Molecular detection and characterization of antagonistic and *in vitro* adhesion property of lactic acid bacteria

A THESIS

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Dedicated to my family, friends and Department of Biotechnology, IIT Guwahati

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STATEMENT

I do hereby declare that the research findings of this thesis is the result of research work carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Aiyagari Ramesh.

As per the general norms of reporting research findings, due acknowledgements have been made wherever the research findings of other researchers have been cited in this thesis.

Date: 14th December, 2009 **Atul Kumar Singh**

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CERTIFICATE

It is certified that the work described in this thesis entitled "***Molecular detection and characterization of antagonistic and in vitro adhesion property of lactic acid bacteria***" by Mr. Atul Kumar Singh for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for the award of degree in any form.

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ABBREVIATIONS

A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ABC	ATP-binding cassette
AFLP	Amplified fragment length polymorphism
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
AP-PCR	Arbitrarily primed-PCR
ATP	Adenosine triphosphate
AU	Arbitrary unit
Bac^+	Bacteriocin producer
BHI	Brain Heart infusion medium
BLAST	Basic local alignment search tool
BLIS	Bacteriocin-like inhibitory substance
bp	Base pair
CF	Culture filtrate
cFDA-SE	5(and 6)- carboxyfluorescein diacetate succinimidyl ester
cF-SE	5(and 6)- carboxyfluorescein succinimidyl ester
cfu	Colony forming unit
cps	Counts per second
CTAB	Cetyl trimethylammonium bromide
DGGE	Denaturing gradient gel electrophoresis
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EMP	Embden-Meyerhof-Parnas
ERIC	Enterobacterial repetitive intergenic consensus sequence

FBS	Fetal bovine serum
FDA	Food and Drug Administration
FSC	Forward scattering
GRAS	Generally regarded as safe
IGS	Intergenic spacer region
kDa	Kilo-dalton
kV	Kilo-volt
ITS	Internal transcribed sequence
LAB	Lactic acid bacteria
LB	Luria Bertani medium
LH-PCR	Length-heterogeneity polymerase chain reaction
M	Molarity
MIC	Minimum inhibitory concentration
MKC	Minimum killing concentration
MRS	de Man, Rogosa and Sharpe medium
MTCC	Microbial Type Culture Collection
M_r	Relative molecular weight
MWCO	Molecular weight cut off
N	Normality
NASBA	Nucleic acid sequence based amplification
NB	Nutrient broth medium
NCBI	National Center for Biotechnology Information
NCFB	National Collection of Food Bacteria
NCIM	National Collection of Industrial Microorganism
NICE	Nisin-controlled expression system
NIH	National Institute of Health
Non-LAB	Non-lactic acid bacteria
NRRL	Northern regional research laboratory
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween 20

PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction-Denaturing gradient gel electrophoresis
PFGE	Pulsed-field gel electrophoresis
pH	Power of hydrogen
PI	Propidium iodide
PTS	Phosphotransferase system
SDS-PAGE	Sodium-dodecyl sulphate- polyacrylamide gel electrophoresis
SSC	Side scattering
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
REA	Restriction enzyme analysis
REP	Repetitive extragenic palindrome
rep-PCR	Repetitive element sequence-PCR
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TAP-PCR	Triplicate arbitrarily primed-PCR
TEM	Transmission electron microscopy
TGGE	Temperature gradient gel electrophoresis
UDSC	University of Delhi, South Campus
UPGAMA	Unweighted pair group method using arithmetic averages
λ_{em}	Emission wavelength (nm)
λ_{ex}	Excitation wavelength (nm)
µl	Microliter
µM	Micromolar

CHAPTER 1

Introduction and Literature review

Introduction

Lactic acid bacteria (LAB) – A brief background on the bacterium and current research trends

Lactic acid bacteria (LAB) are widely regarded as one of the most versatile groups of microorganisms enjoying a GRAS (generally regarded as safe) status and have profound food fermentation and healthcare applications. Safe application of LAB for health benefits to mankind has long history since the time of a Russian bacteriologist Professor Elie Metchnikoff in 1910. There is a growing interest amongst researchers in the field of molecular microbiology to work with LAB owing to its fundamental applications in food fermentation processes and its immense potential in biomedicine as a cargo for mucosal vaccination (Galvez *et al.,* 2007; Wells and Mercenier, 2008).

