bacteriocin activity. Untreated cell-free supernatants with above enzyme and detergents were used as control.

3.10.2 Thermal stability

Thermal stability of the partially purified bacteriocin was determined by incubation of partially purified bacteriocin solutions at 60°C for 20 min, at 100°C for 15 min, and at 121°C for 15 min. Residual activity was determined for bacteriocin using sensitive strain as indicator.

3.10.3 pH stability

The effect of pH on the activity of bacteriocin was tested by adjusting cell-free supernatants from pH 2.0 to 12.0 (at increments of two pH units) with sterile 1 M NaOH or 1 M HCl. After 2 h incubation at 37°C, pH of all sample were readjusted to 6.0. Antimicrobial activity was tested by using the well diffusion method as described above. Untreated samples were used as the control.

3.10.4 Purification by chromatography

Bacteriocin from *Lactobacilli casei* LAM-1 was purified further by using gel filtration chromatography Sephadex G-50 and 50 mM phosphate buffer (pH 6.5). Sephadex-G-50 (5 g) was soaked in 200 ml of 50 mM phosphate buffer (pH 6.5) containing 0.1 g of sodium azide and incubated for 72 h at room temperature. After soaking the gel was deaerated and poured in a 0.9 x 60 cm column. Void volume was determined by passing blue dextran (2000 kDa) through the column. The sample was loaded to the column 2.0 ml at a time. The above mentioned buffer was used to elute the sample fractions each of 3.0 ml, which were collected at a flow rate of 0.2 ml/min and read at 280 nm using spectrophotometer. Antimicrobial assay was performed against indicator organism. The active fractions were pooled and subjected to lyophilization (Modulyod-230, Thermo, USA). The lyophilized sample was used for further analysis.

3.10.5 Purification of the bacteriocin from the bacterial culture

A cell-free solution (CFS) was obtained by centrifuging the 1 l culture at 12,000 x g for 10 min. The pH of the CFS was then adjusted to 6.5 with 1 N NaOH. The proteins of 500 ml CFS was precipitated with ammonium sulphate (45% saturation) overnight at 4°C with gentle stirring and centrifuged at 10,000 x g for 30 min. The precipitate fraction was resuspended in 0.5 ml sodium phosphate buffer (pH 7.2).

3.11 Molecular characterization of Bacteriocins

3.11.1 Determination of molecular weight of bacteriocin

The molecular weight of the bacteriocin was determined by Tricine-SDS polyacrylamide gel electrophoresis (Schagger and Jagow, 1987). After 20 h cultivation, the cells were harvested by centrifugation (9,660 x g, 15 min at 4°C) and proteins were precipitated from cell free supernatant with 70% saturated ammonium sulfate. The precipitate was resuspended in 0.1 of 20 mM sodium phosphate buffer (pH 6.0), desalted against distilled water by using a dialysis membrane (MwCO =1.2 kD, Sigma-Aldrich, MO, USA). Desalted bacteriocin containing sample was separated by gel electrophoresis (Biorad, USA), the low molecular weight size marker (2.5-45 kD) was used (Biogene, USA). After electrophoresis, one half of the gel was stained with Coomassie Blue; the other unstained half was used to determine the position of active antimicrobial peptide. The indicator strain *Listeria monocytogenes* ATCC 19111 embedded in BHI agar (10^5 CFU/ml) was used for the positioning of active band.

3.11.2 N-terminal amino acid sequence analysis

The activity of the purified bacteriocin was confirmed on the SDS-PAGE gel, and the gel was then blotted onto polyvinylidene difluoride membranes and stained with CBB R-250 (Biorad, USA). The objective bands were cut out and analyzed, and the N terminal amino

acid sequence was determined by Edman degradation on a protein sequencer (model 491; Applied Biosystems, Foster City, CA).

3.12 Mechanism of Bacteriocin activity

Ten ml filter-sterilized cell-free supernatant (2844 AU/ml) of *L. casei* LAM-1 was added to the 50 ml culture of *L. monocytogens* ATCC 19111 at early exponential phase and then incubated at 37 °C. Samples were withdrawn hourly to record the optical density at 600 nm and to determine the viable cells (CFU/ml) on TSYE agar plates. Control cells were treated with the inactive bacteriocin (treated for 20 min at 121°C).

3.12.1 Transmission and Scanning electron microscopy

Overnight grown culture of *S. typhimurium* ATCC 19585 and *Shigella flexneri* 2a (10^8 CFU/ml) were treated with the bacteriocin (100 µg/ml) of *L. casei* LAM-1 at 37°C for 12 h. After centrifugation (5500 x g, 10 min, 4°C), the pathogens were washed with PBS. The cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 mol/l sodium phosphate buffer (pH 7.4) for 1 h at room temperature. For transmission electron microscopy (TEM), the specimens were observed using a transmission electron microscope (LEO1430VP, Carl Zeiss, India). For scanning electron microscopy (SEM), the treated cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 mol/ml Sodium phosphate buffer (pH 7.4), post fixed for 2 h with 4% (w/v) OsO₄ in 0.1 mol/l sodium phosphate buffer (pH 7.4), washed 2 times with 0.1 mol/l sodium phosphate buffer (pH 7.4), and dehydrated in 20%, 40%, 60%, 80%, 90%, and 100% ethanol. The cells were dried in a critical-point dryer and coated with gold. The specimens were then examined with a Scanning electron Microscope (Model: JSM-7100F JEOL, Japan) (Drosinos *et al.*, 2005).

3.12.2 Membrane permeability

Permeability intactness of *S. typhimurium* ATCC 19585 was measured using the Live/Dead BacLight™ Bacterial Viability Kit (L13152, Molecular Probes, Invitrogen,

USA) according to the manufacturer's instructions. Cells were energized with glucose (20 mM final concentration) for 10 min at 37°C prior to treatment with test compounds. The pore former nisin (25 mg/l final concentration) was used as a positive control and untreated cells served as negative controls. Cell suspensions (0.5 ml) were treated with bacteriocin preparation (~ 0.5 mg/l final concentration), and positive and negative controls at 37°C and 300 µl aliquots of each sample were removed at various intervals over 60 min. Cells were kept on ice during sampling. Cells were stained by addition of 100 µl live/dead BacLight™ staining reagent to 100 µl samples in triplicate in 96-well flat-bottomed microtitre plates. Samples were mixed thoroughly by pipetting and the Microtiter plate was incubated in the dark at an ambient temperature for 15 min. The fluorescence emission of green (excitation at 485 nm, emission at 530 nm) to red (excitation at 485 nm, emission at 630 nm) ratio was measured with a Fluorescence Spectrophotometer. The effect of bacteriocin on membrane integrity was calculated by taking the green to red fluorescence ratios of the untreated samples as 0% permeable and the nisin treated samples as 100% permeable. Membrane permeability was expressed as a green to red fluorescence ratio (530/630 nm) (Cleveland *et al.*, 2001).

3.12.3 Measurement of intracellular K⁺ content

The intracellular K⁺ concentration of bacteriocins treated *S. Typhimurium* ATCC 19585 cells was determined as described previously (Olivia *et al.*, 1998) with the following modification. Cells were energized with glucose (20 mM final concentration) for 10 min at 37°C prior to treatment with test compounds. Nisin (25 mg/l final concentration) was used as a positive control while untreated cells served as negative controls. Cells were treated with either bacteriocin (20 mg/l final concentration) or the positive and negative controls at 37°C, 0.5 ml samples were taken in duplicate after 10 min incubation. These samples were centrifuged (13000 x g, 5 min, 4°C) through 0.3 ml of silicon oil (1:1 ratio, BDH, UK). The

micro centrifuge tubes were frozen and the bottom of the tubes containing cell pellets was removed. Cell pellets were digested with 1 ml of 3 M HNO₃, vortexed vigorously and incubated at an ambient temperature overnight. Concentrations of intracellular K⁺ in the cell pellets were determined by ICP (Perkin Elmer-Sciex Elan DRC Plus, USA).

3.12.4 Measurement of intracellular ATP content

Extracellular ATP levels of bacteriocins treated *S. typhimurium* ATCC 19585 was determined as described previously (Bruno, 1993) with some modifications. Cells were suspended in 2.5 mM sodium phosphate buffer (pH 7.0) with 10 mM glucose and 100 µg/ml of bacteriocin (2,800 AU/ml). At various times, 20 ml samples were taken to determine the extracellular ATP concentrations, respectively. 20 ml samples were immediately mixed with 80 ml of dimethyl sulfoxide. 50 ml samples were then spun down immediately for 2 min, and 20 ml of the supernatant was removed and mixed with 80 ml of dimethyl sulfoxide. All samples were diluted with 5 ml of sterile deionised water. ATP concentrations were determined by inactivation the luciferin-luciferase enzyme assay.

3.13 Inhibition of pathogen by bacteriocin in a simulated /laboratory prepared food matrix

Fresh vegetable samples like cucumber, radish, carrot and tomato were purchased at local supermarkets and kept under refrigeration for no longer than 24 h until use. These vegetables were sliced (ca. 1.0 cm in size) and artificially contaminated (10 µl per piece) with a sterile saline solution suspension of *S. typhimurium* ATCC 19585 (2.0 x10⁸ CFU/ml) previously grown overnight in brain heart infusion infusion broth at 37 °C. Following inoculation, vegetables were allowed to dry for 1 h at room temperature and then were treated by immersion for 5 min at room temperature in 5 ml of sterile distilled water (controls) or distilled water containing bacteriocin (25-100 µg/ml). After immersion treatments, excess immersion solution was drained on sterile filter paper, and samples were stored in sterile

capped 50 ml polypropylene test tubes placed in refrigerator. At each step, duplicate samples (3 g each) were mixed with 5 ml of sterile saline solution (0.85% NaCl) and pummeled for 3 min in a Seward stomacher (Seward, UK) before they were serially diluted in sterile saline solution and spread in triplicate on plates of BHI agar. Plates were incubated at 37°C for 48 h, and the number of colonies was determined to calculate viable cell counts.

3.14 Post processing stability

The effect of extended storage at low refrigerated temperature on bacteriocin stability was evaluated by placing supernatants for 4°C up to 15 days. To further test the stability of bacteriocin during three freeze-thaw circles, the bacteriocin were frozen at -40°C during 24 h and thawed at 25°C. In all cases, a positive control, consisting of freshly prepared bacteriocin was tested in parallel.

3.15 Statistical analysis

All the experiments were performed in triplicate. The data were analyzed by Analysis of Variance (ANOVA) and the means were compared using Tukey's honestly significant difference test at $P < 0.05$. All the analyses were performed using GraphPad Prism (5.03) software.

Chapter IV
RESULTS

IV. RESULTS

4.1 Isolation of *Lactobacilli*

A general agreement among those in favour of the classical definition of probiotics refers to the need of live microorganisms exerting health-promoting effects, thus the need of survival to host conditions to reach the site of action. The general criteria for selection of strains to be used as probiotics include: safety and origin of the bacteria, their tolerance to the hostile conditions of the stomach and the small intestine, and their ability to adhere to gut epithelial tissue. GRAS (generally recognised as safe) microorganisms include *Lactobacillus* spp. and *Bifidobacterium* spp., with a long history of safe use as they are being consumed by humans for centuries. Selection of the strain regarding host species and location specificity plays an important role if colonisation is essential for achieving the desired effect of the probiotic.

The bacterial isolates screened for potential probiotics in this study were isolated from traditionally fermented food and beverage products (namely mango pickle, garlic-chilli pickle, chilli pickle, teak pickle, Kharoli (fermented mashed mustard), Bhaati jaanr (fermented rice), Mahula liquor and human faeces. In the present study, a total of two hundred and ninety six selected samples especially from traditionally fermented food were collected from different locations of India and subjected for isolation of predominant probiotic cultures. Predominant colony types were selected and purified by continuous streaking on MRS media. Two hundred thirty four strains of lactic acid bacteria (LAB) were isolated on this non selective media (MRS) from traditionally fermented food. Further selective media Rogosa Agar was used for selective enumeration of *Lactobacillus* spp. from these LAB isolates. By selective enumeration, 96 out of 436 strains were found to be species

belonging to the genus *Lactobacillus*. These isolates were subcultured in order to obtain pure isolates and further characterized for their morphology by microscopy and gram staining.

4.2 Morphological and Biochemical Characterization of *Lactobacilli*

Ninety six strains were isolated on MRS medium from different samples of traditionally fermented food and identified as *Lactobacillus* spp. by Gram staining. All the bacterial isolated (LAM, LKH, HKT and T) found to be short, smooth, convex, single or paired square bacilli, Gram positive and formed opaque creamy colony without any pigmentation

The colony morphology on MRS agar was off white, rod, with smooth edges and raised from center. All of the isolated *Lactobacillus* spp. were Gram positive rod with arrangement of singles cells to clusters for *Lactobacillus* spp. LAM-1, LAM-2, LAM-5, LAM-18, while *Lactobacillus* spp. LKH-2, LKH-3 and LKH-5 were found in single or in pairs. *Lactobacillus* spp. LAM-1 and LAM-2 were isolated from pickled mango, while *Lactobacillus* spp. LKH-2, LKH-3 and LKH-5 were isolated from Kharoli and Bhaati jaanr samples respectively (Table 4.1).

None of the isolates were able to ferment sorbitol, maltose and arabinose. *Lactobacillus* spp. LKH-2, LKH-3 and LKH-5 were able to ferment galactose but didn't fermented raffinose and a similar result of fermentation was obtained for *Lactobacillus* spp. LAM-1 and LAM-2. All the isolates showed negative catalase activity (Table 4.1 and 4.2). Phenotypic, genotypic identification and *in vitro* probiotics properties were carried out only for the cultures, having ability to survive in detectable numbers upon successive passage through solutions mimicking saliva, gastric juice and intestinal juice.

Table 4.1: Morphological and Biochemical characterization

S.no	Isolate No.	Gram's Reaction	Shape	Catalase test	Carbohydrate fermentation								Source
					Ga	Ra	La	So	Ma	De	Ar	Mo	
1	LAM-1	+	Rods	-	+	-	+	-	-	+	-	-	MP
2	LAM-2	+	Rods	-	+	-	-	-	-	+	-	-	MP
3	LAM-3	+	Rods	-	+	+	+	-	-	-	-	-	MP
4	LAM-4	+	Rods	-	+	-	-	-	-	+	-	-	MP
5	LAM-5	+	Rods	-	+	-	-	-	-	+	-	-	MP
6	LAM-6	+	Rods	-	+	+	+	-	-	+	-	-	MP
7	LAM-7	+	Rods	-	-	-	+	-	-	+	-	-	MP
8	LAM-8	+	Rods	-	+	-	+	-	-	-	-	-	MP
9	LAM-9	+	Rods	-	-	-	-	-	-	+	-	-	MP
10	LAM-10	+	Rods	-	-	-	-	-	-	+	-	-	MP
11	LAM-11	+	Rods	-	-	-	-	-	-	-	-	-	MP
12	LAM-12	+	Rods	-	-	-	-	-	-	+	-	-	MP
13	LAM-13	+	Rods	-	+	-	+	-	-	-	-	-	MP
14	LAM-14	+	Rods	-	+	-	+	-	-	+	-	-	MP
15	LAM-15	+	Rods	-	+	-	+	-	-	-	-	-	MP
16	LKH-1	+	Rods	-	-	-	-	-	-	-	-	-	BJ
17	LKH-2	+	Rods	-	+	-	-	-	-	-	-	-	BJ
18	LKH-3	+	Rods	-	+	-	+	-	-	+	-	-	BJ
19	LKH-4	+	Rods	-	+	-	+	-	-	+	-	-	BJ
20	LKH-5	+	Rods	-	+	-	+	-	-	+	-	-	BJ
21	LKH-6	+	Rods	-	-	-	-	-	-	-	-	-	BJ
22	LKH-7	+	Rods	-	-	-	-	-	-	+	-	-	BJ
23	LKH-8	+	Rods	-	+	+	+	-	-	-	-	-	BJ
24	LKH-9	+	Rods	-	-	-	-	-	-	+	-	-	BJ
25	LKH-10	+	Rods	-	-	-	-	-	-	-	-	-	BJ
26	LKH-11	+	Rods	-	-	-	-	-	-	+	-	-	BJ
27	LKH-12	+	Rods	-	-	-	-	-	-	+	-	-	BJ
28	LKH-13	+	Rods	-	+	+	+	-	-	+	-	-	BJ
29	LKH-14	+	Rods	-	+	+	+	-	-	+	-	-	BJ
30	LKH-15	+	Rods	-	+	-	+	-	-	+	-	-	BJ
31	LKH-16	+	Rods	-	-	-	-	-	-	-	-	-	BJ
32	LKH-17	+	Rods	-	+	+	+	-	-	+	-	-	BJ
33	LKH-18	+	Rods	-	-	-	-	-	-	-	-	-	BJ
34	LKH-19	+	Rods	-	-	-	-	-	-	+	-	-	BJ
35	LKH-20	+	Rods	-	-	-	-	-	-	-	-	-	BJ
36	LKH-21	+	Rods	-	-	+	+	-	-	+	-	-	BJ
37	LKH-22	+	Rods	-	-	-	+	-	-	-	-	-	BJ
38	T-38	+	Rods	-	-	-	-	-	-	+	-	-	TP
39	T-39	+	Rods	-	+	-	-	-	-	+	-	-	TP
40	T-40	+	Rods	-	+	+	+	-	-	-	-	-	TP
41	T-41	+	Rods	-	-	+	-	-	-	+	-	-	TP
42	T-42	+	Rods	-	-	-	-	-	-	-	-	-	TP
43	T-43	+	Rods	-	+	-	+	-	-	+	-	-	TP
44	T-44	+	Rods	-	-	+	-	-	-	-	-	-	TP
45	T-45	+	Rods	-	+	-	+	-	-	+	-	-	TP
46	T-46	+	Rods	-	-	-	-	-	-	-	-	-	TP
47	T-47	+	Rods	-	+	+	+	-	-	+	-	-	TP
48	T-48	+	Rods	-	-	+	-	-	-	-	-	-	TP

MP:Mango pickle;BJ:Bhaat jaanr;T:Galic chilly;HKT:Fermented mustard

Ga:Galactose;Ra:Raffinose;La:Lactose;So:Sorbitol;Ma:Maltose;De:Dextrose;Ar:Arabinose

Table 4.2: Morphological and Biochemical characterization

S.no	Isolate No.	Gram's Reaction	Shape	Catalase test	Carbohydrate fermentation								Source
					Ga	Ra	La	So	Ma	De	Ar	Mo	
49	T-49	+	Rods	-	-	+	-	-	-	-	-	-	TP
50	T-50	+	Rods	-	+	+	+	-	-	-	-	-	TP
51	T-51	+	Rods	-	+	-	-	-	-	+	-	-	TP
52	T-52	+	Rods	-	+	-	-	-	-	-	-	-	TP
53	T-53	+	Rods	-	-	+	-	-	-	+	-	-	TP
54	T-54	+	Rods	-	+	+	+	-	-	-	-	-	TP
55	T-55	+	Rods	-	+	-	+	-	-	+	-	-	TP
56	T-56	+	Rods	-	+	-	+	-	-	-	-	-	TP
57	T-57	+	Rods	-	+	-	+	-	-	+	-	-	TP
58	T-58	+	Rods	-	-	+	-	-	-	-	-	-	TP
59	T-59	+	Rods	-	+	-	+	-	-	+	-	-	TP
60	T-60	+	Rods	-	-	-	-	-	-	+	-	-	TP
61	T-61	+	Rods	-	-	-	+	-	-	-	-	-	TP
62	T-62	+	Rods	-	+	+	+	-	-	-	-	-	TP
63	T-63	+	Rods	-	+	-	-	-	-	-	-	-	TP
64	T-64	+	Rods	-	+	-	-	-	-	+	-	-	TP
65	T-65	+	Rods	-	+	+	-	-	-	-	-	-	TP
66	T-66	+	Rods	-	-	+	-	-	-	-	-	-	TP
67	T-67	+	Rods	-	+	-	+	-	-	+	-	-	TP
68	HKT-1	+	Rods	-	+	-	+	-	-	+	-	-	HM
69	HKT-2	+	Rods	-	+	+	-	-	-	+	-	-	HM
70	HKT-3	+	Rods	-	-	+	-	-	-	+	-	-	HM
71	HKT-4	+	Rods	-	-	-	-	-	-	+	-	-	HM
72	HKT-5	+	Rods	-	-	-	-	-	-	-	-	-	HM
73	HKT-6	+	Rods	-	+	-	+	-	-	+	-	+	HM
74	HKT-7	+	Rods	-	+	-	+	-	-	+	-	-	HM
75	HKT-8	+	Rods	-	-	-	-	-	-	-	-	-	HM
76	HKT-9	+	Rods	-	-	-	-	-	-	+	-	-	HM
77	HKT-10	+	Rods	-	+	+	+	-	-	+	-	-	HM
78	HKT-11	+	Rods	-	-	-	-	-	-	-	-	-	HM
79	HKT-12	+	Rods	-	+	-	+	-	-	-	-	-	HM
80	HKT-13	+	Rods	-	+	+	-	-	-	-	-	-	HM
81	HKT-14	+	Rods	-	+	-	-	-	-	-	-	-	HM
82	HKT-15	+	Rods	-	-	-	-	-	-	+	-	-	HM
83	HKT-16	+	Rods	-	-	-	-	-	-	-	-	-	HM
84	HKT-17	+	Rods	-	-	-	-	-	-	-	-	-	HM
85	HKT-18	+	Rods	-	-	-	-	-	-	-	-	-	HM
86	HKT-19	+	Rods	-	-	-	-	-	-	+	-	-	HM
87	HKT-20	+	Rods	-	+	-	+	-	-	+	-	-	HM
88	HKT-21	+	Rods	-	-	-	-	-	-	+	-	-	HM
89	HKT-22	+	Rods	-	-	-	-	-	-	-	-	-	HM
90	HKT-23	+	Rods	-	-	-	-	-	-	-	-	-	HM
91	HKT-24	+	Rods	-	-	-	-	-	-	+	-	-	HM
92	HKT-25	+	Rods	-	-	-	-	-	-	-	-	-	HM
93	HKT-26	+	Rods	-	-	-	-	-	-	-	-	-	HM
94	HKT-27	+	Rods	-	-	-	-	-	-	+	-	-	HM
95	HKT-28	+	Rods	-	-	-	-	-	-	+	-	-	HM
96	HKT-29	+	Rods	-	-	-	-	-	-	+	-	-	HM

MP:Mango pickle;BJ:Bhaat jaanr;TP:Galic chilly;HM:Fermented mustard

Ga:Galactose;Ra:Raffinose;La:Lactose;So:Sorbitol;Ma:Maltose;De:Dextrose;Ar:Arabinose

4.3 Survival after the successive passages through artificial saliva, gastric and intestinal juice

Ninty-four isolates from pickle mango, garlic–chilli, Bhaati jaanr, Mahula liquor and children’s faeces were screened for their survival under simulated gastrointestinal conditions. Strains of *Lactobacillus casei* (LAM-1 and LAM-2), *Lactobacillus delbrueckii* (LKH-2 and LKH-3), *Lactobacillus helveticus* (LKH-5), and *Lactobacillus fermentum* (Lamec-29), with good gastrointestinal survival were then chosen for further studies of probiotic properties (Fig. 4.1). All other strains, which did not survive under gastrointestinal conditions were not investigated further. The viability of the *Lactobacillus casei* (LAM-1 and LA-1M) strains upon successive passages through artificial saliva and gastric duodenum juices is shown in Figure 4.1 A. Strain LAM-1 showed notable survival (75%) in simulated gastric and intestinal transit, whereas 62% LAM-2 cells survived these conditions.