Lactic fermented food products are widely consumed all over the world and many of them belong to the category of fermented functional food. Fermented foods with functional attributes basically constitute uncontaminated food products with high nutritional value and increased shelf-life providing health benefits to the consumer. Food fermentation processes controlled by defined strains of LAB has drawn major attention of consumers as they impart definite aroma, flavor and sensorial qualities. Essentially the controlled fermentation with LAB imparts desirable characteristic to the fermented products by virtue of its various physiological and technological attributes (Coolbear *et al.,* 2008; Leroy and De Vuyst, 2004).

Food fermentation applications of LAB demands techniques for rapid identification of LAB starter strains with high fidelity. The identification of starter culture with culture-dependent method poses serious limitations in the rapid analysis and characterization of

strains, rendering the method arduous and time-consuming (Temmerman *et al.,* 2004). Thus the current trend has been to develop nucleic acid based culture-independent method for strain identification (Juste *et al.,* 2008). Amongst culture-independent methods polymerase chain reaction (PCR)-based methods offer rapid and convenient options for LAB strain identification, monitoring of starter cultures, detection and molecular fingerprinting of LAB strains (Coudeyras *et al.,* 2008; Nielsen *et al.,* 2007; Settani *et al.,* 2005; Yost and Nattress, 2002). A major challenge for the current food industry includes reduction of food spoilage based economic losses, lowering the food processing costs and providing uncontaminated food products to the consumers and satisfying their growing demands for ready-to-eat, fresh, nutrient and vitamin rich and minimally-processed and preserved food products. LAB used as starter culture in various fermentation process increases the shelf-life of fermented food product. The biopreservation of food product has been a common practice in the history of mankind and reflects empirical use of microorganisms and/or their metabolic products. LAB produce an array of antimicrobial substances such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides, and bacteriocins and thus contribute as a potent biopreservative agent. Amongst the antimicrobial agents produced by LAB, bacteriocins which are essentially antimicrobial peptides have drawn considerable interest. A motivating factor leading to a growing interest in bacteriocins produced by LAB has been the increasing incidence and detection of food-borne pathogens, which offers the possibility of using the bacteriocin produced by LAB to eliminate the pathogens in food.

Lactic acid bacteria are also known for their probiotic attributes and have been shown to influence the activity and composition of the intestinal microbiota (Ouwehand *et al.,* 2002). A probiotic has been defined as “live microorganism which when administered in

adequate amounts confer a health benefit on the host" (Salminen *et al.,* 1999). Probiotic LAB are selected on the basis of their ability to adhere to the intestinal mucosa, acid and bile resistance, and antagonism against pathogenic bacteria. The ability to adhere to intestine is one of the main selection criteria, because it is considered to be paramount in persistence, enhanced healing of the damaged mucosa (Elliott *et al.,* 1998), antagonism against pathogens (Reid and Burton, 2002) and immunomodulation (Zhou and Gill, 2005). Probiotics have also been reported to alleviate problems associated with inflammatory bowel diseases (Isolauri *et al.,* 2002). One of the prerequisites of a good probiotic is adhesion to mucus and epithelial cells (Salminen *et al.,* 1999). Hence the current research in LAB has been focused towards studies on *in vitro* models using mammalian cell culture techniques to evaluate the adhesion of probiotic LAB to epithelial cells.

Overall the research trends are encouraging and there is immense potential in studying antagonistic LAB specially bacteriocin producing strains for food fermentation applications. Additionally there is an increasing interest in probiotic LAB and thus a need to identify LAB strains with adhesion potential. It is envisaged that research which addresses these above mentioned issues would pave the way to develop tailor made competitive LAB strains for food fermentation applications as well as provide novel therapies for infections caused by intestinal bacterial pathogens.