The slight decline in the live counts of *Lactobacillus casei* strain seemed to be due to the effect of gastric juice (determined after 1 h incubation with simulated acid gastric juice and before addition of artificial duodenum juice), because in the following stages of incubation, cell counts did not changes more than approx. 1 log units. Tolerance of *L. casei* LAM-1 towards duodenum juice was higher than that of *L. casei* LAM-2 which was more sensitive to artificial duodenum juice. Strains belonging to the *Lactobacillus fermentum* group showed a low variability in their survival. Some strains presented the best survival rates in this study, even higher than those observed for the *L. casei* strains, for e.g., Lamec-29 with 82% survival rate (Fig. 4.1 C). Other strains (results not shown) failed to survive under simulated gastric and intestinal condition, these strains were therefore not chosen for further studies.

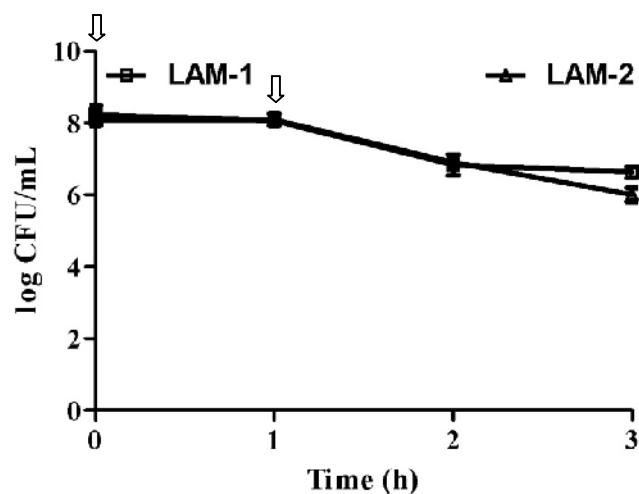


Fig. 4.1 (A) Effect of simulated gastric and intestinal transit on viability of *L. casei*. Arrows indicate addition of simulated gastric juice at time 0 h and simulated duodenum juice after 1 h, respectively. The values shown mean of three experiments. Bar represent the standard error

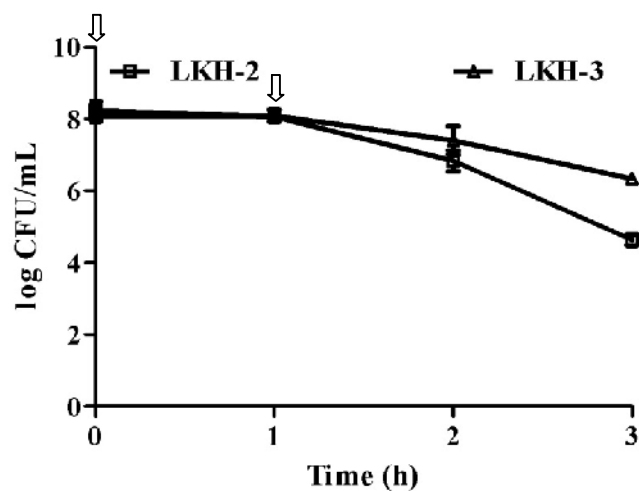


Fig. 4.1 (B) Effect of simulated gastric and intestinal transit on viability of *L. delbruckeii*. Arrows indicate addition of simulated gastric juice at time 0 h and simulated duodenum juice after 1 h, respectively. The values shown mean of three experiments. Bar represent the standard error

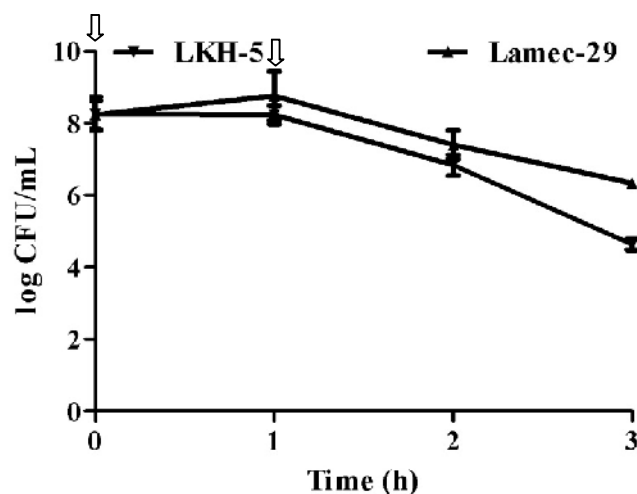


Fig. 4.1 (C) Effect of simulated gastric and intestinal transit on viability of *L. helveticus* and *L. fermentum*. Arrows indicate addition of simulated gastric juice at time 0 h and simulated duodenum juice after 1 h, respectively. The values shown mean of three experiments. Bar represent the standard error.

4.4 Phenotypic identification of strains

Gram-positive, catalase-negative and rod-shaped bacteria which were able to survive the passage through simulated gastric and duodenum juice in the *in vitro* gastrointestinal model were further identified, and their probiotic characteristics were studied.

The selected strains were identified to the species-level by both phenotypic and genotypic investigations. Two strains (LAM-1 and LAM-2) were selected for further studies which stemmed from mango pickle, and they showed phenotypic properties typical of *Lactobacillus casei* strains. Accordingly, these bacteria were rod-shaped, produced DL-lactate, possessed *mDAP* in the cell walls and did not produce gas from glucose fermentation (Table 4.3). These strains were able to ferment ribose, a pentose sugar, which implies that the strains were facultatively heterofermentative *Lactobacilli*. In addition, two strains from (Kharoli) fermented mashed mustard (LKH-2 and LKH-3) also showed similar characteristics to *L. delbrueckii* indicating that these could also be characterised as presumptive *L. delbrueckii* strains. These two strains not only fermented ribose, but also an additional pentose sugar, i.e., arabinose (Table 4.3). Two other strains (LKH-5 and Lamec-29) which

stemmed from Hukutti mass and children meconium exhibited characteristics typical of *Lactobacilli* of the *L. helveticus* and *L. fermentum*-group respectively. These strains produced gases from glucose and also fermented the pentose sugars tested (Table 4.3), indicating obligately homofermentative *Lactobacilli*. Furthermore, they lacked mDAP in the cell wall and produced DL- lactate (Table 4.3).

4.5 Enzyme assay

None of the six isolates produced enzymes such as alkaline phosphatase, esterase (C₄), esterase lipase (C₈)-lipase (C₁₄), trypsin, cystine acrylamidase, α -mannosidase, and α -fucosidase (Table 4.4). However, leucin acrylamidase, valine acrylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α , β -galactosidase, β -glucosidase, and N-acetyl- β -glucosaminidase were detected. The carcinogen enzyme, β -glucuronidase was not produced by any of the isolated *Lactobacilli*.

Table 4.3 Enzyme profile of *Lactobacillus* spp.

Enzyme	LAM-1	LAM- 2	LKH-2	LKH-3	LKH-5	Lamec-29
Control	0	0	0	0	0	0
Alkaline phosphatase	0	0	0	0	0	0
Esterase (C ₄)	0	0	0	0	0	0
Esterase lipase (C ₈)	0	0	0	0	0	0
Lipase (C ₁₄)	0	0	0	0	0	0
Leucine acrylamidase	5	5	5	5	5	2
Valine arylamidase	5	5	5	5	5	4
Cystine acrylamidase	0	0	0	0	0	0
Trypsin	0	0	0	0	0	0
α -Chymotrypsin	0	0	0	0	0	4
Acid phosphotase	2	2	2	2	2	5
Napthol-AS-BI-phosphohydrolase	3	3	3	3	3	3
α -Galactosidase	4	4	4	4	4	4
β - Galactosidase	4	4	4	4	4	4
β -Glucuronidase	0	0	0	0	0	0
α -Glucosidase	4	4	4	4	4	2
N-acetyl-B-	2	2	2	2	2	2
α -Mannosidase	0	0	0	0	0	0
α -Fucosidase	0	0	0	0	0	0

4.6 Molecular characterization of the bacterial isolates

The 16S rDNA sequence of all the isolates was obtained by sequencing the 16S rDNA amplicon with the primers 16Sseqfw (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16Sseqrev (5'-GGN TAC CTT GTT ACG ACT TC-3'). Sequences of all the respective cultures were deposited in the GenBank database with accession number (Table 4.5). To identify these six bacterial isolates, all were subjected to 16S rRNA amplification using primers, and about 1.5 Kb amplicon was observed in all isolates (Fig. 4.2). 16S rRNA PCR products were sequenced using Applied Biosystems automatic sequencer. Sequencing reactions were performed with the primers.

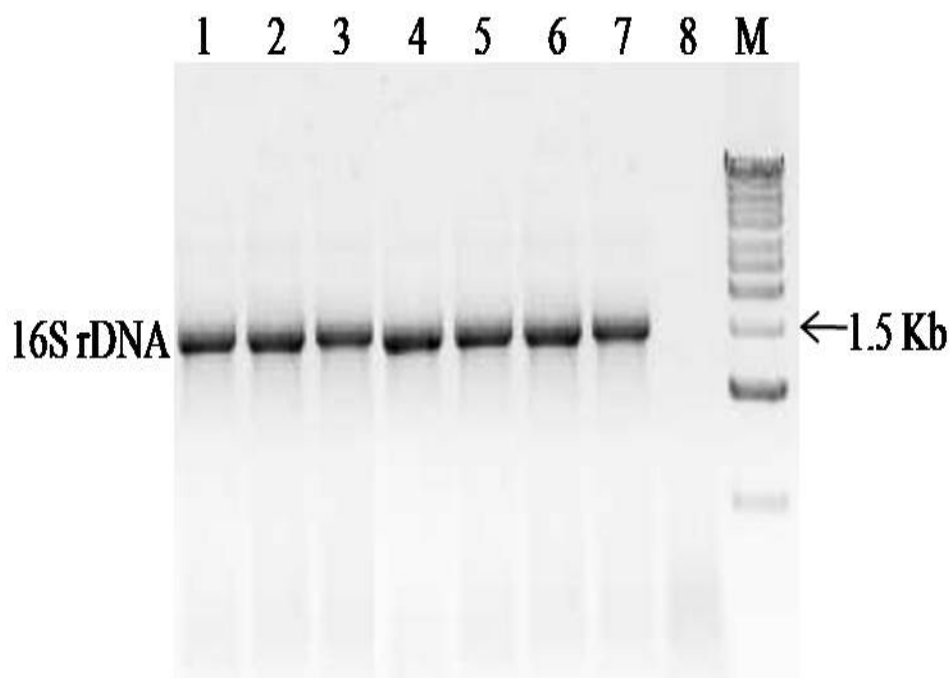


Fig. 4.2 16S rDNA amplification of bacterial isolates. Lane 1-7: LAM-1, LAM-2, LKH-2, LKH-3, LKH-5, HKT-9 and Lamec-29; Lane 8: Control and Lane M: 1 Kb marker.

Table 4.4 Phenotypic characterization of *Lactobacillus* strains (only those relevant for obligately homofermentative and facultatively heterofermentative *Lactobacilli* are included):

Strain	Source	Gas from Glucose	mDAP	Lactic Acid Isomer	NH ₃ from Arginine	Growth (°C) 15/45	Ribose	Cellobiose	Galactose	Lactose	Maltose	Mannitol	Mannose	Melibiose	Raffinose	Salicin	Sucrose	Trehalose	Arabinose
<i>L. casei</i> LAM-1 ^a	Mango Pickle	-	+	DL	-	+/-	+	+	-	+	-	-	-	-	-	+	+	+	-
<i>L. casei</i> LAM-2 ^a	Mango Pickle	-	+	DL	-	+/-	+	+	-	+	-	-	-	-	-	+	+	+	-
<i>L. delbrueckii</i> LKH-2	Fermented Mashed Mustard	+	+	DL	-	+/+	+	+	+	+	+	+	+	+	+	+	+	-	+
<i>L. delbrueckii</i> LKH-3	Fermented Mashed Mustard	+	+	DL	-	+/-	+	+	+	+	+	+	+	+	+	+	+	-	+
<i>L. helveticus</i> LKH-5 ^b	Fermented Mashed Mustard	+	+	DL	-	+/+	+	+	+	+	+	+	+	+	+	+	+	-	-
<i>L. fermentum</i> ^b Lamec-29	Children's meconium	+	+	DL	-	-/+	+	+	+	-	-	-	-	-	-	+	+	-	+

^a : obligately homofermentative ^b : facultatively heterofermentative

Table 4.5 Probiotic isolated from respective samples with accession number from NCBI

Source	Isolates	Isolates designation	Accession number
Mango Pickle	<i>Lactobacillus casei</i>	LAM-1	JN620211
Mahua liquor	<i>Lactobacillus casei</i>	LAM-2	JN618457
Fermented Mashed Mustard	<i>Lactobacillus delbrueckii</i>	LKH-2	JN620214
Bhhati jaanar	<i>Lactobacillus delbrueckii</i>	LKH-3	JN620213
Kharoli	<i>Lactobacillus helveticus</i>	LKH-5	JN620212
Children's meconium	<i>Lactobacillus fermentum</i>	Lamec-29	JN620215

4.6.1 Sequence alignment of *Lactobacillus* spp.

The sequences were analyzed by multiple sequence alignment to check the similarities among the isolates. The homologies among the sequences varied from 91 to 98% between isolates. Minimum of 91% similarity was found in LKH-3 with LKH-5 and maximum 98% similarity was found between LKH-2 and LAM-1 (Table 5). None of these isolates had shown 99% or more similarity among themselves. Mango pickle and mahua liquor isolates LAM-1 and LAM-2 had shown 95% similarities.

Table 4.6 Percentage similarity of 16S rRNA sequences of bacterial isolates using multiple sequence alignment (ClustalW)

SeqA	Name	Len (nt)	SeqB	Name	Len (nt)	Score
1	LAM-1	1445	2	LAM-2	1520	95
1	LAM-1	1445	3	LKH-2	1357	93
1	LAM-1	1445	4	LKH-3	1349	93
1	LAM-1	1445	5	LKH-5	1429	92
1	LAM-1	1445	6	Lamec-29	1489	93
2	LAM-2	1520	3	LKH-2	1357	92
2	LAM-2	1520	4	LKH-3	1349	92
2	LAM-2	1520	5	LKH-5	1429	92
2	LAM-2	1520	6	Lamec-29	1489	91
3	LKH-2	1357	4	LKH-3	1349	92
3	LKH-2	1357	5	LKH-5	1429	93
3	LKH-2	1357	6	Lamec-29	1489	98
4	LKH-3	1349	5	LKH-5	1429	93
4	LKH-3	1349	6	Lamec-29	1489	92
5	LKH-5	1429	6	Lamec-29	1489	94

The phylogenetic tree (Fig. 4.3) constructed with sequences of representative bacteria revealed that all the six isolates clustered with other members of the phylum firmicutes and were associated with *Bacillus* group. The related sequences showing similarity in BLAST were retrieved from GenBank and aligned using the program CLUSTAL W (Thompson *et al.*, 1994). The resulting multiple alignments were optimized visually and the evolutionary distance were calculated by Kimura 2 parameter. Phylogenetic dendrogram were constructed

by neighbor-joining method using MEGA 5 package (Tamura *et al.*, 2007). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses. The complete 16S rRNA sequence analysis revealed that bacterial isolates had 77% to 100% similarity with the sequences of NCBI database. The isolate LKH-3 was identified as *Lactobacillus helveticus* as it showed 100% resemblance with it. On the basis of phylogenetic data, isolate Lamec-29 had close resemblance with members of the genus *Lactobacillus*. The percentage similarities were calculated between Lamec-29 and *Lactobacillus fermentum* and found to be similar approximate 86%. The isolate LAM-1 had shown maximum similarity with *Lactobacillus casei* which is known to probiotic bacterium. The isolate LKH-2 showed 97% similarity with two different *Lactobacillus* species, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The percentage differences calculated between isolate LAM-2 and three different species of *Lactobacillus casei*, viz., *Lactobacillus casei* AB605428, *Lactobacillus casei* HQ117896 and *Lactobacillus casei* JN560922 suggest that this may represent a new species of genus *Lactobacillus*.