In the following section a detailed literature review pertaining to lactic acid bacteria (LAB), their general characteristics, methods of identification, bacteriocin producing traits, food application potential and adhesion property is discussed.

Literature review

1.1. Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) belong to microaerophilic, non-sporulating, catalase-negative, non-motile and gram-positive group of bacteria with low G+C content and primarily producing lactic acid as a metabolic product (Vandamme *et al.,* 1996). LAB are generally associated with habitats rich in nutrients, such as various food products (milk, meat, beverages, vegetables). However, some are also found as members of the normal microflora of mouth, intestine and vagina of mammals (Stiles and Holzapfel, 1997). In food fermentation processes, LAB play a pivotal role as starter culture and also impart flavor to food products (Coolbear *et al.,* 2008; Di Cagno *et al.,* 2008; Leroy and De Vuyst, 2004; van Hylckama Vlieg and Hugenholtz, 2007). LAB exhibit antimicrobial activity which is attributed to its secreted metabolites like organic acids, hydrogen peroxide, carbon dioxide, diacetyl and low molecular weight antimicrobial agents. Amongst the secreted antimicrobial agents produced by LAB, low molecular weight antimicrobial agents known as bacteriocin has drawn major attention. Bacteriocins are ribosomally synthesized proteinaceous molecules produced by LAB which confer a selective advantage and a competitive edge to the producer strains over other competing microbe in the same ecological niche (Riley and Wertz, 2002). Bacteriocins produced by LAB are categorized into different classes and subgroups based on their size, heat sensitivity, signal sequence and post-translational modification based structural difference (Ennahar *et al*., 2000; Drider *et al.,* 2006; Jack *et al.,* 1995). Amongst the bacteriocins produced by LAB nisin, produced by *Lactococcus lactis* has been approved by United States Food and Drug Administration (FDA) for food application as a supplement to functional foods (Cotter *et al.,* 2005; de Arauz *et al.,* 2009).

1.2. Physiology of LAB

Lactic acid bacteria (LAB) have the ability to pursue their metabolically active physiological state in different ecological niche (Konings *et al.,* 2000). The essential feature of LAB physiology includes efficient carbohydrate fermentation coupled to substrate-level phosphorylation. LAB as a group exhibit tremendous capacity to degrade different carbohydrates and related compounds and has been also reported to accommodate in various physiological conditions and change their metabolic pathways and thus the products accordingly. Application of LAB in fermented food samples could be attributed to the arsenal of enzymes and other metabolites produced at various metabolic states under different physiological conditions as reported in the study of Hugenholtz *et al.* (1999). LAB possesses ATP-binding cassette (ABC) transporter proteins and sugar phosphotransferase system (PTS), which provides the capacity to utilize nutrients and survive in varying ecological niche (Davidson *et al.,* 2008). LAB physiology crucial to food fermentation primarily involves the pathways responsible for hexose fermentation and the metabolite produced at the end of the fermentation. The main fermentation pathways involved are Embden-Meyerhof-Parnas (EMP) and pentose phosphate pathway. LAB produces free amino acid through proteolysis and converts them into flavor forming compounds through amino acid catabolism (Gobbetti *et al.,* 2005). The amino acids are further converted into flavor compounds via two different routes viz. transamination and elimination. Aminotransferases enzymes catalyze the transamination of amino acid into their corresponding α-keto acids. The α-keto acids are then further converted into aldehydes, alcohols and esters as aroma compounds (Liu *et al.,* 2008).

1.3. LAB classification

The classification of LAB was primarily based on classical microbiological methods as reported by Orla-Jensen (1919). However, LAB has been classified by polyphasic methods considering both phenotypic as well as genotypic similarities amongst different members of LAB (Vandamme *et al.,* 1996). The phenotypic characteristic includes morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid bacteria, ability to grow at high salt concentrations and acid or alkaline tolerance. Primarily genotypic trait based classification includes the study of similarities in 16S rRNA gene sequence (Case *et al.,* 2007; Mignard and Flandrois, 2006) and β subunit of DNA-dependent RNA polymerase coding gene sequence (Dahllof *et al.,* 2000) and application of other molecular tools.