The isolate LKH-3 showed 96% homology with two different *Lactobacillus* species, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. The phylogenetic tree identified this isolate as *Lactobacillus* spp. It is interesting that the sources of all the isolates of NCBI database which were used to construct phylogenetic tree were probiotic bacteria. It matches well with the fact that isolates of present study were also isolated from traditional food and human gut and possess probiotic attributes. It is noteworthy that isolates Lamec-29, LKH-2, LKH-3 and LAM-2 16S rRNA did not show high identity with sequences in the database. The sequences that share an identity below 98% are usually considered to be part of the same genus (Sadowsky *et al.*, 1996). On this basis, these isolates described here probably represent a new member of a known genus (*Lactobacillus*), which

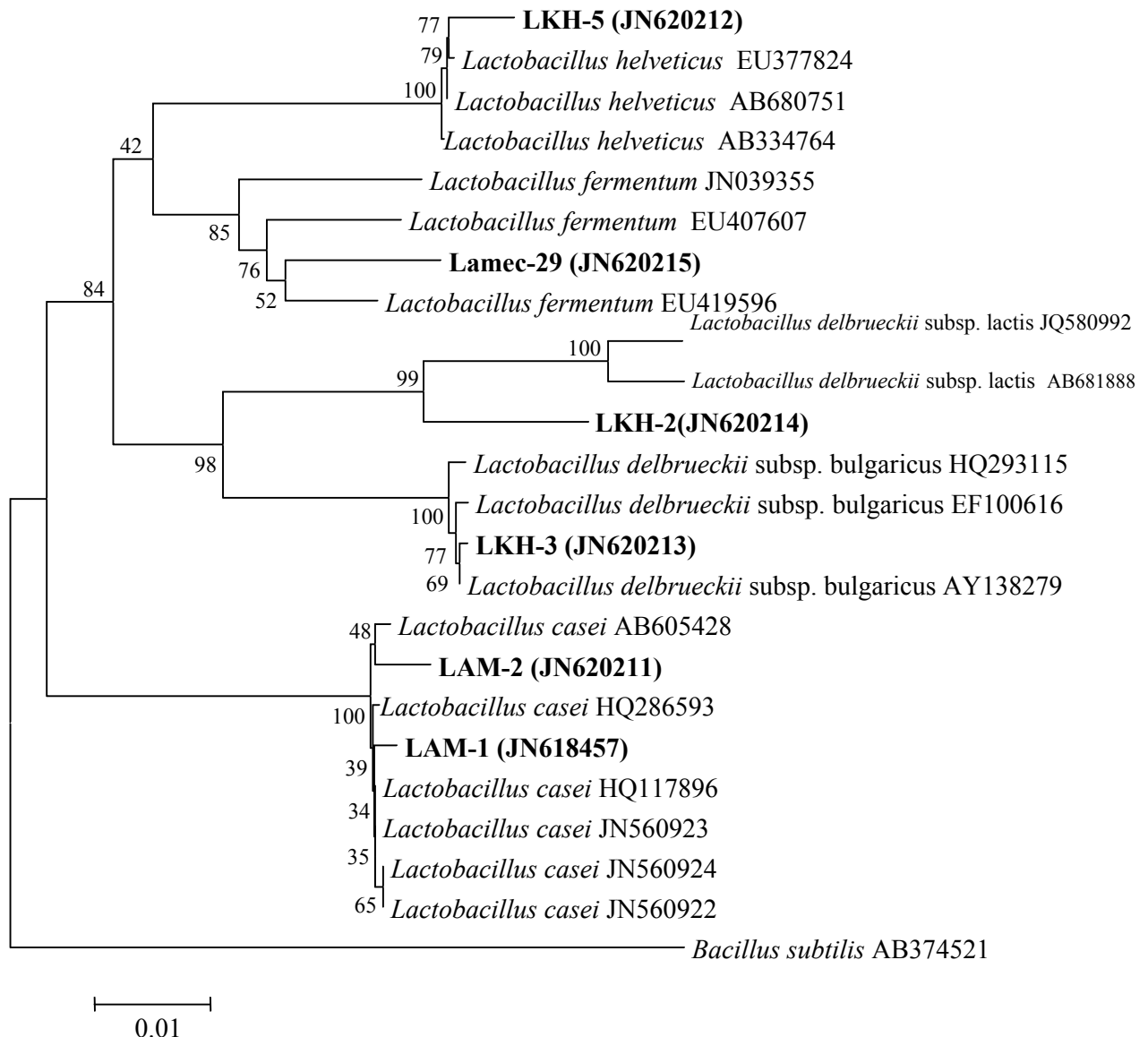


Table 4.7 Aligned Sequence Data of *Lactobacillus casei* strain LAM-1

LOCUS	JN620211	1445 bp	DNA	linear	BCT 15-NOV-2011
DEFINITION	<i>Lactobacillus casei</i> strain LAM-1 16S ribosomal RNA gene, partial sequence.				
ACCESSION	JN620211				
VERSION	JN620211.1 GI:356817736				
KEYWORDS					
SOURCE	<i>Lactobacillus casei</i>				
ORGANISM	Lactobacillus casei Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae; Lactobacillus.				
REFERENCE	1 (bases 1 to 1445)				
AUTHORS	Mukesh, S.K. and Abhijit, G.				
TITLE	Direct Submission				
JOURNAL	Submitted (10-AUG-2011) Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab 147004, India				
FEATURES	Location/Qualifiers				
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Table 4.8 Aligned Sequence Data of *Lactobacillus casei* strain LAM-2

LOCUS	JN618457	1520 bp	DNA	linear	BCT 23-NOV-2011
DEFINITION	<i>Lactobacillus casei</i> strain LA-1M 16S ribosomal RNA gene, partial sequence.				
ACCESSION	JN618457				
VERSION	JN618457.1 GI:357540867				
KEYWORDS					
SOURCE	<i>Lactobacillus casei</i>				
ORGANISM	Lactobacillus casei Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae; Lactobacillus.				
REFERENCE	1 (bases 1 to 1520)				
AUTHORS	Singh, M.K., Singla, R. and Ganguli, A.				
TITLE	Isolation and characterization of beneficial microbes from traditional food of India				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 1520)				
AUTHORS	Singh,M.K., Singla,R. and Ganguli,A.				
TITLE	Direct Submission				
JOURNAL	Submitted (25-AUG-2011) Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab 147004, India				
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Table 4.9 Aligned Sequence Data of *Lactobacillus delbrueckii* strain LKH-2

LOCUS	JN620214	1357 bp	DNA	linear	BCT 15-NOV-2011
DEFINITION	<i>Lactobacillus delbrueckii</i> strain LKH-2 16S ribosomal RNA gene, partial sequence.				
ACCESSION	JN620214				
VERSION	JN620214.1 GI:356817803				
KEYWORDS	.				
SOURCE	<i>Lactobacillus delbrueckii</i>				
ORGANISM	Lactobacillus delbrueckii Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae; Lactobacillus.				
REFERENCE	1 (bases 1 to 1357)				
AUTHORS	Mukesh, S.K. and Abhijit, G.				
TITLE	Direct Submission				
JOURNAL	Submitted (10-AUG-2011) Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab 147004, India				
FEATURES	Location/Qualifiers				
source	1..1357 /organism="Lactobacillus delbrueckii" /mol_type="genomic DNA" /strain="LKH-2" /isolation_source="fermented mashed mustard (Kharoli) seed" /host="Brassica campestris var. toria" /db_xref="taxon:1584" /country="India: Kajipet, Aruranchal Pradesh" /collection_date="13-Mar-2008" /collected_by="Mukesh Kumar Singh" rRNA <1..>1357 /product="16S ribosomal RNA"				
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Table 4.10 Aligned Sequence Data of *Lactobacillus delbrueckii* strain LKH-3

LOCUS	JN620213	1349 bp	DNA	linear	BCT 15-NOV-2011
DEFINITION	<i>Lactobacillus delbrueckii</i> strain LKH-3 16S ribosomal RNA gene, partial sequence.				
ACCESSION	JN620213				
VERSION	JN620213.1 GI:356817779				
KEYWORDS	.				
SOURCE	<i>Lactobacillus delbrueckii</i>				
ORGANISM	Lactobacillus delbrueckii Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae; Lactobacillus.				
REFERENCE	1 (bases 1 to 1349)				
AUTHORS	Mukesh, S.K. and Abhijit, G.				
TITLE	Direct Submission				
JOURNAL	Submitted (10-AUG-2011) Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab 147004, India				
FEATURES	Location/Qualifiers				
source	1..1349 /organism="Lactobacillus delbrueckii" /mol_type="genomic DNA" /strain="LKH-3" /isolation_source="fermented mashed mustard (Kharoli)" /host="Brassica campestris var. toria" /db_xref="taxon: 1584 " /country="India: Manipur, Assam" /collection_date="23-Mar-2009" /collected_by="Mukesh Kumar Singh" /identified_by="Mukesh Kumar Singh" rRNA <1..>1349 /product="16S ribosomal RNA"				
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Table 4.11 Aligned Sequence Data of *Lactobacillus helveticus* strain LKH-5

LOCUS	JN620212	1429 bp	DNA	linear	BCT 15-NOV-2011
DEFINITION	Lactobacillus helveticus strain LKH-5 16S ribosomal RNA gene, partial sequence.				
ACCESSION	JN620212				
VERSION	JN620212.1 GI:356817760				
KEYWORDS	.				
SOURCE	Lactobacillus helveticus				
ORGANISM	Lactobacillus helveticus Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae; Lactobacillus.				
REFERENCE	1 (bases 1 to 1429)				
AUTHORS	Mukesh, S.K. and Abhijit, G.				
TITLE	Direct Submission				
JOURNAL	Submitted (10-AUG-2011) Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab				
147004,	India				
FEATURES	Location/Qualifiers				
source	1..1429 /organism="Lactobacillus helveticus" /mol_type="genomic DNA" /strain="LKH-5" /isolation_source="fermented mashed mustard (Kharoli)" /db_xref="taxon: 1587 " /country="India: Guwahati, Assam" /collection_date="29-Mar-2009" /collected_by="Mukesh Kumar Singh" rRNA <1..>1429 /product="16S ribosomal RNA"				
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Table 4.12 Aligned Sequence Data of *Lactobacillus fermentum* strain LAmec-29

LOCUS	JN620215	1489 bp	DNA	linear	BCT 15-NOV-2011
DEFINITION	Lactobacillus fermentum strain LAmec-29 16S ribosomal RNA gene, partial sequence.				
ACCESSION	JN620215				
VERSION	JN620215.1 GI:356817817				
KEYWORDS	.				
SOURCE	Lactobacillus fermentum				
ORGANISM	Lactobacillus fermentum Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae; Lactobacillus.				
REFERENCE	1 (bases 1 to 1489)				
AUTHORS	Mukesh, S.K. and Abhijit, G.				
TITLE	Direct Submission				
JOURNAL	Submitted (18-AUG-2011) Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab				
147004,	India				
COMMENT	Sequences were screened for chimeras by the submitter using Bellerophon 3.0.				
FEATURES	Location/Qualifiers				
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4.7 Probiotic Attributes of *Lactobacillus* spp.

4.7.1 Survival of *Lactobacillus* strains under acidic condition

Different regions of the gastrointestinal tract have varying acid levels. Stomach and the regions after stomach have the highest acidity and the pH of these areas may fall as low as pH 2.0. In order to be used as beneficial adjuncts, *Lactobacillus* must be able to survive these harsh conditions and colonise in the gut and therefore tolerant acidity.

Survivals of six different *Lactobacillus* strains under acidic conditions (pH 2.0) are illustrated in Table 4.13. In general, the number of survivors of all the cultures during 3 h of incubation decreased under acidic conditions. The viable count log CFU/ml substantially decreased at pH 2.0. LAM-1 showed the highest viability followed by LKH-2, Lamec-29 LKH-5; LAM-2 and LKH-2 had also shown moderate activity at pH 2.0. As shown in Table 4.13 at pH 2.0, LKH-3 had lowest viability.

Table 4.13 Survival of *Lactobacillus* strains in MRS broth at pH 2.0 at 37°C, as determined by viable count.

Strains	Viable count ^a (log ₁₀ CFU/ml)		
	0 h	3 h	% inhibition ^b
LAM-1	9.23 ± 0.07	9.20 ± 0.39	- ^c
LKH-2	9.71 ± 0.12	6.50 ± 0.47	33.0
LKH-3	9.71 ± 0.08	3.96 ± 0.36	59.2
LKH-5	9.76 ± 0.23	7.79 ± 0.81	20.1
LAM-2	9.56 ± 0.03	7.23 ± 1.12	24.3
Lamec-29	9.25 ± 0.08	7.96 ± 0.04	13.9

^a log mean counts of three trials (average ± s.d.); -no inhibition; n = 3; observations comes from three replicate assays; data are represented as mean ± sd; ^b % inhibition = [(cfu/ml initial – cfu/ml final)/ cfu/ml initial] x 100.

4.7.2 Survival of *Lactobacillus* strains in the presence of bile

Gastrointestinal systems have varying concentrations of bile. The rate of secretion of bile and the concentration of bile in different regions of the intestine vary, depending mainly on the type of food consumed and it may not be possible to predict the bile concentration in the intestine at any given moment. The viable count of six different *Lactobacillus* strains in the bile concentrations of 0.3% was presented in Tables 4.14. All six *Lactobacillus* strains showed different degrees of sensitivity towards this compound up to 3 h of incubation. LAM-1 showed highest growth followed by Lamec-29 and LKH-2 at 0.3% bile concentration. Remaining *Lactobacillus* strains were observed to have fair growth up to 3 h of incubation. Results of 0.3% bile concentration were presented in (Table 4.14). The isolate LKH-5, LAM-2 and LKH-3 showed reasonable growth during incubation.

Table 4.14 Ability of *Lactobacillus* strains to grow in MRS broth in the presence of bile 0.3% at 37°C

Strains	Viable count ^a (log ₁₀ CFU/ml)		
	0 h	3 h	% inhibition ^b
LAM-1	9.83 ± 0.07	8.20 ± 0.39	16.58
LAM-2	9.06 ± 0.03	6.23 ± 1.12	31.29
LKH-2	9.21 ± 0.12	6.50 ± 0.47	15.39
LKH-3	9.81 ± 0.08	5.96 ± 0.36	39.2
LKH-5	9.96 ± 0.23	5.79 ± 0.81	41.9
Lamec-29	9.05 ± 0.08	7.96 ± 0.04	12.0

^a log mean counts of three trials (average ± s.d.); -cno inhibition; n = 3; observations comes from three replicate assays; data are represented as mean ± sd; ^b % inhibition = [(cfu/ml initial – cfu/ml final) / cfu/ml initial] x 100

4.7.3 Resistance of *Lactobacillus* strains to 0.4% phenol

Resistance to phenol was tested as an additional indicator for survival under intestinal conditions (Xanthopoulos *et al.*, 2000). Six probiotic candidate strains (*L. casei* LAM-1, LAM-2, *L. delbrueckii* LKH-2, LKH-3, *L. helveticus* LKH-5 and *L. fermentum* Lamc-29) which survived the *in vitro* gastrointestinal passage as described above, were tested and showed different degrees of sensitivity towards phenol. All *L. casei* strains were less sensitive to phenol. Five of the six strains tolerated 0.4% phenol for 24 h, as their numbers did not decrease (initial inoculum of approx. log 7.6 to 8.0). However, five of the strains were able to grow in the presence of phenol during this incubation time (Table 4.15).

Table 4.15 Ability of *Lactobacillus* strains to grow in the presence of phenol 0.4% at 37°C

Strains	Viable count ^a (log ₁₀ CFU/ml)					
	MRS broth			MRS broth + phenol 0.4%		
	0 h	24 h	Increase ^b	0 h	24 h	Increase ^b
LAM-1	7.95±0.06	10.09±0.02	2.14	8.05±0.14	8.75±0.34	0.70
LAM-2	8.17±0.16	10.65±0.07	2.48	8.12±0.13	8.52±0.40	0.40
LKH-2	8.09±0.04	9.88±0.11	1.79	7.98±0.43	8.64±0.51	0.66
LKH-3	7.87±0.10	8.87±0.24	1.00	8.05±0.08	8.63±0.09	0.58
LKH-5	7.07±0.03	7.29±0.12	0.22	7.40±0.11	7.42±0.02	0.02
Lamc-29	7.36±0.12	9.12±0.03	1.76	7.24±0.21	5.65±0.28	-1.59

^a log mean counts of two trials (average ± s.d.), bincrease = log₁₀(final population)-log₁₀(initial population) n = 3; observations comes from three replicate assays; data are represented as mean ± sd

4.7.4 Antagonistic activity against pathogens

In the agar spot test, the indicator strains, *S. aureus* ATCC 9144, *A. hydrophila* ATCC 35654, *Y. enterocolitica* ATCC 9610, *E. sakazakii* ATCC 51329, *S. flexneri* 2a, *S. typhimurium* ATCC 19585, *L. monocytogenes* ATCC 1911, *E. coli* 0157:H7, *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 showed weak to strong inhibition (zone of inhibition of more than 1 mm from edge of producer colony up to 23 mm) as listed in Table 4.16. *L. acidophilus* ATCC 4356 was not inhibited at all by five of the *Lactobacillus* strains tested but weakly inhibited by *L. fermentum* Lamec-29 strain. The indicator strains belonging to the *Lactobacillus* genus were weakly or not inhibited (inhibition zone of less than 2 mm) except in the case of *L. monocytogenes* ATCC 1911, where as the maximum inhibition was observed in the case of *E. sakazakii* ATCC 51329 by *L. fermentum* Lamec-29.

The inhibitory activity was not due to bacteriocin production, as neutralised, cell-free supernatant of the producer culture did not exhibit any antimicrobial activity when compared to the effect of live cells in the agar spot test. Therefore, the inhibitory activity observed probably depended on production and diffusion of organic acids into the medium. Hydrogen peroxide could hypothetically also act as an inhibitory substance, but the incubation of the plates under anaerobic conditions overrules this as a possible cause for the observed inhibition. Production of H_2O_2 under aerobic conditions was also investigated and it was observed that two of the *L. delbrueckii* strains tested (LKH-2 and LKH-3) and one strain of *L. helveticus* (LKH-5) were able to produce H_2O_2 (Table 4.17)

Table 4.16 Agar spot test for detection of antagonistic activity

Probiotic candidates	Strain	<i>S. aureus</i> ATCC 9144	<i>A. hydrophila</i> ATCC 35654	<i>Y. enterocolitica</i> ATCC 9610	<i>E. sakazakii</i> ATCC 51329	<i>S. flexneri</i> 2a	<i>S. typhimurium</i> ATCC 19585	<i>L. monocytogenes</i> ATCC 1911	<i>E. coli</i> 0517:H7	<i>L. acidophilus</i> ATCC 4356	<i>L. plantarum</i> ATCC 8014
<i>L. casei</i>	LAM-1	13	10	4	18	6	18	16	13	0	0
	LAM-2	10	14	4	19	8	16	18	10	0	0
<i>L. delbrueckii</i>	LKH-2	8	6	2	16	6	6	16	8	0	2
	LKH-3	6	8	4	16	4	6	17	6	0	0
<i>L. helveticus</i>	LKH-5	6	8	4	14	6	6	12	6	0	0
<i>L. fermentum</i>	Lamec-29	16	8	6	23	10	12	16	4	2	2

^a: Inhibitory activity expressed as zone of inhibition surrounding the colony in mm

4.7.5 Bile salt hydrolase and β -galactosidase activities

All the candidate probiotic strains were tested for their ability to hydrolyse the sodium salt of taurodeoxycholic acid. All strains possessed bile salt hydrolase activity (Table 4.17), and in connection with this, it was noted that all these strains also exhibited high resistance to duodenum juice containing 0.3% bile salts in the gastrointestinal passage model, which may be connected with this Bsh activity as suggested by some authors (De Smet *et al.*, 1995; De Smet *et al.*, 1998; De Boever, 2000). All six strains which were able to grow on lactose as the sole carbon source tested on this medium were further investigated for β -galactosidase activity. The highest activity (8.7 $\mu\text{mol/ml/min}$) was determined for the *L. casei* LAM-1 strain (Table 4.17). This strain was followed by the reference strain *L. plantarum* ATCC 8104, which also exhibited a high β -galactosidase activity of 9.3 $\mu\text{mol/ml/min}$.

Table 4.17 H₂O₂ production and enzymatic activities of selected potentially probiotic *Lactobacillus* strains

Strain		<i>B-galactosidase</i>		
		H ₂ O ₂	<i>B-galactosidase</i> activity	<i>Bsh</i>
Probiotic candidates				
<i>Lactobacillus casei</i>	LAM-1	-	8.7	+
	LAM-2	-	5.45	+
<i>Lactobacillus delbrueckii</i>	LKH-2	+	6.29	+
	LKH-3	+	8.2	+
<i>Lactobacillus. helveticus</i>	LKH-5	+	2.8	+
<i>Lactobacillus fermentum</i>	Lamec-29	-	8.31	+
Reference strain				
<i>L. plantarum</i>	ATCC 8104	+	9.3	+

4.7.6 Antibiotic Resistance

All strains tested were resistant to streptomycin and gentamicin, as well as to ciprofloxacin (Table 4.18). In contrast, all strains were considered as susceptible to the antibiotics erythromycin, ampicillin, penicillin, benzylpenicillin, tetracycline and chloramphenicol (Table 4.18), from the MIC breakpoint values as suggested by SCAN (Chesson *et al.*, 2002).

Table 4.18 Antibiotic Resistance of selected potentially probiotic *Lactobacillus* strains

Strain		Minimum inhibitory concentration (µg/ml)							
		EM	GM	AB	TC	CL	SM	CI	PG
Probiotic Candidates									
<i>L. casei</i>	LAM-1	1	64	0.064	6.0	2.0	2.0	32	0.38
	LAM-2	1	96	0.064	6.0	2.0	2.0	32	0.38
<i>L. delbrueckii</i>	LKH-2	0.75	n.d	0.032	3.0	2.0	12	8	0.75
	LKH-3	0.75	n.d	1.0	0.25	1.5	12	16	0.125
<i>L. helveticus</i>	LKH-5	1	32	0.064	3	2.0	16	n.d	0.25
<i>L. fermentum</i>	Lamec-29	32	256	25	48	32	8	256	32
Breakpoint value		4	1	2	16	16	16	4	2

em: erythromycin, gm: gentamicin, ab: ampicillin, tc: tetracycline, cl: chloramphenicol, sm: streptomycin, ci: ciprofloxacin, pg:

benzylpenicillin. n.d.: not determined. ^a: breakpoints according to scan (2002).

4.8 Adhesive properties

4.8.1 Microbial adhesion to solvents

The use of three solvents allowed the evaluation of the hydrophobic/hydrophilic cell surface properties of *Lactobacilli* and their Lewis acid-base (electron donor and acceptor) characteristics (Pelletier *et al.*, 1997; Briandet *et al.*, 1999). As shown in Table 4.19, strains of *L. delbrueckii* and *L. helveticus* had a low partitioning percentage in the apolar solvent n-hexadecane, indicating that these strains possess a hydrophilic surface. All *L. casei*, and *L. fermentum* strains were characterised by a high affinity to n-hexadecane, indicating the hydrophobic nature of their surfaces (Table 4.19).

In order to measure the Lewis acid-base properties of the bacterial surface, two solvents (chloroform and ethyl acetate) with the same van der Waals properties as n-hexadecane (Pelletier *et al.*, 1997) were used in order to be sure that the affinity for each solvent tested was not due to van der Waals forces. The results of the microbial adhesion to chloroform showed that *L. delbrueckii* and *L. helveticus* strains (LKH-2, LKH-3 and LKH-5) had a low affinity for this acidic solvent, whereas all other strains had a strong affinity for it. When ethyl acetate, an electron donor, was employed for determining the microbial adhesion to solvents, adhesion values were noticeably high (ranging from 16.8 to 67.8%) for *L. delbrueckii* and *L. fermentum* strains, indicating that these bacteria also have an acidic surface character. *L. casei* LAM-1 strains also showed relatively good adherence value (30%) to ethyl acetate.

Table 4.19 Adhesion of potential probiotic *Lactobacillus* strains to solvents

Probiotic candidates	Strain	% Adhesion		
		n-Hexadecane	Chloroform	Ethyl Acetate
<i>Lactobacillus casei</i>	LAM-1	60.8 ± 4.4	55.5 ± 4.6	30.4 ± 4.3
	LAM-2	67.6 ± 1.8	77.8 ± 3.4	8.7 ± 3.4
<i>Lactobacillus delbrueckii</i>	LKH-2	14.7 ± 1.4	20.6 ± 0.7	16.8 ± 0.7
	LKH-3	12.0 ± 2.3	22.7 ± 0.4	6.1 ± 1.4
<i>Lactobacillus helveticus</i>	LKH-5	7.3 ± 0.4	21.8 ± 0.5	8.3 ± 1.1
<i>Lactobacillus fermentum</i>	Lamec-29	45.9 ± 5.4	60.8 ± 2.8	67.8 ± 0.7

n = 3; observations comes from three replicate assays; data are represented as mean ± sd

4.8.2 Auto-aggregation of *Lactobacillus* strains

Auto-aggregation was studied on the basis of the sedimentation characteristics of the strains because when cells aggregate, they sediment and clear the supernatant. *L. delbrueckii* LKH-3 showed aggregation values of less than 20% (Fig. 4.4), whereas the *L. delbrueckii* LKH-2 and *L. helveticus* LKH-5 auto-aggregated with a value higher than 20%. Although these strains both known to adhere well to intestinal epithelial cells, a clear difference in auto-aggregation could be observed as *L. casei* showed much higher auto-aggregation values (more than 80%), while those of *L. fermentum* were below 60% (Fig. 4.4). A variation in aggregation values obtained using acidic (pH 4.0) and neutral (pH 7.4) PBS was not significant. Only in the case of *L. casei* strains LAM-1 and LAM-2, the auto-aggregation was enhanced when tested in their overnight spent supernatants and it was noticeably higher than that observed in PBS at pH 4.0 (Fig. 4.4).

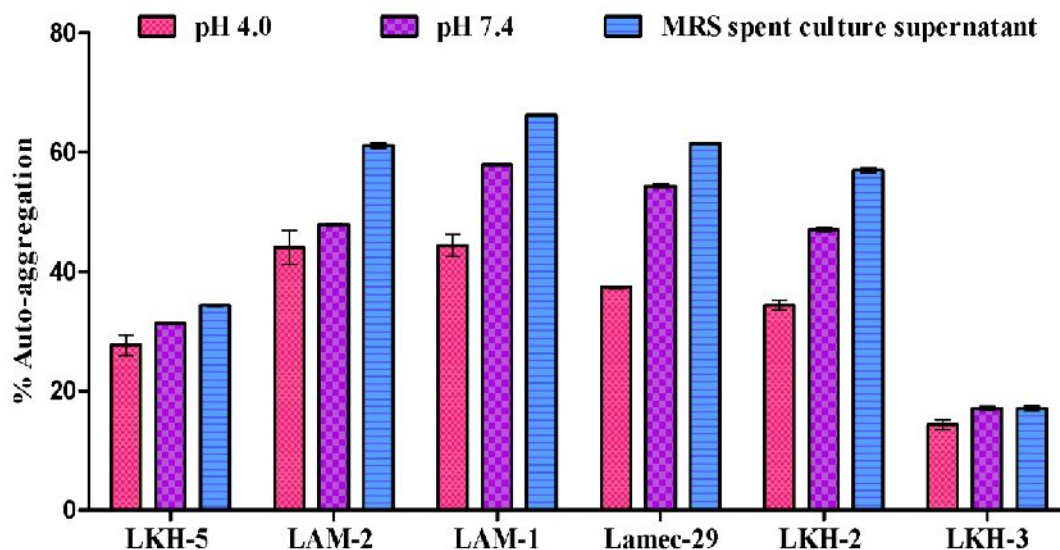


Fig. 4.4 Auto-aggregation of *Lactobacillus* strains

Overnight cultures were washed and resuspended in PBS pH 7.4, in PBS pH 4 or in MRS spent culture supernatant. Absorbance (580 nm) was measured at the beginning (A_0) and after a 2 h incubation (A_1) at 23°C. Auto-aggregation % was calculated as: $(1 - A_1/A_0) \times 100$. Each point represents the mean $\pm$ standard error (SE) mean of three independent experiments.

4.8.3 Co-aggregation of *Lactobacillus* strains with foodborne pathogens

The scoring system used for the co-aggregation test is illustrated in Fig. 4.5. *E. coli* 0517: H7 and *L. monocytogenes* ATCC 1911 were able to auto-aggregate with scores of 4 and 3, respectively in Table 4.20. For co-aggregation of pathogens with *Lactobacillus* spp., the scores were reduced by at least one unit in all cases, except for *L. casei* LAM-1. When supernatant of overnight cultures of *Lactobacilli* was added to the coaggregation system (1:10) with *L. monocytogenes* and *S. typhimurium*, the coaggregation with *L. casei* LAM-1 and LAM-2 increased by one unit (result not shown).

Table 4.20 Coaggregation^a of potential probiotic *Lactobacillus* strains with intestinal pathogens

Strain		<i>S. aureus</i> ATCC 9144	<i>E. coli</i> 0517:H7	<i>E. sakazakii</i> ATCC 51329	<i>S. flexneri</i> 2a	<i>S. typhimurium</i> ATCC 19585	<i>L. monocytogenes</i> ATCC 1911
Probiotic candidates							
<i>Lactobacillus casei</i>	LAM-1	4	2	4	2	1	3
	LAM-2	4	1	4	2	1	3
<i>Lactobacillus delbrueckii</i>	LKH-2	1	1	2	1	1-2	1
	LKH-3	1	1	2	1-2	1-2	1
<i>Lactobacillus helveticus</i>	LKH-5	1	1	2	2	1	2
<i>Lactobacillus fermentum</i>	Lamec-29	1-2	2	2	2	4	3
Foodborne pathogen							
<i>S. aureus</i>	ATCC 9144	4	0	0	0	0	0
<i>E. coli</i>	0517: H7	0	2	0	0	0	0
<i>E. sakazakii</i>	ATCC 51329	0	0	4	0	0	0
<i>S. flexneri</i>	2a	0	0	0	3	0	0
<i>S. typhimurium</i>	ATCC 19585	0	0	0	0	4	0
<i>L. monocytogenes</i>	ATCC 1911	0	0	0	0	0	4

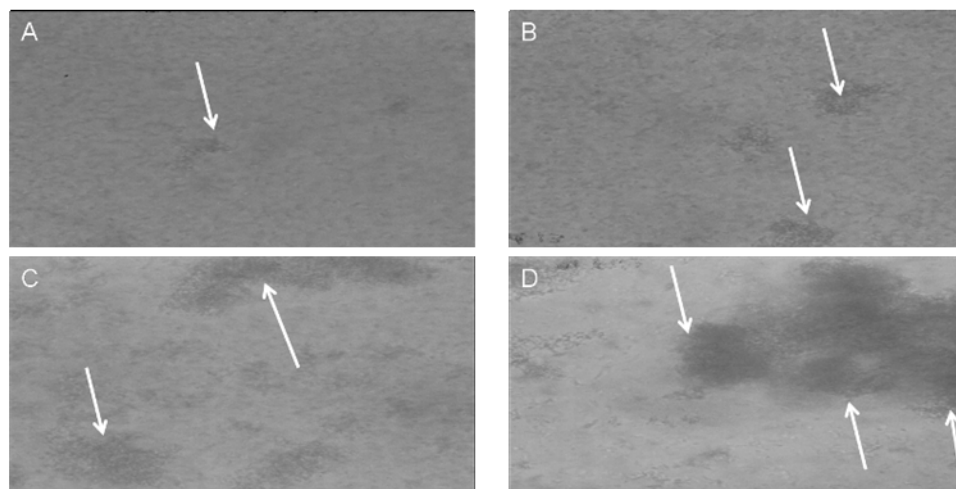


Fig. 4.5 Scoring system for the coaggregation assay. Organisms used to illustrate the different scores were *S. typhimurium* ATCC 19585 together with *L. casei* LAM-1. (A): *S. typhimurium* ATCC 19585 together with *L. casei* LAM-1, (B): *L. casei* LAM-1 together with *E. coli* 0517: H7, (C): *L. monocytogenes* together with *L. casei* LAM-1 and (D): *L. casei* LAM-1 together with *S. aureus*. Photographs were taken on a microscope (200 x magnification).

In that way, *L. casei* LAM-1 reached the highest score (4) in co-aggregation with these foodborne pathogen strains. *L. fermentum* co-aggregated with *S. typhimurium* with a score of 3 (Table 4.20). After Gram staining, it was verified by light microscopy that aggregates comprised of Gram-positive as well as Gram-negative bacteria.

4.8.4 Adhesion of *Lactobacillus* strains to Caco₂ cells

The probiotic *Lactobacillus* strains were investigated for their ability to adhere to the human intestinal epithelial CaCo₂ cells. *L. casei* (LAM-1) possesses high adhesion. Gram staining of the slides enabled adherent bacterial strains to be clearly visible as dark purple rods on a pale pink cell background (Fig. 4.6).

L. casei LAM-1 was characterised as non-adhesive whereas *L. delbruckeii*, showed moderate adherence. Interestingly, the *L. casei* LAM-1 and LAM-2 isolated from mango pickle showed a much higher adhesive index.

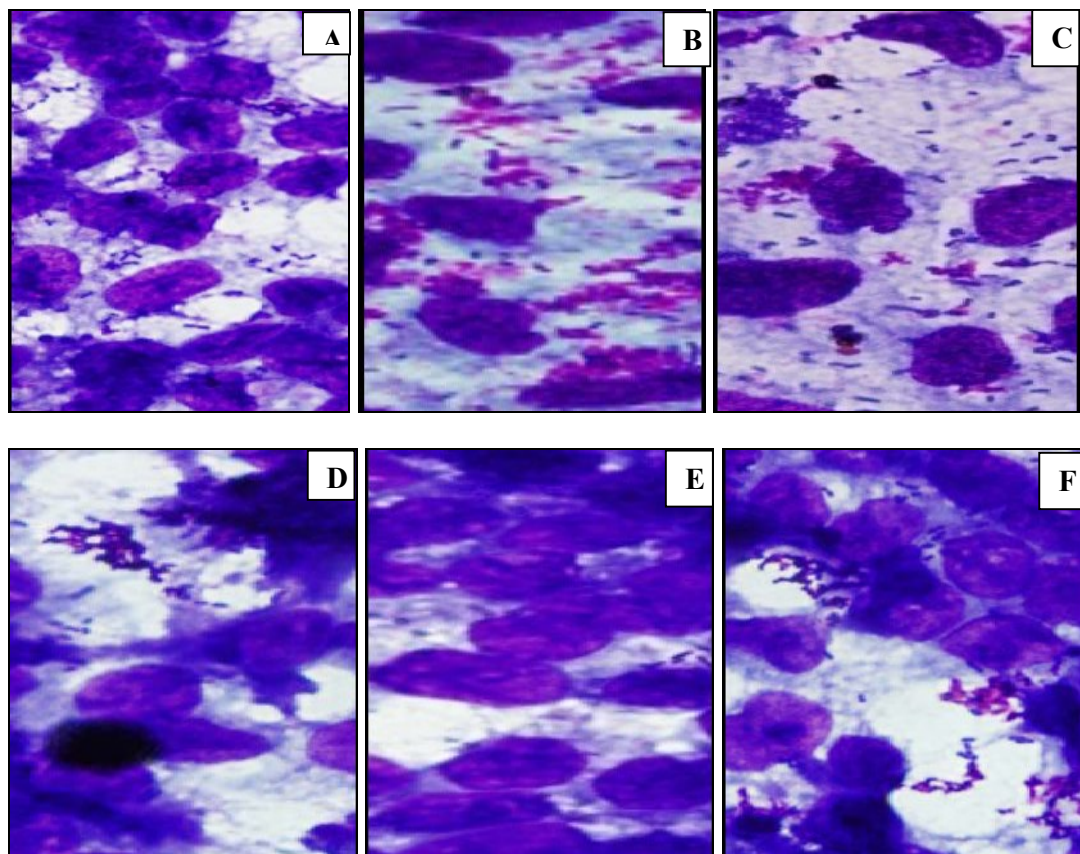


Fig. 4.6 Adhesion of *Lactobacillus* strain to Caco₂ Cells: (A): *L. casei* LAM-1, (B): *L. delbrueckii* LKH-2, (C): *L. delbrueckii* LKH-3, (D): *L. helveticus* LKH-5, (E): *L. casei* LAM-2, (F): *L. fermentum* Lamec-29. Photographes were taken on 1000 x magnification.

4.9 Cholesterol removal in the growth medium

The cholesterol removed by these *Lactobacillus* strains under anaerobic condition is presented in fig.4.7. The variations among Six *Lactobacillus* strains (LAM-1, LAM-2, LKH-2, LKH-3, LKH-5 and Lamec-29) removed cholesterol ranged from 40% to 10%. Strains *L. casei* LAM-1 showed moderate cholesterol reduction in presence of the mixture of bile salts, however, little cholesterol was removed by LAM-2. The results demonstrated that no cholesterol could be removed by these strains without bile salts (data not shown).

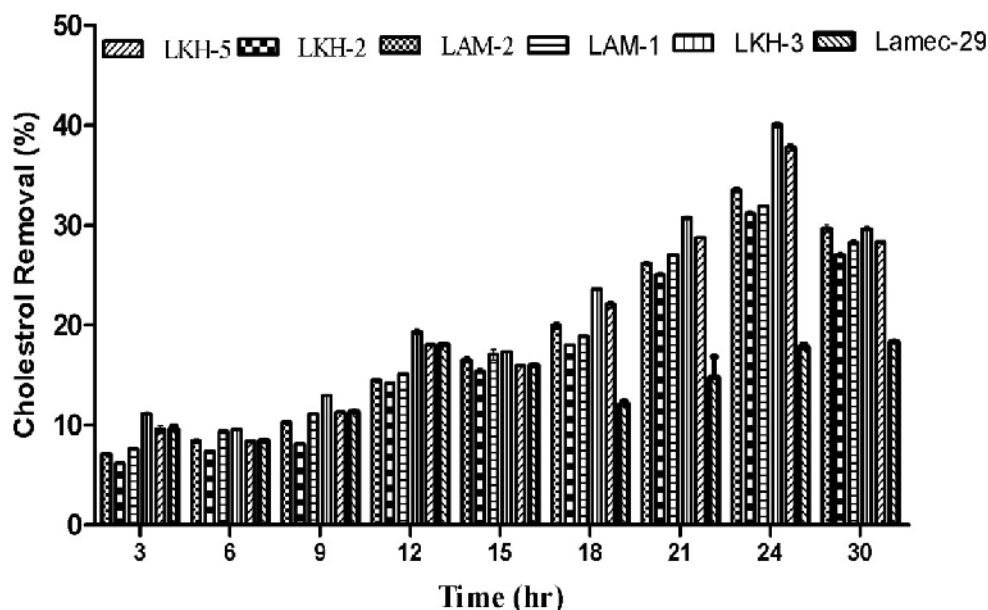


Fig. 4.7 Chloestrol removal by *Lactobacillus* strain. Each point represents the mean $\pm$ standard error (SE) mean of three independent experiments.

4.10 Detection of antimicrobial peptide (bacteriocin)

A total of six *Lactobacillus* isolated from traditionally food and meconium was evaluated for the production of bacteriocins or antimicrobial peptides. In the agar spot test, the *Enterobacter* (Chronobacter) *sakazakii* ATCC 51329, *L. monocytogenes* ATCC 19111, *S. flexneri* 2a, *A. hydrophila* ATCC 35654, *Y. enterocolitica* ATCC 9610 and *S. typhimurium* ATCC 19585 indicator strains showed weak to strong inhibition (zone of inhibition of more than 1 mm from edge of producer colony up to 23 mm). The results are shown in Table 4.21, which depicts strains with antagonistic activity, as demonstrable by the broad range of pathogen inactivation profile. The bacteriocin elaborated by *L. casei* LAM-1 was judged to be most potent, amongst the six isolates. The bacteriocin was thus further purified and characterized.

Table 4.21 Antimicrobial spectrum of *Lactobacillus* spp. by agar well diffusion assay

Organism	LAM-1	LAM-2	LKH-2	LKH-3	LKH-5	Lamec-29
<i>Aeromonas hydrophila</i> , MTCC 646	+++	++	++	+++	++	+++
<i>Salmonella typhimurium</i> ATCC 19585	+++	++	+++	++	++	++
<i>Escherichia coli</i> 0517:H7	+++	-	++	++	++	--
<i>Staphylococcus aureus</i> ATCC 9144	+++	++	++	+	++	+++
<i>Yersinia enterocolitica</i> MTCC 840	+++	-	+	+++	++	++
<i>Enterobacter sakazakii</i> MTCC 659	+++	++	++	++	+	++
<i>Shigella flexneri</i> 2a	+++	++	++	++	++	+
<i>Listeria monocytogenes</i> ATCC 19111	+++	++	+	+	++	+

atcc: american type culture collection, mtcc: microbialtype culture collection

-, no antimicrobial activity; +, inhibition zone < 10 mm; ++, inhibition zone > 11 mm; +++, inhibition zone > 20 mm

4.10.1 Sensitivity of bacteriocin to enzymes, pH and temperature

The antimicrobial activity showed no significant differences ($P < 0.05$) at temperatures 60, 80, and 100°C for 15 and 30 min respectively and activity was not lost upon autoclaving (Table 4.23). The heat stability could be a very useful characteristic as food preservative, because many food-processing procedures usually involve a heating step. Also the activity of bacteriocin remained stable following incubation at pH values between 2.0 and 10.0, but inactivated at pH 12.0, the observed reductions were found significant ($P < 0.05$) (Table 4.23).

Loss of bacteriocin activity was not observed upon treatment with catalase, indicating that H_2O_2 was not responsible for inhibitory activity. Treatment of bacteriocin with proteolytic enzymes proteinase K and pepsin, resulted in complete loss of bacteriocin activity, indicating that active component of bacteriocin was proteinaceous in nature while a significant ($P < 0.01$) bacteriocin activity was observed with non treated bacteriocin. The

presence of α -amylase, DNase and RNase were slightly affected bacteriocin activity (Table 4.22). These results revealed that the antimicrobial substance was of proteinaceous nature and its activity depended on glycosylation, which required both the glycol portion and the protein portion for activity.

The detergents used were: sodium dodecyl sulphate (SDS), Tween 80, Tween 20, EDTA and urea. Sodium dodecyl sulphate (SDS), Tween 80 and Tween 20 could stimulate the bacteriocin production detergents may be due to non-denaturation of its association with other molecules, having a stabilizing effect on bacteriocin activity. Bacteriocin showed a significant decrease ($P<0.05$) in their activity when the assay was carried out after treatment with EDTA and urea (Table 4.22).

Table 4.22 Effect of enzymes and detergents on bacteriocin production

Treatment	Bacteriocin production	
	Concentration	(mg/mL)
<u>AU/mL</u>		
Enzymes		
Control*	-----	7350±390
Proteinase K	0.1	Nil
α -amylase	0.1	6540±288
Dnase	0.1	6210±366
Rnase	1.0	5430±360
Pepsin	0.1	Nil
Detergent		
SDS	1.0	5430±299
Tween 80	2.0	4540±442
Tween 20	0.1	4210±456
EDTA	5.0	Nil
Urea	1.0	Nil

*without addition of enzymes; n = 3; observations comes from three replicate assays; data are represented as mean $\pm$ sd

Table 4.23 Effect of pH and Temperature on bacteriocin activity

	Treatment	Bacteriocin activity
pH	2-10	+
	12	-
Temperature	60, 80, 100°C for 15 min	+
	60, 80, 100°C for 30 min	+
	121°C for 15 min	+

+ Inhibition zone of at least 5 mm in diameter; -, no inhibition zone recorded, Data represent the means of three sets.