Based on the phenotypic and genotypic characteristics the LAB genera has been divided into *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Stiles and Holzapfel, 1997). Bifidobacterium often considered as LAB, is phylogenetically unrelated to other LAB genera as it has unique mode of sugar fermentation and high G+C genomic content. The detailed insights of phenotypic and genotypic classification of LAB have been discussed in the subsequent section.

1.4. Phenotypic and biochemical method of LAB classification

Phenotypic or biochemical method of LAB classification is primarily based on the chemotaxonomic techniques. The classical phenotypic classification relies on morphological,

physiological and biochemical features of bacteria. Some of the salient tests which aid genus level identification of LAB are indicated in Table 1.1.

Table 1.1

Distinguishable phenotypic characteristic of major genus of lactic acid bacteria*

LAB Genus	Character								
	Tetrad formation	CO_2 from glucose[#]	Growth at 10 °C	Growth at 45 °C	Growth in 6.5% NaCl	Growth in 18% NaCl	Growth at pH 4.4	Growth at pH 9.6	Lactic acid[$]
Carnobacterium	–	–	+[a]	–	ND[b]	–	ND	–	L
Lactobacillus	–	±	±	±	±	–	±	–	D, L, DL[c]
Aerococcus	+	–	+	–	+	–	–	+	L
Enterococcus	–	–	+	+	+	–	+	+	L
Lactococcus	–	–	+	–	–	–	±	–	L
Vagococcus	–	–	+	–	–	–	±	–	L
Leuconostoc	–	+	+	–	±	–	±	–	D
Oenococcus	–	+	+	–	±	–	±	–	D
Pediococcus	+	–	±	±	±	–	+	–	L, DL[c]
Streptococcus	–	–	–	–	–	–	–	–	L
Tetragenococcus	+	–	+	+	+	+	–	+	L
Weissella	–	+	+	+	±	–	±	–	D, DL[c]

+, positive; –, negative; ±, response varies between species; ND, not determined.
[#]Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.
[$]Configuration of lactic acid produced from glucose.
[a]Small amounts of CO2 can be produced, depending from media.
[b]No growth in 8% NaCl has been reported.
[c]Production of D-, L-, or DL-lactic acid varies between species.
*Data adapted from Seppo Salminen et al., (2004) *Lactic acid bacteria Microbiological and Functional Aspects*, Third edition Marcel Dekker, Inc.

The morphology of a bacterium includes both cellular (shape, endospore, flagella, inclusion bodies and gram-staining) and colony (color, dimensions, forms) characteristics. Vandamme et al. (1996) and Stiles and Holzapfel (1997) have reported in their review that physiological and biochemical features include data on growth at different temperature, pH values, salt concentrations, atmospheric conditions, growth in the presence of various substances like antimicrobial agents and data on the presence or activity of various enzymes and metabolic products. The phenotypic method of bacterial classification also includes analysis of cell wall composition (Schleifer *et al.,* 1972), cellular fatty acid, whole-cell protein profile (Descheemaeker *et al.,* 1994) and electrophoretic mobility of lactate dehydrogenase protein

(Fujisawa *et al.,* 1992). Phenotypic methods of LAB classification are simple and do not require state of the art equipments for experiments. However, the procedures are arduous and often provide poor discrimination amongst the LAB genus as most of the LAB share common metabolic process and the enzymes involved in metabolism. The inadequate discriminatory potential of phenotypic methods necessitates the application of molecular methods in the classification and taxonomy of lactic acid bacteria.

1.5. Molecular methods for LAB classification and identification

Increasing number of LAB species with very closely related and similar metabolic functions lead to inappropriate taxonomic affiliations, when they are classified on the basis of phenotypic and biochemical properties. Molecular approaches which encompass nucleic acid-based techniques offer reliable solutions for classification and identification of LAB strains (Stiles and Holzapfel, 1997). Molecular method of bacterial taxonomy and classification includes the genotypic methods directed towards the analysis of homology in the DNA and RNA molecules. The traditional phenotypic identification of LAB is rather tedious and not reliable all the time. Phenotypic response of bacterial strains also varies with environmental factors and thus essentially emphasizes the need of more reliable method of bacterial classification. Schleifer *et al.* (1995) has reported the use of nucleic acid probes for LAB taxonomy and classification.

Recently with the availability of rapid and automated DNA sequencing techniques, direct sequencing of 16S ribosomal RNA (rRNA) gene has emerged as a powerful tool for classification of bacteria. The use of rRNA and their encoding genes as target molecules for the detection and classification of microbes has gained immense significance owing to their

universal distribution and high nucleotide sequence conservation. The number of rRNA (*rrn*) operons located on a genome varies considerably between species and it has been correlated to the rate with which microbes can respond to available resources. In lactic acid bacteria, complete genome sequences of *Lactobacillus plantarum*, *Lactococcus lactis*, and *Lactobacillus johnsonii* were used to compare location, sequence, organisation, and regulation of the ribosomal RNA (*rrn*) operons (de Vries *et al.,* 2006).

The polymerase chain reaction (PCR) technique is a widely used tool for LAB classification purpose. One of the targets for such experiments involving PCR has been the rRNA gene. With automated sequencing systems and availability of PCR-based direct sequencing tools, the 16S rRNA sequence has become an extremely significant handle for reliable identification and classification of LAB strains. Previous studies have reported the application of 16S rRNA primers to detect a specific LAB genus or species using conventional and multiplex PCR approach (Chagnaud *et al.,* 2001; Dubernet *et al.,* 2002; Settanni *et al.,* 2005). Research efforts have also been directed on the design of 16S rRNA primers and its application to monitor LAB dynamics and community development in food system (Delbe's *et al.,* 2007; Randazzo *et al.,* 2006). The rRNA heterogeneity among LAB species has also been judiciously exploited in PCR-DGGE (denaturing gradient gel electrophoresis), resulting in powerful analysis of LAB population in a variety of food matrices (Cocolin *et al.,* 2006; Cocolin *et al.,* 2007; Randazzo *et al.,* 2009).

1.6. Molecular fingerprinting methods to study LAB strain variations and phylogeny

Genotypic techniques based on fingerprinting analysis exhibit various levels of discriminatory power, from species level to differentiation of individual strains. Essentially

signature banding patterns are generated for various strains and these patterns are then utilized for cluster analysis to establish the phylogeny (Ehrmann *et al.,* 2005; Temmerman *et al.,* 2004;). Many fingerprinting methods are based on the principle of PCR, which enables the selective amplification of targeted DNA fragments with oligonucleotide primers under controlled reaction conditions. Theoretically, PCR primers can be designed to amplification and characterize the bacterial genome at any taxonomic level.

DNA-based fingerprinting or typing methods have primarily been used to subdivide species at strain level. DNA-based typing methods include whole-genome restriction fragment analysis also called as restriction fragment length polymorphism (RFLP) or restriction enzyme analysis (REA), plasmid DNA analysis, randomly amplified polymorphic DNA (RAPD), arbitrarily primed polymerase chain reaction (AP-PCR), amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE). A number of PCR-based fingerprinting techniques have been developed for detecting strains variations in LAB. RAPD has been one of the most commonly used techniques for distinguishing LAB strains. RAPD has been shown to be useful in discriminating strains belong to *Lactobacillus acidophilus* (Du Plessis *et al.,* 1995; Gancheva *et al.,* 1999). Several other studies conducted by various research groups report the application of RAPD for LAB strain identification (Bouton *et al.,* 2000; Rossetti and Giraffa, 2005; Pulido *et al.,* 2005; Ruiz *et al.,* 2008). The facile nature of molecular typing makes RAPD an attractive option but to obtain reproducible results standardization of the procedure is a stringent requirement. An alternative fingerprinting PCR-based method is the repetitive-element based PCR (rep-PCR), which is based on conserved repetitive DNA sequence in the bacterial genome. The targets used in rep-PCR include various conserved elements and accordingly the method is known