The observed non reduction or loss of bacteriocin activity, following treatment with detergents may be due to non denaturation of its association with other molecules, having a stabilizing effect on bacteriocin activity. Bacteriocin remained stable after incubation at 37°C from pH 2.0 to 10.0 ($P < 0.05$). This indicates that, bacteriocin may be useful in acidic as well as non acidic food processing conditions. Further, the antimicrobial activity of bacteriocin was not inactivated at high temperature viz. boiling temperature (60°C, 30 min) or upon boiling (100°C, 45 min) (Table 4.23).

4. 10.2 Determination of molecular weight of bacteriocin

Molecular weight of the bacteriocin was determined by Tricine-SDS polyacrylamide gel electrophoresis (Fig. 4.8). The protein band was observed when stained with Coomassie blue and the antimicrobial activity of bacteriocin was associated with a band having an apparent molecular weight of about 2.5 kDa.

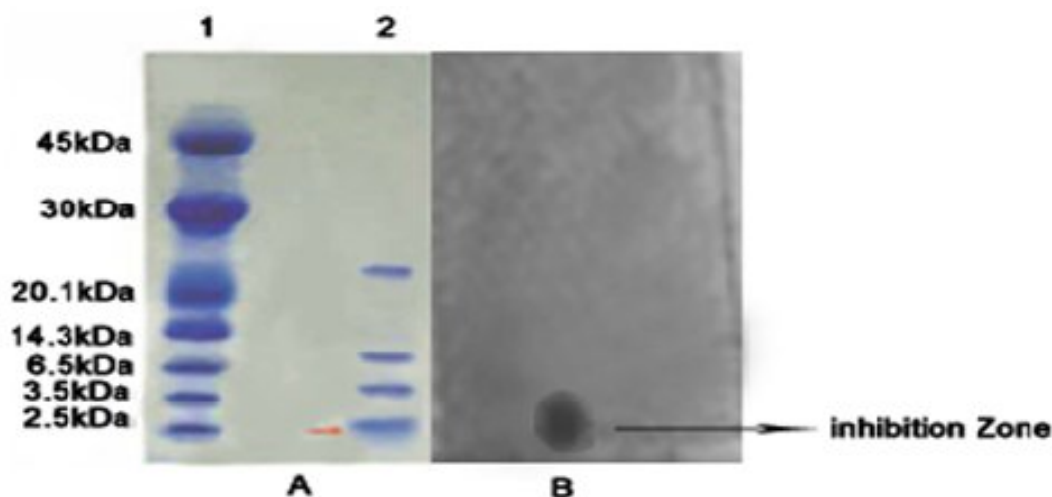


Fig. 4.8 Tricine SDS-PAGE of the bacteriocin: Lane 1, molecular weight markers; lane 2, Bacteriocin, (B), Zone of growth inhibition, corresponding to the position of the peptide band in lane A2. The gel was overlaid with *L. monocytogens* (approx. 10^5 CFU/ml), embedded in BHI soft agar

4.10.3 N-terminal amino acid sequence of bacteriocin

The N-terminal amino acid sequence of purified bacteriocin, indicated that the N terminus starts with alanine and end with glycine. The amino acid analysis of this band revealed the following partial sequence: “ARSYGNGVYCGNKKCWGRGEATEG”. Amino acids 1 to 12 and 14 to 24 revealed that the 21st amino acid was alanine (A) and the 24th amino acid was glycine (G). The molecular mass was 2563.94 Da and the isoelectric point was at pH 8.77. The bacteriocin possess the net charge 2 and had 4 hydrophobic residues.

Table 4.24: The physiochemical properties of the peptide sequence

AA	N°	%	AA	N°	%	AA	N°	%
Ala	2	8.33	Ile	0	0.00	Arg	2	8.33
Cys	2	8.33	Lys	2	8.33	Ser	1	4.17
Asp	0	0.00	Leu	0	0.00	Thr	1	4.17
Glu	2	8.33	Met	0	0.00	Val	1	4.17
Phe	0	0.00	Asn	2	8.33	Trp	1	4.17
Gly	6	25.00	Pro	0	0.00	Tyr	2	8.33
His	0	0.00	Gln	0	0.00			
Total	24							



Fig. 4.9 CLUSTAL X alignment of bacteriocin from *L. casei* LAM-1 with *Pisciocin* V1b, from *Carnobacterium piscicola* (1), Lactococcin MMFII from *Lactococcus lactis* (2), Sakacin-A from *L. sakei* (3), Curvacin-A from *L. curvatus* (4), Enterocin P from *E. faecium* (5). Asterisks below the aligned sequences indicate amino acids present in all seven sequences. Positions with colons below contain a residue of the strongly conserved groups in all seven sequences, and periods indicate more weakly conserved groups in all seven sequences

The physiochemical properties of the peptide sequence the molecular weight, theoretical isoelectric point (pI), amino acid composition, atomic composition and grand average of hydropathicity (GRAVY) predicted by Bactibase database (Table 4.24).

4.11 Batch fermentation for bacteriocin production *L. casei* LAM-1

Production of bacteriocin by *L. casei* LAM-1 was studied under batch culture conditions as shown in Fig. 4.10 and 4.11. Cell density of *L. casei* LA-1 increased from 0.1 to 3 (OD_{600 nm}) during 48 h of growth at 37°C. The pH of the medium decreased from 6.0 to 3.5 over the same period. Bacteriocin production started at the late exponential phase and reached its maximum at the early stationary phase suggesting that the antimicrobial peptide to be a secondary metabolite, whereas maximum biomass occurred at 18 h. Results showed that *L. casei* LAM-1 produced bacteriocin in MRS broth and exhibited higher bacteriocin activity of 2844 AU/ml at pH 6.5 and temperature around 37°C at 20 h.

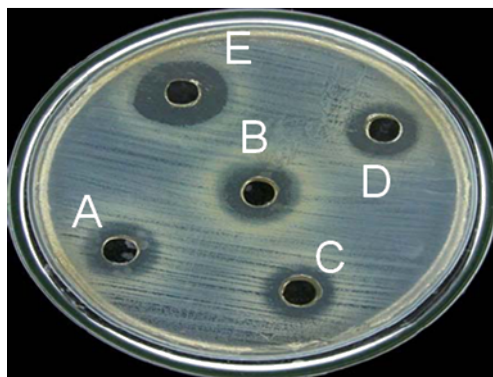


Fig. 4.10 Antimicrobial activity of *Lactobacillus casei* LAM-1 against *Listeria monocytogenes* at incubation period of 4 h (A), 8 h (B), 12 h (C), 16 h (D) and 20 h (E)

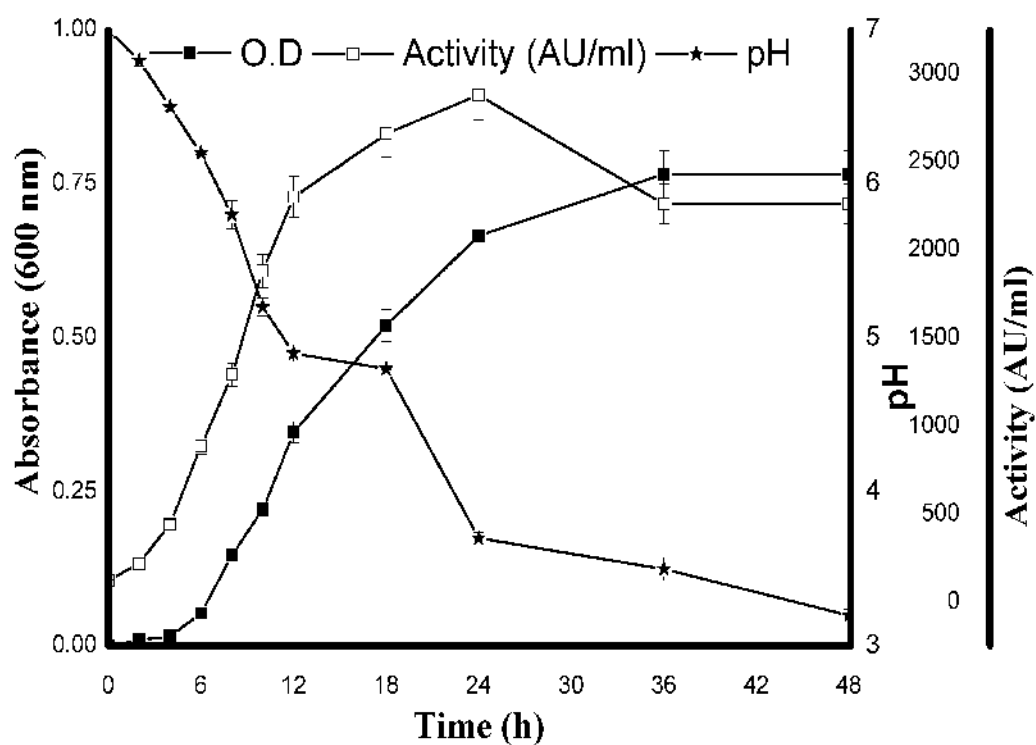


Fig. 4.11 Bacteriocin production during the growth of *L. casei* in MRS broth at 37°C. The optical density (absorbance at 600 nm: ●) and pH (▲) of culture were measured at the time intervals as indicated. The antibacterial activity (□) was also assayed and expressed as AU/ml. The values shown represent averages from triplicate experiments. Error bars represent the standard deviation.

4.11.1 Effect of incubation temperature, initial pH and inoculums size on bacteriocin production

The effect of incubation temperature on bacteriocin production by *L. casei* LAM-1 in MRS broth is shown in Fig 4.12. Bacteriocin activity was studied when the producer strain was incubated at 25, 30, 35 and 40°C, but maximum production of bacteriocin was obtained at a temperature of 35°C. When different initial pH values in MRS broth were assayed (Fig. 4.13), it was observed that at pH of 6.7, bacteriocin production was maximum after 20 h incubation. Inoculum size was varied accordingly and it was found that inoculums corresponding to 1.8 O.D. resulted in maximum bacteriocin production (data not shown). Dunn's multiple comparison test showed that all the factors were significant ($P < 0.05$). These values suggest that these factors have a direct relationship on the production of bacteriocin production.

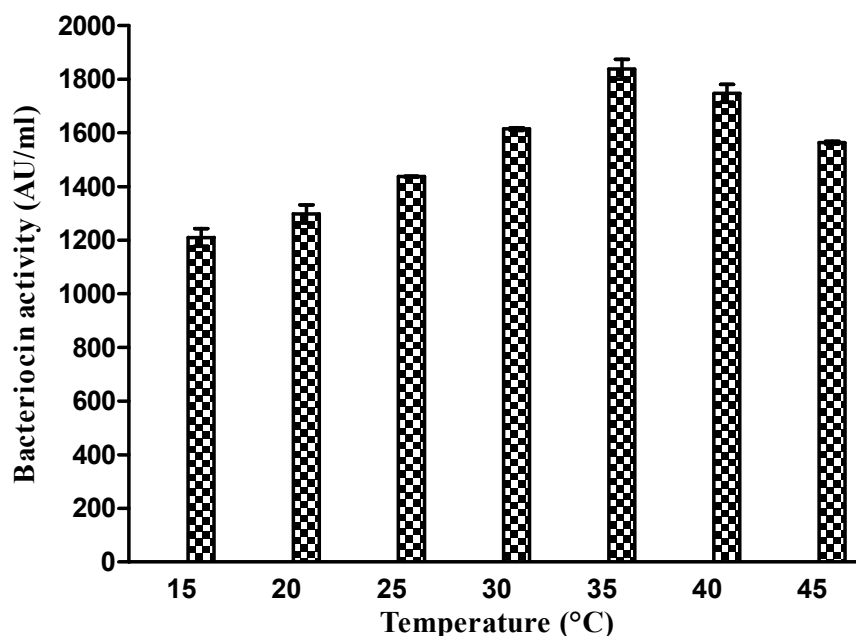


Fig. 4.12 Effect of incubation temperature on bacteriocin production by *L. casei*-LAM-1. The values shown represent mean from triplicate experiments. Bars represent the standard error.

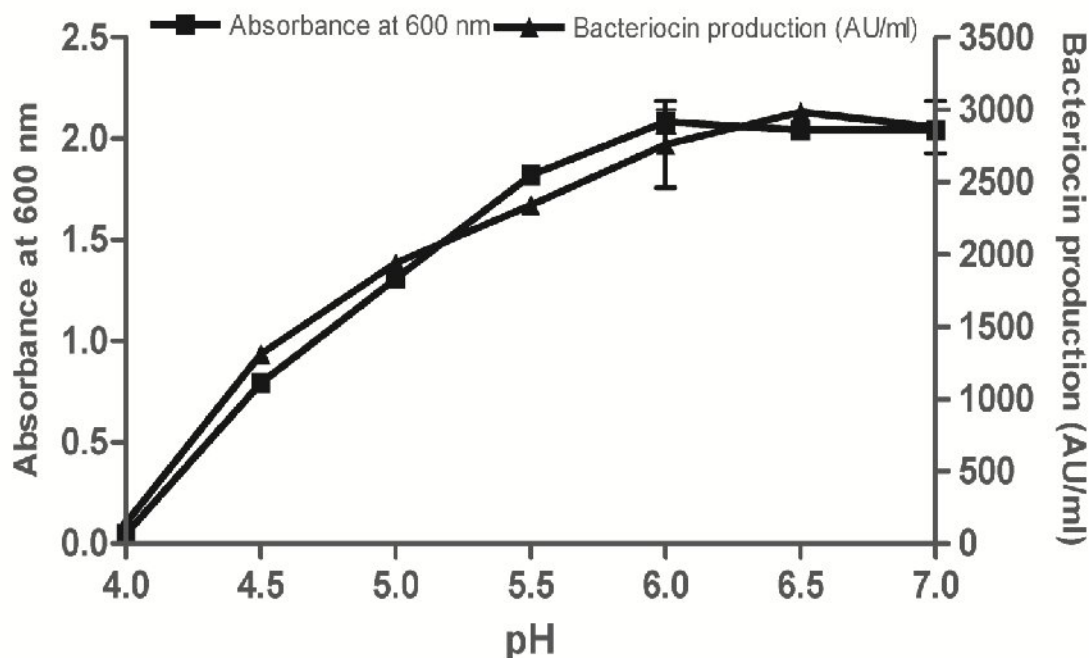


Fig. 4.13 Effect of pH of medium on bacteriocin production by *L. casei* LAM-1. The values shown represent mean from triplicate experiments. Bars represent the standard error

4.12 Bacteriocin mode of action

The effect of bacteriocin on the growth of *L. monocytogenes* ATCC 19111 was examined to establish whether it has a bactericidal or a bacteriostatic mode of action. The addition of bacteriocin resulted in the high reduction of respective cell populations for 10 h. The addition of 2844 AU/ml bacteriocin to a 3 h old (early exponential phase) culture of *L. monocytogenes* ATCC 19111 ($OD_{600\text{ nm}} = 0.1$) resulted in the decrease of optical density of BHI broth from 0.1 to 0.033. After 8 h, as compared to control without bacteriocin, the viable cell count (log CFU/ml) of *L. monocytogenes* ATCC 19111 decreased from 7.8 to 2.8 (approximately 3 fold). Bacteriocin led to marked decreases in both optical density and viable cell count of *L.*

monocytogens ATCC 19111 suggesting that the mode of action of bacteriocin as bactericidal (Fig. 4.14).

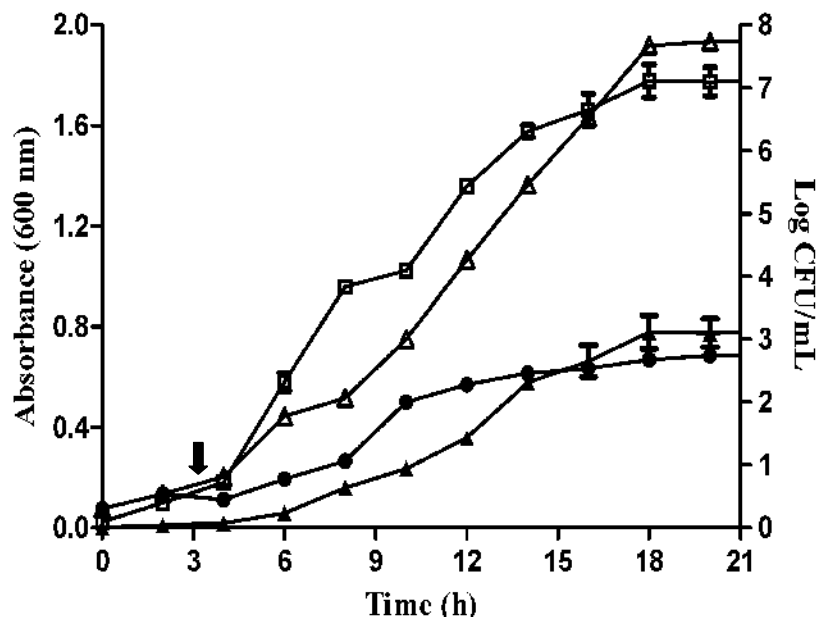


Fig. 4.14 Mode of bacteriocin of *L. casei* LAM-1 against *L. monocytogens* ATCC 19111. (●) and (▲) optical density of the cells measured at 600 nm treated with the bacteriocin and inactive bacteriocin, respectively; (□) and (Δ), Log CFU/ml counts of the indicator strain treated with the bacteriocin and the inactive bacteriocin, respectively. The arrow indicates the time of addition of the bacteriocin (3 h). The values shown represent mean from triplicate experiments. Bars represent standard error

4.13 Mechanism of pathogen inactivation by bacteriocin

The impact of bacteriocin on the morphology of log-phase *S. typhimurium* and *S. flexneri* 2a cells was investigated with a scanning electron microscopy. The cells maintained typical rods and intact status before treatment with bacteriocin (Fig. 4.11 A). When held in contact with bacteriocin for 12 h, *S. typhimurium* and *S. flexneri* 2a cells showed an alteration in morphology (Fig. 4.11 B). Extensive damage, including ruptured cells, shrinkage and

surfacing pitting was observed (Fig. 4.11 B). Further morphological investigation of all strains was performed with transmission electron microscopy.

The cells of *S. typhimurium* had changes in morphology before and after treatment with bacteriocin (Fig. 4.15 A and 4.15 B). *S. typhimurium* had the typical structure of Gram-negative bacteria, with a uniform wall to which the cytoplasmic membrane tightly adhered. The outer membrane and cell wall circumscription was observed (Fig. 4.15 A and B). Exposure to bacteriocin induced a dramatic change in *S. typhimurium* and *S. flexneri* 2a cells (Fig. 4.16 A, B, C and D). Following treatment with bacteriocin for 12 h, almost all cells showed some degree of alteration. In *S. typhimurium* cells, outer membrane was destroyed (a), Mesosome-like membranous formations were observed protruding into the cytoplasm (b), several cells displayed ruptures and loss of cytoplasm (c), indicating that the structure of the cytoplasmic membrane was severely affected by the bacteriocin from *L. casei* LAM-1.

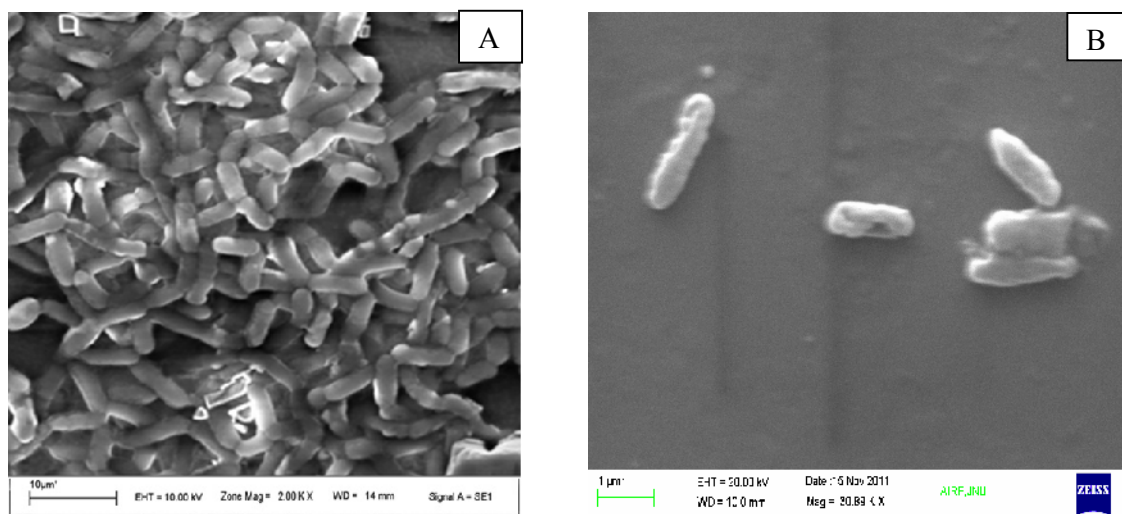


Fig. 4.15(A & B). SEM micrographs of treated and untreated *S. typhimurium* . In untreated, the cells are long, intact, and evenly shaped (A) After bacteriocin treatment, the cells appear shorter and more compact. Morphology of exponentially growing cells visualized by scanning electron microscopy at 20,000 x magnification

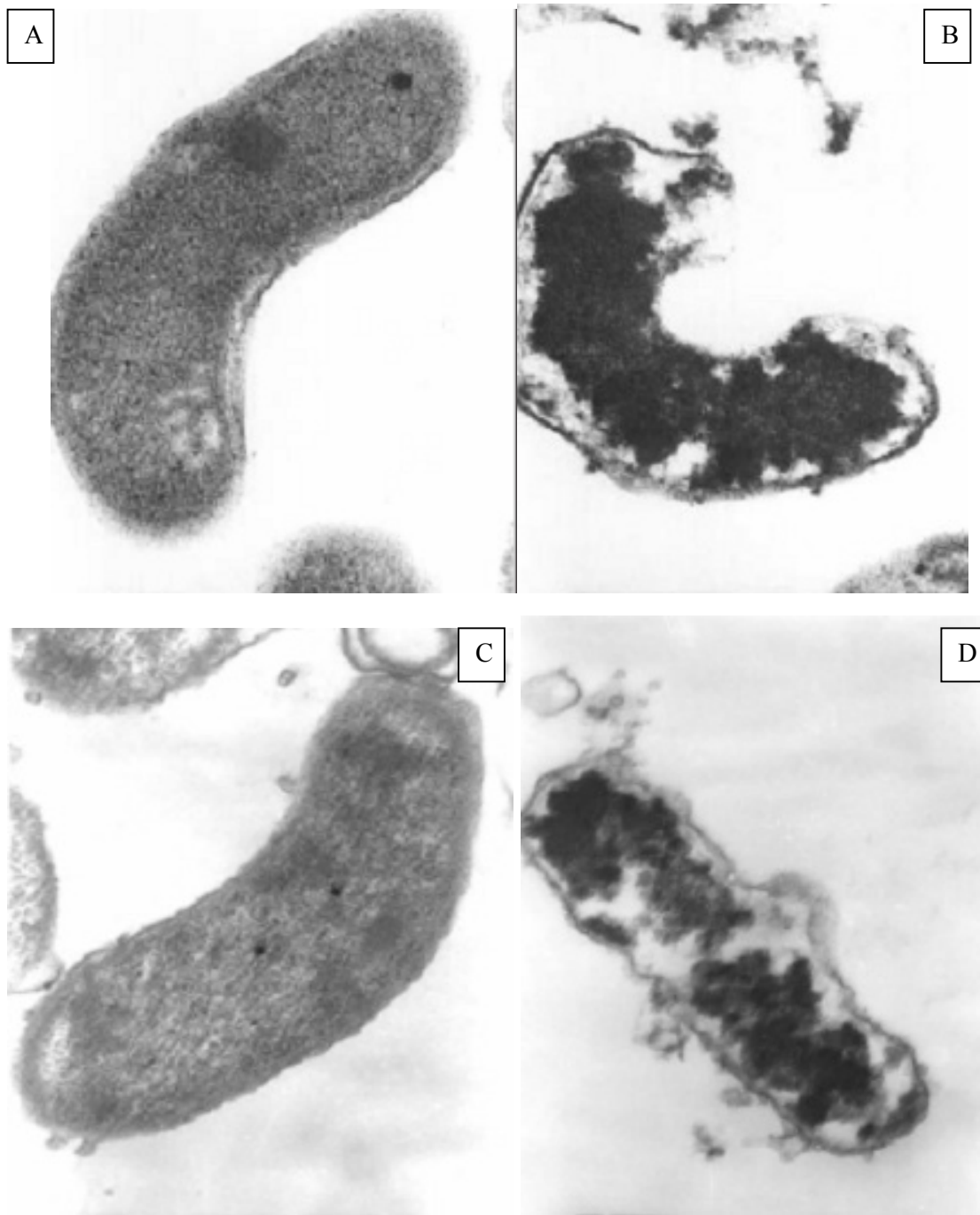


Fig 4.16 (A, B, C & D). Transmission electron Micrograph of bacteriocin treated and untreated cells of *S. typhimurium* cells. The treatment of *S. typhimurium* and *S. flexneri* 2a strains with the semi purified bacteriocin of *L.casei* LAM-1 led to the formation of necrotic cells. TEM analysis showed that the pathogens treated with bacteriocin exhibited destruction of the cell membrane and extrusion of cell contents.

4.13.1 Measurement of intracellular K⁺ content

The bactericidal action of bacteriocin results in the leakage of K⁺ ions from susceptible cells. In the control cells (absence of the bacteriocin), an intracellular concentration of K⁺ of approximately 118 mM. The subsequent addition of nisin caused a dramatic loss of cellular K⁺ (37.8 mM). The measurement of the K⁺ ions from susceptible cells treated with bacteriocin had induced massive leakage of K⁺ (concentration reduced upto-18.7 mM). The efflux of K⁺ was immediate, and after 15 min of treatment with 2,800 AU/ml of bacteriocin.

4.13.2 Measurement of intracellular ATP content

The impact of bacteriocin on the energetic condition of sensitive cells was determined by measuring the cellular ATP levels. On the addition of bacteriocin (2,800 AU/ml) and nisin resulted into no decrease in the cellular ATP levels. After 10 min of incubation with the bacteriocin, no ATP decrease or imcrease could be detected in the extracellular ATP concentration in Table 4.25. These results suggest that the pores formed by the bacteriocin are not large enough to allow the leakage of large compounds, such as ATP.

Table 4.25 ATP content of *Salmonella thyphurium* treated with bacteriocin

Time(min)	Untreated	Treated: nisin	Treated: bacteriocin
0	8.70	8.69	8.05
10	8.68	8.15	8.03
20	8.70	8.15	8.0
30	8.70	8.14	8.0
40	8.66	8.14	8.01
60	8.68	8.15	8.01

4.13.3 Membrane permeability

The fluorescence ratios of the nisin-treated samples were taken to represent 100% permeability in comparison with the untreated cells. Pathogen cell suspensions treated with bacteriocin showed an increase in membrane permeability after 5 min of exposure (35% increase), which remained unchanged after 60 min (Fig. 4.17 A & B). These results indicate that the bacteriocins may not be a pore-former *per se* like nisin, but it does appear to have a destabilizing effect on the integrity of the cell membrane.

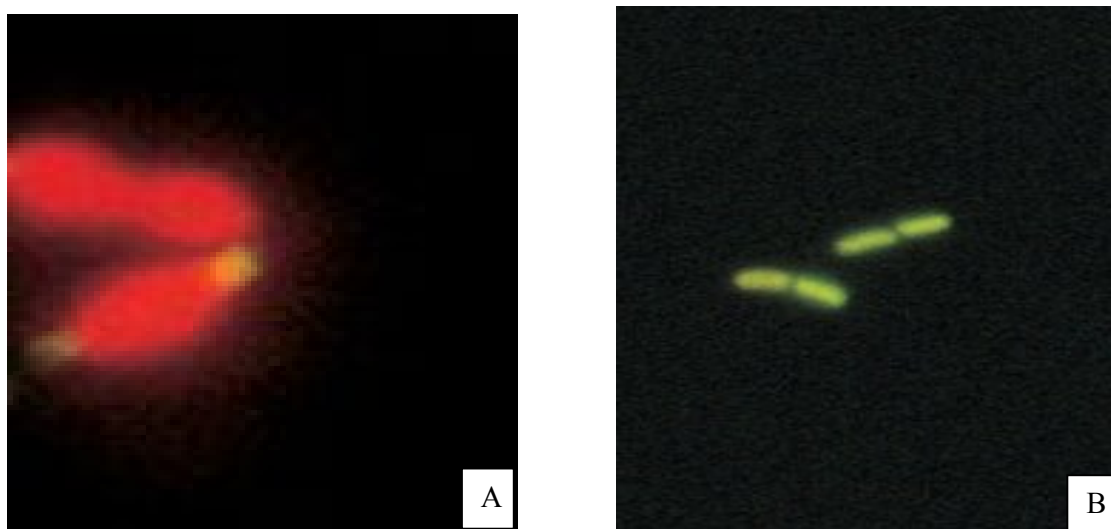


Fig 4.17(A & B) Live and dead cells of *Salmonella typhimurium*. A represents the cells treated with bacteriocin and B represents the untreated cells

4.14 Inhibition of pathogen by bacteriocin in a simulated /laboratory prepared food matrix and following processing

Storage of the active substance at 4°C for 15 days did not influence the activity bacteriocin were subjected to frozen at -20°C during 1 day and thawed for 20 min at 25°C. After three freezing and thawing cycles, in general, culture supernatants maintained similar antagonistic properties. The fact that bacteriocin demonstrated freezing and thawing stability has technological importance and may broaden the potential application of such compounds

as biopreservatives. Bacteriocin was active on *S. typhimurium*, with a bactericidal effect proportional to its concentration (25–100 $\mu\text{g/mL}$) (Fig. 4.18). After bacteriocin dosing (100 $\mu\text{g/ml}$), total bacterial populations in vegetable were below detection limit (1 log CFU/g) after 24 h of incubation. The samples dosed with bacteriocin at 25–50 $\mu\text{g/ml}$ showed a partially recovered growth after 24 h of incubation. These results could be useful for preservation of vegetables from pathogenic microorganism because no bacterial growth was observed in vegetable sample due to death of the bacteria under high doses of bacteriocin.

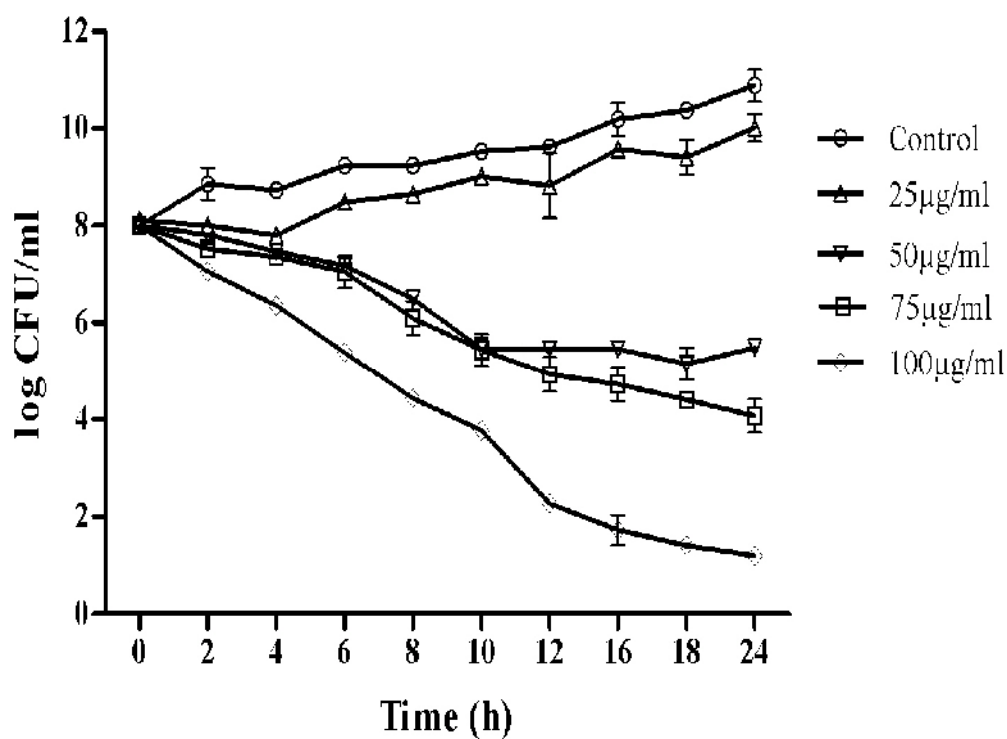


Fig 4.18 Effect of different concentrations of bacteriocin on *S. typhimurium* food model. The values shown represent mean from triplicate experiments. Bars represent standard error.

Chapter V
DISCUSSION

V. DISCUSSION

Recent studies have demonstrated that adhesion or colonization of probiotic lactic acid bacteria in gastrointestinal region varies in relation to age, gender and country, due to difference in gut ecology and food habits (Mueller *et al.*, 2007). It may be presumed that LAB cultures with probiotic properties, originating from either indigenous fermented food or human origin might be efficient for host gut due to longer transit time and also colonization ability in gut which attributes to the optimal functionality to host. New types or species of LAB with probiotic properties from traditional fermented food in India are not well documented. Therefore, it is possible that the strains from these products might be better suited in Indian population.

The first criteria of this study was the selection of new potential probiotic strains from non dairy traditional fermented food such as mango pickle, garlic-chilli pickle, chilli pickle, bhaati jaanr, mahua liquor and mashed mustard (Kharoli) which can survive under simulated gastrointestinal conditions. This ability should ensure that these strains reach the small intestine, which is the intended site of action. According to the guidelines for the evaluation of probiotics in food reported by a Joint FAO/WHO working group (Chesson *et al.*, 2002), two of the currently used *in vitro* tests are resistance to gastric acidity and bile salts, as based on both survival and growth studies. The predictability of these *in vitro* tests is limited, but the use of sophisticated and dynamic, computer models of the gastrointestinal tract, like the one developed by Marteau *et al.* (1997), is beyond the scope of most laboratories. Most of the reported studies have been done in acidified MRS (pH 2, 2.5, 3 and 3.5) (Chou and Weimer, 1999; Kociubinski *et al.*, 1999; Xanthopoulos *et al.*, 2000; Saito, 2004) and in MRS broth containing 0.15 to 0.5% bile salts (Chou and Weimer, 1999; Zarate *et al.*, 2000; Fernandez *et*

al., 2003). Instead of evaluating the effect of each component in separate experiments, it would be important to evaluate all components (enzymes, low pH, bile salts, duodenum secrete and food vehicle) in one system, as the two successive stresses of stomach transit and small intestinal transit might interact and thereby affect the survival of these strains in a synergistic way. Therefore, for the viability of bacteria under *in vitro* gastrointestinal model of the stomach, it is necessary not only to test the tolerance at low pH, but also the action of enzymes like pepsin and lysozyme. It is also important to consider the food vehicle in which the probiotic would be included, as it might exert a protective role and enhance the viability of bacteria. For this reason, not only the effect of pH, but also that of pepsin and lysozyme must be included in the stomach passage model. After 1 h incubation under simulated gastric conditions, a further treatment was followed with simulated intestinal fluids containing bile salts and pancreatin, which was a pool of pancreatic enzymes (amylases, trypsin, lipase, ribonuclease and proteases). In this way, both gastric and intestinal stresses were successfully combined in a simulated *in vitro* gastrointestinal model.

Using this *in vitro* simulated stomach-duodenum passage, only 96 strains were able to survive under the conditions of this study. The fact that only 6 strains out of 96 isolates tested survived the passage through this model validates the model, since gastrointestinal conditions are considered to be detrimental to most of food-associated lactic acid bacteria, but the probiotic control strain which indicated survival under the conditions tested. Highest rate of survival to the gastrointestinal conditions was observed for *L. casei* LAM-1. The present study was based on a ‘worst case’ scenario to determine the most resistant strains as the conditions selected in the *in vitro* model were even harsher than that of the real situation, where the gastric pH after a meal was of approx. 3, and there was a continual removal of the bile salts, leading to fluctuating and often lower bile levels. As already pointed out by various

workers in the field (Havenaar *et al.*, 1992; McCracken and Gaskins, 1999; Bezkorovainy, 2001; Dunne *et al.*, 2001), as well as a Joint FAO/WHO working group (Chesson *et al.*, 2002), *in vitro* studies can only partially mimic the actual *in situ* conditions in the gut ecosystem. Nevertheless, such *in vitro* systems are powerful tools especially for screening numerous samples.

Six new potentially probiotic isolates were selected on the basis of their good survival properties under *in vitro* simulated stomach-duodenum passage. Further phenotypic and genotypic characterization of six strains revealed two strains as *L. casei* (LAM-1 and LAM-2), the other two as *L. delbruckeii* (LKH-2 and LKH-3), one as *L. helveticus* (LKH-3) and one as *L. fermentum* (Lamec-29). These strains were further investigated for their functional and probiotic attributes. Two *L. casei* strains (LAM-1 and LAM-2) stemmed from mango pickle, indicating the safe origin of the strains and also the ability of these strains to survive the passage through the harsh conditions in the gastrointestinal tract. The other selected strains (two *L. delbruckeii* and one *L. helveticus*) were isolated from traditional beverages Kharoli and Bhaati jaanr, respectively. A previous study conducted with several Asian fermented foods such as “suan cai”, “kimchi”, “natto”, “miso”, “Nham” and “Miang” reported bioactive properties as well as the occurrence of LAB with interesting probiotic characteristics (Ramadan *et al.*, 2005). However, pickled vegetables/fruits as well as beverages widely consumed in the Indian subcontinent especially as adjunct to main meals, have been rarely investigated for probiotic strains of LAB. Mahula liquor is prepared by the fermentation of flowers of *Madhuca latifolia*, claimed to have extensive medicinal properties, whereas Bhaati jaanr is made by fermenting rice. Both are extensively consumed in several rural areas of North Eastern, Eastern and Northern India.

Lactobacillus is a normal inhabitant of the gut ecosystem (Tannock, 1999), and therefore it is not surprising, that they possess the capability to sustain conditions of gut. The strain *L. casei*, which was originally isolated from the fermented milk, is a known probiotic strain that is commercialized in country including india. This could also be an interesting alternative for developing a probiotic product without milk constituents using the *L. casei* strains characterized in this study. However, the two selected *L. delbruckeii* strains isolated from Kharoli, were present in low numbers in this product and were not representatively important as predominant species in the microbiota of this traditional fermented food.

L. casei LAM-1 survived under low pH conditions for five hours and well tolerated bile acids under *in vitro* conditions even at concentrations higher than those previously used by other authors (Jacobsen *et al.*, 1999; Fernández *et al.*, 2003). Acid tolerance of bacteria is an important factor to assure their resistance of gastric stresses and also for their use as dietary adjuncts in acid foods such as yoghurt.

Resistance to bile salts is considered as an important property for strains to be used as probiotics, however, there is still no consensus about the precise concentration to which the selected strain should be tolerant. The physiological concentration of bile acids in the small intestine varies from 5 to 20 mM/l (Hofmann, 1991). Therefore, a concentration of 0.5% ox-gall, equivalent to 12.25 mmol/l bile acids was used in this study, as was also previously done by Kociubinsky *et al.* (1999). This concentration is higher than the ones (0.3% and 0.15%) previously used by other investigators for judging bacterial probiotic properties (Chou and Weimer, 1999; Zarate *et al.*, 2000; Fernandez *et al.*, 2003).

Another test for resistance to intestinal conditions used in this study was resistance to 0.4% phenol. Phenols may be formed in the intestine as a result of the bacterial deamination of some aromatic amino acids derived from dietary and endogenous proteins. These

compounds are known to have bacteriostatic properties at least *in vitro* (Suskovic *et al.*, 1997). Hence, if they also present this bacteriostatic activity *in vivo*, bacteria tolerant to phenols might have more chances of survival than those which were not. In contrast to the high phenol resistance (resistance to 0.4% phenol) which was previously reported by Xanthopoulos *et al.* (2000) and Suskovi *et al.* (1997) for *L. acidophilus* strains, the *L. casei* LAM-1 strain was highly resistant towards this compound. The results of this study on the phenol resistance of all *Lactobacilli* strains suggested that they were moderately tolerant.

A major requirement for probiotic strains is that they should be safe for human consumption. In order to assure the safety of bacteria used in food, the European Food Safety Authority (EFSA) has initiated a ‘Qualified Presumption of Safety’ concept, similar to the GRAS system in the USA, has the purpose of allowing strains with long history of safe use and status to enter the market without extensive testing requirements (EFSA, 2004). Antibiotic resistance is one of the safety concerns included in the QPS concept to determine a strain’s QPS status. The reason for this is the hypothesis that food bacteria may act as reservoirs for antibiotic resistance genes (Chesson *et al.*, 2002; Danielsen and Wind, 2003; Franz *et al.*, 2005). In a survey of 62 *Lactobacillus* starter strains, Danielsen and Wind (2003) found a high level of resistance to aminoglycosides for all investigated *Lactobacilli*. Similar results were also found by Charteris *et al.* (1998) and Katla *et al.* (2001). These authors concluded that resistance to aminoglycosides among *Lactobacilli* is natural, i.e. intrinsic trait, which has been attributed to the absence of a cytochrome-mediated electron transport which mediates the uptake of aminoglycosides (Charteris *et al.*, 2001). Danielsen and Wind (2003) also suggested that resistance to ciprofloxacin appeared to constitute a natural or intrinsic resistance. In view of these deliberations, antibiotic resistances observed for the strains in this study, i.e. resistance towards ciprofloxacin and aminoglycosides may consider being intrinsic

or natural resistances. The strains selected in this study did not contain any of the transferable, acquired resistances which were known to occur among LAB and include resistances towards e.g., chloramphenicol, erythromycin and tetracycline (Danielsen and Wind, 2003). Therefore, the selected strains do not possess any risk for consumers with respect to antibiotic resistance transfer to other gut-associated bacteria.

All the probiotic candidates in this study were able to inhibit pathogenic strains associated with food, as indicated by the agar spot test. This antagonistic activity was due to the production of a bacteriocin, since they inhibited related strains and the neutralized supernatants shown inhibitory activity. As the plates were incubated anaerobically, the antimicrobial effect was not thought to be due to H₂O₂ production. Thus, the inhibition of the indicator strains most probably was due to the production of bacteriocin. Under aerobic conditions, both *L. casei* strains LAM-1 and LAM-2 strains and *L. delbruckeii* strains LKH-2 and LKH-3 were able to produce H₂O₂, showing that this was a strain-specific trait, because other strains belonging to the same species did not produce H₂O₂. Production of this antimicrobial compound by *Lactobacilli* has been described before (Annuk *et al.*, 2003; Servin, 2004), but its specific role is still unclear. H₂O₂ producing *Lactobacilli* are especially found in the vagina of healthy women and the presence of these bacteria have been associated with a lower frequency of vaginosis as they can, for example, inhibit gonococci (Servin, 2004). Hydrogen peroxide-producing *lactobacilli* are able to co-aggregate with pathogens that may exert an antagonistic effect. Therefore, it would be interesting to test *in vivo*, if the lactobacilli selected in this study also display these properties (co-aggregation and H₂O₂ production) in the vaginal microenvironment. The significance of this property in the small intestine has not been reported yet, but we hypothesize that this could be possible in a

transitional segment of the GIT, such as the duodenum, where the conditions are microaerophilic, rather than anaerobic.

Whether, Bsh activity is correlated with high tolerance to bile salts is still under debate. While some researchers argue that deconjugation of bile salts might be a detoxification mechanism of vital importance to the *Lactobacillus* cell, and thus play an important role during colonization in the gastrointestinal tract (Tannock *et al.*, 1989; De Smet *et al.*, 1995; Usman and Hosono, 1999; De Boever *et al.*, 2000), others infer that the higher toxicity of the deconjugated salts might more strongly affect the viability of the bacterium (Grill *et al.*, 2000). Bsh activity is a controversial probiotic property, particularly because the primary bile salts can be hydroxylated by 7 α -hydroxylating bacteria in the gut (e.g. clostridia) and thus converted to secondary bile salts which are pro-carcinogenic (Marteau *et al.*, 1995). Tanaka *et al.* (1999) observed a correlation between the habitat of lactic acid bacteria species and the presence of Bsh activity. For instance, lactic acid bacteria isolated from human intestine or faeces were Bsh positive, whereas those of food-origin were mostly Bsh negative (Tanaka *et al.*, 1999). In contrast, all selected strains of this study exhibited Bsh regardless of their origin (traditionally fermented food or human meconium). Bsh activity is considered a functional property, which has also been suggested to be important in lowering serum cholesterol levels (du Toit *et al.*, 1998; Pereira *et al.*, 2003). Accordingly, deconjugation of bile acids to primary bile salts by Bsh positive bacteria leads to an increased demand of cholesterol from which bile salts are synthesised *de novo* in the liver, and thus may lead to decreased serum cholesterol. All the six isolated strains possessed Bsh activity, and therefore their potential in lowering cholesterol levels should be further investigated. This is a valuable trait for probiotic bacteria, which may be connected with either survival or colonization in the gastrointestinal tract. However, the significance of their Bsh activity is not yet clear and this

trait may play a positive role in reduction of serum cholesterol levels, would need to be further investigated *in vivo*.

Lactose mal-digestion may be improved with therapy, utilizing bacteria from fermented milk products which contain the lactose cleaving enzyme β -galactosidase (Hove *et al.*, 1999; Szilagyi, 2002). Probiotic bacteria characterized in this study might also be used for compensation of lactase insufficiency. Some authors sustain that yogurt bacteria are more efficient for treatment of lactose intolerance, because they do not survive gastrointestinal conditions as well as probiotic bacteria. Thus, the yoghurt bacteria release the lactase enzyme after disruption of the cell wall as a result of bile sensitivity in the small intestine (Gilliland and Kim, 1984; Schrezenmeir and de Vrese, 2001). On the other hand, Zarate *et al.* (2000) reported that β -galactosidase activity was severely affected at pH 2. Therefore, if the enzyme is not released during the passage through the stomach, the activity of the enzyme is preserved until it reaches its site of action i.e. small intestine. This is also supported by a study in humans on the improvement of lactose intolerance with fermented milks (Mustapha *et al.*, 1997), where it was shown that a *L. acidophilus* strain, which exhibited the lowest β -galactosidase activity and lactose transport but the greatest bile and acid tolerance among the strains tested, was the most effective in improving lactose digestion and tolerance. Thus, Mustapha *et al.* (1997) proposed that bile and acid tolerance may be other important factors to consider when *Lactobacillus* strains are selected for improving lactose digestion and tolerance. Another aspect is that lactose can also be used as carbon source for growth by β -galactosidase-positive bacteria, and it has therefore been proposed as a potential prebiotic sugar (Szilagyi, 2002). However, the use of this sugar as a prebiotic in persons with lactose intolerance would obviously not be recommended. In this study, strains such as *L. casei* LAM-1 or LAM-2 possessed good tolerance to gastrointestinal conditions and high β -

galactosidase activity should be considered for further *in vivo* studies as they may contribute to alleviate symptoms of lactose intolerance.

Bacterial attachment to cells of the intestinal mucosa is considered crucial for selection of probiotic strains, since it allows bacterial strains to at least prolong their transit time through the intestine, allowing them to exert their beneficial interactions or possibly colonize the intestine (Lehto and Salminen, 1997a; Blum *et al.*, 1999). Intestinal epithelial cell lines have been used extensively to perform comparative studies of adhesion properties of probiotic strains (Lehto and Salminen, 1997a; Blum *et al.*, 1999; Lee *et al.*, 2000). The human colon carcinoma Caco₂ cell line used in this study was established in 1964 by (Rousset, 1986). This cell line expresses two important differentiation features which are characteristic of mature intestinal cells: tight junctions and a typical brush border. When cells are grown in the absence of serum, they secrete lysozyme and half of the cells differentiate as goblet-like cells (Rousset, 1986). In addition, the time course of the differentiation process, with exponentially dividing cells being undifferentiated and the differentiation taking place when cells stop dividing, mimics the situation found in the small intestine with dividing crypt cells being undifferentiated and with the differentiation taking place during the crypt to villus migration of non-dividing cells. However there are, limitations to this model, since cells are not normal but malignant cells, and they are not derive from the small intestine, but from the colon. In spite of these facts, these cells have been proven to be useful tools for the investigation of intestinal cell differentiation, function, the adhesion and invasion of bacteria and parasites, to name but a few (Rousset, 1986; Kerneis *et al.*, 1992; Jung *et al.*, 1995; Blum *et al.*, 1999; Lammers *et al.*, 2002; Lee *et al.*, 2005). Although mucus-producing epithelial cells in tissue culture are available, this study focused on the cell-cell interaction of eukaryotic cells with bacteria from two different points of view, i.e. the binding interaction

and the elicitation of a cytokine response as a result of this binding interaction. For this reason, mucus-secreting cells were not used, as it was also the case in most other binding studies, because of the possible interference of mucus with binding and signal transduction (Lehto and Salminen, 1997a; Blum *et al.*, 1999; Del Re *et al.*, 2000; Lee *et al.*, 2000). There are several direct and indirect methods for studying adhesion potential *in vitro*, but there is still no consensus about the optimal method that can actually predict microbial adhesion *in vivo* (Blum *et al.*, 1999).

Nevertheless, it is widely accepted that the level of adhesion of a probiotic strain under a given assay condition (often expressed as percentage adhesion) does not constitute an absolute value and has to be evaluated in relation to non-adherent strains tested under the same conditions. Another relevant point when comparing the binding of different strains is to maintain the amount of bacteria added to the system at similar levels, because the number of bound bacteria is influenced by the number of bacteria added to the assay (Tuomola and Salminen, 1998; Lee *et al.*, 2000). Both factors, the inclusion of a negative control and the maintenance of the same inoculum of probiotic bacteria or probiotic candidates (as determined by plating) were considered in the adhesion assay in this study. Two *L. casei* strains (LAM-1 and LAM-2) isolated from mango pickle were strongly adhering. Adhesion of bacteria to animal cells is a much more complex phenomenon than adhesion of bacteria to inanimate surfaces. This is because of the complexity of both microbial cell surfaces and eukaryotic membranes, and the ability of living cells to regulate the expression of molecules on their surface in response to changes in the environment. Thus, this process involves non-specific, as well as specific ligand-receptor mechanisms (Gordon *et al.*, 1985). Hydrophobic interactions contribute in the initial adhesion of numerous pathogens to tissues (Doyle, 2000). Mudd and Mudd (1924) demonstrated that bacteria can vary considerably in their degree of

hydrophobicity. Furthermore, it was suggested that bacteria with high hydrophobic surfaces might reversibly adhere to intestinal cells (Del Re *et al.*, 2000; Ehrmann *et al.*, 2002). However, other authors have reported no correlation between hydrophobicity and adhesion (Conway and Reginald, 1989; Vinderola *et al.*, 2004). The values of the microbial adhesion to solvents test obtained with n-hexadecane reflected the hydrophobicity of the bacterial surface, whereas the values obtained with chloroform and ethyl acetate in this test can be regarded as a measure of the electron donor (basic) and electron acceptor (acidic) characteristics of the cell walls, respectively (Bellon-Fontaine *et al.*, 1996).

The two selected *L. casei* strains (LAM-1 and LAM-2) studied showed high affinity for the hydrophobic solvent n-hexadecane and for the polar solvents chloroform and ethyl acetate. This indicated, the surface of these strains was able to simultaneously interact with charged (hydrophilic) and non-charged (hydrophobic) molecules. A relationship between adhesion to intestinal mucus and amphiphatic characteristics has been also previously described (Collado *et al.*, 2006) very recently. Thus, it appears that this bivalent nature of the cell surface of the *L. casei* strains (this study) may point towards a high adhesion to intestinal epithelial cells, as indicated by the adhesion assay results.

Adsorption at surfaces is a non-selective process that is only a part of the multistep phenomenon of adhesion. Nevertheless, it is still important, as it brings two surfaces close enough to permit possible adhesions and cell receptors to interact with each other (Gordon *et al.*, 1985; Abraham *et al.*, 1999). A great variability in adhesion to Caco₂ cells was found among *L. casei* strains. One of the strains (*L. helveticus* LKH-5) was non-adhesive, another was moderately adhesive (*L. fermentum* Lamec-29) and two strains (*L. delbruckeii* LKH-2, and LKH-3) were strongly adhesive. Strains which showed poor adhesion to Caco₂ (*L. helveticus* LKH-5 and *L. fermentum* Lamec-29) showed variable values of adhesion to n-

hexadecane and chloroform, but all had low adhesion values to ethyl acetate. The non-adhesive *Lactobacillus* strains in this study, therefore, appeared to have a weak acidic surface character as indicated by an affinity for a basic solvent (ethyl acetate), a variable hydrophobicity and a moderate to strong basic character (as indicated by an affinity for an acidic solvent such as chloroform).

In conclusion, the characterization of the physicochemical properties of a particular strain may help in explaining how this particular strain interacts at the first stage of the multistep process of adhesion to the intestinal cell. Generalization is not possible as it has been shown previously also (Van Loosdrecht *et al.*, 1987; Conway and Reginald, 1989; Del Re *et al.*, 2000; Ehrmann *et al.*, 2002; Vinderola *et al.*, 2004), that there are always exceptions to the rule and no absolute correlation between hydrophobicity and adhesion can be established. Most of these studies targeted only the hydrophobic character of the cell surfaces, but has not investigated the hydrophilic properties (including its acid/base properties). In this study, it was shown that *lactobacilli* with amphiphilic surfaces adhered well to Caco₂ cells. Therefore, it may be inferred, that the amphiphilic character of the surfaces allows bacteria to be more versatile in their interactions with complex surfaces, such as eukaryotic membranes. Pelletier *et al.* (1997) reported similarities among the physicochemical surface properties of strains of the same species and suggested the use of these properties in a taxonomic perspective for microbial classification.

In addition, Del Re *et al.* (2000) and Kos *et al.* (2003) suggested that auto-aggregation can also be correlated to adhesion to intestinal epithelial cells. Cesena *et al.* (2001) reported that the gastrointestinal persistence *in vivo*, as well as the adhesion to epithelial cells *in vitro*, was higher for a *L. crispatus* strain with an aggregating phenotype, than for its non-aggregating mutant. Auto-aggregation and co-aggregation have also been related to the

ability to interact closely with undesirable bacteria (Gusils *et al.*, 1999; Ehrmann *et al.*, 2002).

In this study, strains of *L. casei* auto-aggregated well and also adhered to the intestinal epithelial cells. *L. delbruckeii* strains auto-aggregated to a lesser extent than that of *L. casei* strains. Some authors (Boris *et al.*, 1998; Del Re *et al.*, 2000; Ehrmann *et al.*, 2002) have suggested that auto-aggregation of probiotic bacteria is strongly related to adhesion. Objectives from the present study also suggested that the majority of bacteria which were able to auto-aggregate, also adhered well to epithelial cells. Thus, it may be speculated that auto-aggregation indeed is related to adhesion, but this relationship may not be exclusive, because exceptions were noted. Since a general trend between auto-aggregation and the level of adherence to intestinal epithelial cells was suggested by the results of this study, the auto-aggregation test may serve as an indicator of potentially probiotic strains with good adherence ability in initial screening experiments. However, it was clearly shown that there may be exceptions to this indicative ability, adherence studies in cell cultures remain of great importance and cannot be replaced by auto-aggregation tests.

Apart from possibly serving as an indicator to adherence ability, the auto-aggregation phenotype is an interesting probiotic property, as it plays an important role in colonization in the oral cavity (Kolenbrander, 1995) and the urogenital tract (Boris *et al.*, 1998), as well as in the gastrointestinal persistence of the microorganisms *in vivo* (Cesena *et al.*, 2001). For *L. casei* strains LAM-1 and LAM-2, auto-aggregation was enhanced when tested in their own overnight supernatant (at pH of approx. 4.0), and it was considerably higher than that observed in PBS at pH 4.0. This indicated that auto-aggregation was not a pH-dependent effect but was potentiated by the presence of an auto-aggregating factor in the supernatant, as it has been reported before for other *Lactobacilli* (Schachtsiek *et al.*, 2004). It has also been

hypothesized, that one of the mechanisms through which lactic acid bacteria protect the host from infections is by their ability to co-aggregate with intestinal pathogens and uropathogenic bacteria (Reid *et al.*, 1988; Huis in't Veld *et al.*, 1994).

A strong inclination to auto-aggregation does not imply a strong co-aggregation property, but it has been observed that strains with high co-aggregation ability also show high auto-aggregation (Ehrmann *et al.*, 2002) and our findings also support the same. However, exceptions were noted as *L. casei* LAM-1, which had high auto-aggregation values, did not co-aggregated with *S. aureus* but weakly with *L. monocytogenes*. Previous co-aggregation studies have been limited to bacteria from human origin. Schachtsiek *et al.* (2004) first reported co-aggregation of *Lactobacillus coryniformis*, a food or feed-associated bacterium, with pathogens. *L. coryniformis* was able to coaggregate with *E. coli* K88, *Campylobacter jejuni* and *Campylobacter coli*, but not with *S. typhimurium*, *Clostridium perfringens* and *L. monocytogenes*. In this study, the *L. casei* strains from food origin was able to co-aggregate with human pathogens (*L. monocytogenes*, *S. aureus*, enterotoxigenic *E. coli* and *S. typhimurium*) the co-aggregation derived was higher degree than the commercially used probiotic strains. Strains (especially the *L. casei* strains because of their inherent fermentative versatility) with this property could be of special interest, because co-aggregates may be formed in the food matrix, and thereby prevents the entrapped pathogens from adhering to host cells upon ingestion (Schachtsiek *et al.*, 2004). Decreased numbers of *S. aureus* together with increased numbers in *Lactobacilli* and *Bifidobacteria* in the intestinal microbiota of infants have been associated with atopic dermatitis (Bjorksten *et al.*, 2001; Watanabe *et al.*, 2003).

For these reasons, consumption of products enriched with prebiotics and probiotic bacteria (as the strains presented in this study), may compensate the intestinal microbial

imbalance (by increasing the numbers of *Lactobacilli* and *Bifidobacteria*) in patients with atopic dermatitis. Furthermore, it can also help in exclusion of *S. aureus* through co-aggregation by preventing the pathogen to adhere to IEC and inhibiting the pathogen by production of inhibitory substances such as organic acids in the close proximity resulting in the formation of co-aggregate. Another reason, why exclusion of *S. aureus* would be beneficial is that, nasal and intestinal carriage of *S. aureus* in hospitalized patients has been shown to be a risk for subsequent infections, especially the emergence of methicillin and multiresistant *S. aureus* (Vesterlund *et al.*, 2006). The use of probiotic strains, such as the *Lactobacilli* characterized in this study may also serve as a supportive or preventive treatment.

Diarrhea acquired in developing countries like India is caused mainly by viruses and bacteria such as enterotoxigenic *E. coli*, *Campylobacter*, *Salmonella* spp. (non-typhoid) and *Shigella* spp. (Yates, 2005). Diarrheal diseases results in significant morbidity, as it affects millions of people (Guandalini, 2002). Probiotic preparations have been suggested as preventive/supportive therapy and/or post-therapy after treatment with antibiotics, to help in re-establishing the microbial balance in the GIT and thus prevent antibiotic-associated diarrhoea (Isolauri *et al.*, 1991; Kaur *et al.*, 2002; Yates, 2005). *Enterococcus* spp. on the other hand, has been recognized as major opportunistic pathogens causing bacteraemia, endocarditis, urinary tract infections in the hospital environment and they can also act as potential recipients of vancomycin recipient genes (Franz *et al.*, 1999; Bertuccini *et al.*, 2002; Klein, 2003). Listeriosis is a food-borne disease, which is characterized by meningitis, septicemia and fetal death (Gahan and Hill, 2005). *L. monocytogenes* is an intracellular parasite and the GIT is thought to be the primary site for entry through epithelial cells (Finlay and Falkow, 1997; Gahan and Hill, 2005). Therefore, the inhibition of this invasion step

would prevent the further translocation to the spleen and liver, where *L. monocytogenes* cells multiply, followed by subsequent bacteremia. Because of this background, and because adhesion is considered the first step of pathogenicity (Finlay and Falkow, 1997), two selected *Lactobacillus* strains were tested for their ability to strong competence exclusion to inhibit adhesion to intestinal cells of such important pathogens as *S. typhimurium* ATCC 14028 and *L. monocytogenes* Scott A, which cause considerable morbidity worldwide.

It is expected that a sufficient amount of a probiotic bacteria must be consumed so that the probiotic strains may exert their beneficial effect (Lee *et al.*, 2000). It has also been shown that some probiotic strains able to inhibit adhesion of pathogens only do so, when present at a higher concentration than the pathogen itself (Mack *et al.*, 1999; Lee *et al.*, 2000). This can also be related to the fact that probiotic bacteria are present in probiotic formulations in high numbers, and pathogens usually occurs in low numbers, and in spite of this, they cause disease as their infective dose is generally quite low. For these reasons, higher concentrations of *lactobacilli* were also used in the adhesion inhibition assays of this study. Two strongly adherent strains, *L. casei* LAM-1 and LAM-2, however, were able to significantly reduce the adhesion of the human pathogens like *E. coli* and *L. monocytogenes* in Caco₂ cell culture. In addition, *L. delbruckeii* LKH-2 also reduced the adhesion of the *S. typhimurium* strains used in this study. A slight inhibition of *S. Typhimurium* ATCC 14028 by the non-adhesive *L. casei* LAM-1 was also observed, which does not seems to depend on competitive exclusion for adhesion sites. Instead, it might depend on another antagonistic mechanism such as the production of non-lactic acid molecules with antimicrobial properties as has been described before (Fayol-Messaoudi *et al.*, 2005).

The spectrum and magnitude of the inhibition of adhesion, as well as the auto-aggregation and co-aggregation properties of the selected *L. plantarum* and *L. johnsonii*

strains, suggested that the mechanisms involved in inhibition of adhesion were different. More studies are needed to elucidate if this is due to unspecific steric hindrance or a specific mechanism involving adhesins. Adhesion of pathogenic bacteria to mucosal surfaces is considered as the first step of intestinal infections (Finlay and Falkow, 1997). Overall the findings of this study demonstrated the ability of live *Lactobacilli*, especially *L. casei* LAM-1 and LAM-2, to interfere with pathogens through mechanisms such as production of organic acids and H₂O₂, co-aggregation and competition for adhesion sites. In general, both strains of human origin and strains isolated from food were able to resist simulated gastrointestinal conditions, inhibit pathogens, co-aggregate with pathogens and inhibit their adhesion to IEC, indicating that host-specificity is possibly not required for probiotic activity.

The bacterial isolates were then screened for their potential for bacteriocin production. Six isolates namely, *L. casei* (LAM-1 and LA-1M), *L. delbrueckii* (LKH-2 and LKH-3), *L. helveticus* (LKH-5) and *L. fermentum* (Lamec-29) showed good antimicrobial activities against food borne pathogens *Staphylococcus aureus* ATCC 9144, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Enterobacter (Chronobacter) sakazakii* ATCC 51329, *Shigella flexneri* 2a, *Salmonella typhimurium* ATCC 19585 and *Listeria monocytogenes* ATCC 1911. *Salmonella typhimurium* ATCC 19585. *Lactobacillus casei* (LAM-1 and LA-1M) were capable of inhibiting *Staphylococcus aureus* ATCC 9144, *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Shigella flexneri* 2a, *Salmonella typhimurium* ATCC 19585 and *Listeria monocytogenes* ATCC 1911. *Salmonella typhimurium* ATCC 19585, and *Enterobacter (Chronobacter) sakazakii* ATCC 51329. *L. casei* LAM-1 produced potent bacteriocin with good activities against *Listeria monocytogenes* ATCC 1911. Optimum bacteriocin production was observed at 35°C at constant pH 7.0 after 24 h of incubation for *Lactobacillus casei* LAM-1. *L. casei* LAM-1

gave maximum activity of 2844 AU/ml at 18 h, before and after which a less activity was observed. Maximum bacteriocin production occurred in exponential growth phase in the case of *Lactobacillus casei* (LAM-1) as was typical for bacteriocin production by most of LAB (De Vuyst and Vandamme 1994) therefore, displayed secondary metabolite kinetics. Decreasing bacteriocin activity after 20 h incubation may be explained by bacteriocin degradation due to culture proteases, or low culture pH (Parente and Hill, 1992; Torri Tarelli *et al.*, 1994). In addition, re-adsorption of bacteriocin to the producer cell surface at low pH may contribute to the decrease in the observed bacteriocin in culture medium.

Maximum activity was also observed at 35°C, while 25 and 45°C did not supported bacteriocin production. Criado *et al.* (2006) concluded from their study that temperature has a strong influence on bacteriocin production by their strain and maximal bacteriocin activity was observed at 35°C, whereas, Shin *et al.* (2008) reported 37°C and pH 5-7 as optimum for the bacteriocin production from *Pediococcus pentosaceus* K23-2. Hu *et al.*, (2008) reported enterocin from *Enterococcus duran* over a temperature range 20-43°C.

The bacteriocin activity of *L. casei* LAM-1 in prolonged fermentation dramatically decreased from 28-36 h. Similar pattern had been observed for other LAB bacteriocins (Aasen *et al.*, 2000; Mataragas *et al.*, 2003). For *L. casei* LAM-1, optimum activities were observed between pH 6 and 7 after 18 h. Effect of pH on the bacteriocin production by the selected strains in the present study suggests no growth and production at pH 3.0, although growth was observed at pH 4.0 and 5.0, but no bacteriocin was produced within the 4 h incubation period. According to Van de Berghe *et al.* (2006) bacteriocin production demonstrated primary metabolite kinetics but was limited to the early growth phase. These observations may be explained by critical biomass for switching off bacteriocin production

was dependent on medium pH and incubation temperature, and is inversely correlated with the specific bacteriocin production.

Leroy *et al.* (2003) reported in their study that at constant pH 6.5, high bacteriocin activity was obtained in the temperature range of 20-30°C. Bacteriocin activity was only found between pH 5.5 and 8.0. Kang and Lee (2005) who optimized bacteriocin produced from *E. faecium* GM-1 reported the optimal production of bacteriocin, when the culture pH was 6.0-6.5 and an incubation temperature of 35-40°C was provided.

The *L. casei* LAM-1 differed in behavioral pattern, i.e bacteriocin production occurred in late stage of growth peaking after 18 h. In batch fermentation, *L. casei* LAM-1 produced bacteriocin at the mid log growth phase, and was maximum for upto 2,300 AU/ml at the late stationary phase (Yoon *et al.*, 2005).

Optimization of inoculum size revealed the maximum production of bacteriocin and inhibition of sensitive strain to 1% for all of the isolated strains of *L. casei* (LAM-1). One percent (1% v/v) inoculum was used in bacteriocin production experiments by many authors (Leroy *et al.*, 2003; Moreno *et al.*, 2003; Achemchem *et al.*, 2005).

Plasmid curing revealed that the bacteriocin production genes are mediated by chromosomal DNA. These findings are in accordance with Franz *et al.* 1996 and Du Toit *et al.* 2000, who reported that no plasmid could be isolated from *L. casei* LAM-1 indicating that the gene for bacteriocin production are located on the chromosomal DNA.

The activity of bacteriocins produced by *L. casei* (LAM-1) was thermo-stable and retained their activity even after the heat treatment at 121°C for 15 minutes. These results were consistent with the stability of bacteriocins reported previously. Ying *et al.* (2011) observed that pediocin LB-B1 to be relatively heat stable at moderate temperatures of 100°C

for 90 min and at 121°C for 15 min. Sparo *et al.* (2006) also reported a stable enterocin MR 99 from *E. faecium*. Partially purified bacteriocin appeared stable when pH was adjusted from 4-12. These results are supported by various research findings (Moreno *et al.*, 2003; Sparo *et al.*, 2006; Abriouel *et al.*, 2006; Shin *et al.*, 2008; Ghrairi *et al.*, 2008).

Antibacterial activity of the partially purified bacteriocin was completely destroyed upon treatment with proteolytic enzyme. Bacteriocin activities were not affected by lipase, lysozyme, and catalase and the results were in accordance with the findings of Park *et al.* 2003; Abriouel *et al.* 2006; Cocolin *et al.* 2007 and Ghrairi *et al.* 2008. They identified and characterized enterocin produced by *E. faecium* and reported their inactivation by proteinase K, trypsin, α -chymotrypsin and papain, but not by lysozyme, lipase, catalase or β -glucosidase. Among the detergents, Sodium dodecyl sulphate (SDS), Tween 80 and Tritone X-100 stimulated bacteriocin production, which was strongly inhibited by EDTA and urea. Similar results were observed by Ivanova *et al.* (2000) and Ogunbanwo *et al.* (2003). But, stimulatory effect of Sodium dodecyl sulphate (SDS), Tween 80 and Tritone X-100 on bacteriocin in playing that the detergents act as co-factors, which are required to increase the bacteriocin production. A bacteriolytic effect was noted against *Listeria monocytogenes* for the bacteriocin produced by *L. casei* LAM-1.

The molecular weight of bacteriocins produced by *L. casei* LAM-1 was ~2.5 kDa and a single inhibition zone was observed from the partially purified bacteriocin preparations from the strains of *L. casei* LAM-1, suggesting the production of only one potent bacteriocin. Moreno *et al.* (2003) also reported single inhibitory zone for all bacteriocin after electrophoresis followed by a bioassay. The molecular mass of the bacteriocin was between 2.5 and 6.2 kDa as described by Ying *et al.* 2011. The bacteriocin was stable under different storage temperatures (4, 28 and 37°C as tested up to 6 months. The molecular weight of the

peptide was around 2.5 kDa as observed by Tricine-SDS-polyacrylamide gel electrophoresis. The amino acid sequence of bacteriocin by Edman degradation was TRSGNGVCNNSKCWNVGEAKENIAGIVISGKASGL. Based on available evidence, the bacteriocin was ascribed to the Class IIa Pediocin like bacteriocins.

Moreover 20 pediocin like peptides have been characterized till date (Nes *et al.*, 2001; Nissen-Meyer *et al.*, 1997). They have anti-*Listeria* activity and inactivate target cells by permeabilizing the cell membrane (Moll *et al.*, 1993; Chikindas *et al.*, 1993). Pediocin-like bacteriocins have similar sequences; especially in their N-terminal region. They all have a disulfide bridge and a common YGNGV/L sequence “pediocin box” motif. Besides, they have very similar amino acid sequences, especially in their cationic and hydrophilic N-terminal half. The sequences of their hydrophobic/amphiphilic C-terminal half are somewhat more diverse, and as a consequence the peptides have been grouped into three subgroups, based on the sequence similarities and differences in the C-terminal half (Morisset *et al.*, 2004; Fimland *et al.*, 2002). To understand the mechanism of inactivation, two important food borne pathogens, *S. typhimurium* and *S. flexneri* 2a were chosen. The prevalence of both the pathogens in the Indian subcontinent accords sufficient relevance in examining inactivation methods using the purified bacteriocin. Analysis showed that the pathogens treated with bacteriocin exhibited destruction of the cell membrane and extraction of cell contents. Other studies have reported a change in cell shape from bacilli to coccoid, which has been associated with a loss of infectivity (Rollins and Colwell, 1987). Though a shift to a coccoid form was not observed by electron microscopy, in these sense previous reports have indicated similar differences upon the transformation of bacillary forms to coccoid forms through the processing or degradation of existing proteins (Takeuchi *et al.*, 1998), it is possible that bacteriocin changed the morphology of the pathogens from bacilli to cocci.

To test the hypothesis that bacteriocin interacts with the cytoplasmic membrane of *S. typhimurium* and *S. flexneri* 2a and therefore affects the membrane integrity, the effect of semipurified bacteriocin on the cytoplasmic membrane permeability was determined. A fluorescent technique involving two fluorescent dyes, SYTOw9 (a green fluorescent nucleic acid stain that labels all bacterial cells in a population) and propidium iodide (a red fluorescent nucleic acid stain that only penetrates cells with damaged membranes), was utilized for this purpose. When SYTOw9 and propidium iodide were used in combination, bacteria with intact cell membranes stain fluorescence green, whereas those with damaged membranes stain fluorescence red. The degree of membrane damage can then be estimated from the fluorescence ratio of green to red.

The data obtained suggested that, bacteriocin induces cell death by rendering sensitive cell membranes permeable, allowing for the efflux of K^+ ions and phosphate. This action resulted in the leakage of K^+ ions, and ultimately cell death. It was observed that bacteriocin makes the membranes of sensitive cells permeable, allowing the efflux of K^+ ions and phosphate but not larger compounds, such as ATP. This mechanism of action resembles the broad-spectrum bacteriocins, like nisin, which forms much larger pores (Garcia-Garcera *et al.*, 1993; Konings *et al.*, 1989). There are other possible explanations for the observed variations in the levels of sensitivity to bacteriocin. For example, the ability of the bacteriocin to interact with the cytoplasmic membrane is influenced by factors such as the composition of the cell envelope, including the peptidoglycan layer, and the lipid composition of the membrane, as has been demonstrated with nisin (Gao *et al.*, 1991; Garcia-Garcera *et al.*, 1993).

The exploration of naturally occurring antimicrobials in food preservation received increasing attention due to the consumer awareness of natural food products and growing

concern about microbial resistance toward conventional preservatives. Many studies addressing the bacteriocins application in dairy, meat, fish, milk, and vegetables have been previously reported in the literature (Knoetze, *et al.*, 2008, Albano *et al.*, 2011). Problems concerning maintenance of freezing temperatures during industry storage, distribution and supermarket storage are also increasing with significant damage in food quality and food safety. Also, extended storage at low temperature did not affect bacteriocin stability. This fact may broaden the potential application of such compounds as bio-preservatives.

Therefore, the use of either purified bacteriocin, the producer strain (*in situ* production) or both are of particular interest to the industries such as fish and vegetable fermentation as it may become helpful to ensure the food hygiene and safety of the products. Recently, Molinos *et al.* (2005) tested the effectiveness of immersion solutions containing enterocin AS-48 for decontamination of raw vegetables. Other bacteriocins which were tested in vegetable products include nisin in tinned vegetables and fruit juices (Delves-Broughton, 1990; Alpas, and Bozoglu, 2000; Komitopoulou *et al.*, 1999), pediocin PA-1/AcH in salad and fruit juice (Cleveland *et al.*, 2001; Alpas, and Bozoglu, 2000). Overall in the current study, an attempt was made to characterize the technological and functional attributes of probiotics. The findings suggest that the characterized bacteriocin from the selected strain had a broad range of spectrum against foodborne pathogens. Thus, these probiotic *Lactobacillias* or their bacteriocin(s), have the potential for commercial use. In particular, the bacteriocin of *L. casei* LAM-1 may be exploited further as a bioperservative.

CONCLUSIONS

The main objective of this study was to find new probiotic candidates to be used in functional food and *in vitro* characterisation of selected strains following the criteria for selection of probiotic strains proposed by a Joint FAO/WHO working group of experts in probiotics.

In the present study, six strains of *Lactobacillus* were selected out of ninety six isolates from fermented indigenous foods, beverages and human gut, based on their survival ability under gastrointestinal conditions, the isolates were characterized by phenotypic and genotypic methods.

Each strain presented individual characteristics, which may contribute to their ‘probiotic’ health-promoting effects. Based on the results of their probiotic properties, two strains, *L. casei* (LAM-1 and LAM-2), two *L. delbruckeii* (LKH-2 and LKH-3), one *L. helveticus* (LKH-5) and one strain of *L. fermentum* (Lamec-29) were finally selected.

All six strains were able to tolerate phenol, which indicate that they may reach the site of action, the small intestines unharmed. Because of their metabolic properties, they would probably contribute to the reduction of cholesterol levels due to the presence of Bsh activity. In addition, two of the strains, namely *L. casei* LAM-1 and LAM-2, may also contribute to alleviation of lactose intolerance because of their β -galactosidase activity. All six selected strains have long history of safe use and do not exhibit transferable antibiotic resistance, which implies their acceptability according to the guidelines of the European Food Safety Association.

Based on the antimicrobial, coaggregative and adhesive properties of the selected *Lactobacilli* it was inferred that the isolates were potential for inhibition or exclusion of food pathogens by different mechanisms. Moreover the ability of the probiotic *Lactobacilli* to coaggregate with pathogens, may enhance their (pathogens) clearing from the gastrointestinal

tract, thus preventing them from adhering to IEC and also inhibiting them in this micro-environment by producing organic acids, H₂O₂ and bacteriocin.

Competitive exclusion of two of the selected strains, i.e. *L. casei* LAM-1 and *L. delbruckeii* LKH-2, was observed *in vitro* against representative food-borne pathogens, suggesting a significant role in prevention of enteric infections.

After screening all six strains for their functional properties, strains displaying interesting probiotic properties were then chosen for ability to produce bacteriocin. Amongst all, *L. casei* LAM-1 elaborated a bacteriocin with wide spectrum of action (gram positive and negative food borne pathogens) and stability under high temperature, pH and freeze thaw cycles. Most probably, bacteriocin activity results from the sum of several factors; growth-associated and stress associated mechanisms added to a possible constitutive production. According to the derived polynomial as a function of pH, temperature and time of incubation, it can be observed that production of bacteriocin by *L. casei* LAM-1 is regulated by different physiological factors. The bacteriocin exhibited bactericidal activity through rapid loss of ATP and K⁺ ions by disruption of cell membranes of target pathogens; cell death was evidenced by live dead staining and scanning electron microscopy followed by transmission electron microscopy.

It is recommended that colonization ability of the above mentioned probiotic isolates must be further validated through *in vivo* studies. Overall, this study provides a rationale for the further use of the selected *L. casei* LAM-1 and *L. delbruckeii* as probiotics for therapeutic and preventive purposes; however a prior clinical trial in humans is mandatory. The application of the bacteriocin from *L. casei* LAM-1 for commercial purposes as biopreservative is also suggested.

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Appendix I

MRS (De Man, Rogosa and Sharpe) broth

Ingredients	Quantity (g/L)
Peptone from casein	10.0
Meat extract	8.0
Yeast extract	4.0
D(+)-glucose	20.0
Dipotassium hydrogen phosphate	2.0
Tween 80	1.0
Diammonium hydrogen citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.2
Manganese sulphate	0.04

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

MRS agar: MRS broth containing 15.0 g/l agar.

MRS-Arginine broth

Ingredients	Quantity (g/L)
Peptone from casein	10.0
Yeast extract	5.0
D(+)-glucose	0.5
Dipotassium hydrogen phosphate	2.0
Tween [®] 80	1.0
Diammonium hydrogen citrate	20.0
Sodium acetate	5.0
Magnesium sulphate	0.1
Manganese sulphate	0.05
Arginine	3.0

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

Rogosa agar

Ingredients	Quantity (g/L)
Peptone from casein	10.0;
Yeast extract	5.0;
D(+)-glucose	20.0;
Potassium dihydrogen phosphate	6.0;
Ammonium citrate	2.0;
Tween 80	1.0;
Sodium acetate	15.0;
Magnesium sulphate	0.575;
Iron (II) sulphate	0.034;
Manganese sulphate	0.12;
Agar-agar	15.0;

pH adjusted to 5.5 with acetic acid 96% (v/v). This medium was not autoclaved.

ABTS-Medium for determination H₂O₂ production.

Ingredients	Quantity (g/L)
Fish extract	10.0
Tryptone	10.0
Yeast extract	5.0
Tween 80	1.0
Dipotassium hydrogen phosphate	2.0
Diammonium hydrogen citrate	2.0
Magnesium sulphate	0.2
Manganese sulphate	0.05
Sodium acetate	5.0
D(+)-glucose	15.0
Agar	13.0

pH 6.5. Supplemented with 0.5 mM ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) and 0.3 U/ml horse-radish peroxidase (Sigma, USA).

Basal medium for sugar fermentation test (API50CH)

Ingredients	Quantity (g/L)
Peptone from casein	5.0
Meat extract	5.0
Yeast extract	5.0
Dipotassium hydrogen phosphate	2.0
Tween [®] 80	1.0
Magnesium sulphate	0.2
Manganese sulphate	0.05

Brain heart infusion agar (BHI)

Ingredients	Quantity (g/L)
Protease peptone	10.0
Calf brain	200.0
Beef heart	250.0
Dextrose	2.0
NaCl	5.0
Disodium phosphate	5.0
Agar	15 .0

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

M17 broth

Ingredients	Quantity (g/L)
Peptone from soymeal	5.0
Peptone from meat	2.5
Peptone from casein	2.5
Yeast extract	2.5
Meat extract	5.0
Lactose monohydrate	5.0
Ascorbic acid	0.5
Sodium β -glycerophosphate	19.0
Magnesium sulphate	0.25

BUFFERS AND SOLUTIONS**1. TBE buffer (10X)**

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

2. 0.1M Phosphate buffer

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

3. Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

4. Ethidium Bromide

0.5 μ g mL⁻¹

Denaturing solution.

guanidinium isothiocyanate	4 M
sodium citrate (0.75 M; pH 7)	25 mM
β-mercaptoethanol	0.1 M
N-lauryl sarcosinate	0.5 %

GES solution.

guanidinium thiocyanate	5 M
EDTA	100 mM
Sarkosyl; pH 8	0.5%.

Loading buffer.

bromophenol blue dye	2.5 mg/ml
glycerol in 1 x TE (pH 8.9)	50 % (v/v)

TERMLS.

EDTA	10 mM
D(+)-glucose	0.2 g/l D
lysozyme	0.015 g/ml
mutanolysin	100 U/ml
25 µg/ml RNase	

Tris-HCl. 121.1 g Tris base dissolved in 800 ml H₂O, adjusted to pH 8 with approx. 42 ml HCl, adjusted to 1 and autoclaved.

TBE buffer (10x)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

Plasmid extraction solution I (10X)

Tris-HCl	25 mM (pH 8.0)
Glucose	50 mM
Na ₂ EDTA	10mM

Plasmid extraction solution II

NaOH	5M
SDS	10%

Plasmid extraction solution III

5.0 M K-acetate (pH 4.5)

Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%

Glycerol in water 30.0%

Staining solutions

Alcian Blue	0.1%
3% glacial acetic acid	100.0 mL

DMEM used for culturing of Caco₂ colon carcinoma cells. Dulbecco's modified Eagle's medium (Invitrogen Technologies, USA).

DEPC-H₂O. DEPC (diethylpyrocarbonate) 0.1 %

The solution was agitated overnight and then autoclaved to eliminate DEPC. DEPC-H₂O is free of RNases.

Primers

16S rDNA forward primer	5'-AGAGTTTGATCCTGGCTCAG-3'
16S rDNA reverse primer	5'-ACGGGCGGTGTGTTTC-3'

Appendix II

Fig. 3.1. Standard curve of Bovine serum albuin. Relationship between proteinand absorbance using colorimetric method assay. $R^2 = 0.989$