as ERIC-PCR (Enterobacter repetitive inter consensus region), BOX-PCR (BOX element based) and $(GTG)_5$. A large number of reports indicate the application of rep-PCR methods for molecular typing and strain characterization of LAB (De Angelis *et al.,* 2007; Gevers *et al.,* 2001; Nikolic *et al.,* 2008; Terzic-Vidojevic *et al.,* 2007). With regard to AP-PCR, an 18 mer primer targeting a universally conserved region of 16S rRNA gene with a wobble base at 17^{th} position was used to perform triplicate arbitrary primed PCR (TAP-PCR) and fingerprint various strains of LAB genera (Cusick and O'Sullivan, 2000). Most of the molecular techniques discussed in the aforementioned section do not provide information on the whole-genome level. Pulsed-field electrophoresis (PFGE) is one of the most powerful techniques to extract information on a whole genome level. Application of PFGE for molecular genotyping of LAB strains has been documented (Bouton *et al.,* 2002; Ventura and Zink, 2002). In spite of the powerful taxonomic resolution offered by techniques such as PFGE, routine application of these techniques are hampered due to the cost, level of sophistication involved and practical problems in large scale implementation.

1.7. Role of LAB in food fermentation

The concept of functional food was introduced by Hippocrates, The Father of Medicine long ago and his motto "Let food be your medicine" reflected the importance for the consumption of fermented food products. Based on the reports on fermented food providing increased life span to the consumer and longer preservation of food product, immense research was carried out in the field of fermented food products in early 19^{th} century (Vasiljevic and Shah, 2008). The role of LAB in fermented food was first studied by Louis Pasteur and his co-workers in 1857 and the idea to describe lactic acid bacteria as a group of microorganism involved in

the food and feed fermentation developed in the early period of 19th century based on their unique characteristics.

Food fermentation processes controlled by defined strains of LAB has drawn major attention of consumers for minimally processed food products with definite aroma, flavor and sensorial qualities. LAB has significant contribution in the preparation of healthy and functional foods during fermentation process. LAB plays vital role in food fermentation processes to increase the shelf-life and organoleptic value of fermented food products (Topisirovic *et al.,* 2008). The low pH build by acid and the action of other antimicrobials produced by LAB inhibits the growth of many spoilage and pathogenic bacteria. Besides, various physiological and technological attributes of LAB imparts desirable characteristic to the fermented products such as unique aroma, flavor and texture as (Coolbear *et al.,* 2008; Leroy and De Vuyst, 2004). In food fermentation industry LAB is primarily used as starter cultures to initiate the fermentation process. LAB produces a gamut of antimicrobial metabolites such as organic acid, diacetyl, acetoin, hydrogen peroxide, antibiotics and bacteriocins, which increases the shelf-life of fermented food products. Amongst the anti-microbial metabolites, bacteriocins have drawn major attention for their application in food and feed system as antimicrobials under the regime of natural biopreservatives (Cotter *et al.,* 2005; Drider *et al.,* 2006; O'Sullivan *et al.,* 2002).

LAB also plays a vital role in natural or spontaneous fermentation processes. Inherent LAB population present in a given food or feed substrate is defined as "autochthonous" population, which primarily act as a starter culture and govern the fate of fermentation. Samples used in such fermentation consist of mixed LAB population of different genus and thus form a complex microbial community. Microbial community of LAB, inhabiting a

nutrient rich environment in various food samples has high density, wide diversity and complexity of interactions amongst them, leading to microbial succession (Fontana *et al.,* 2006). It can also be envisaged that metabolic activities of lactic acid bacteria plays major role in successful natural fermentation of carbohydrate-rich food and feed fermented products. Such fermentation process leads to the succession of LAB based on their antagonistic and adaptability attributes (Haruta *et al.,* 2006; Kostinek *et al.,* 2007). Microbial succession of LAB with the emergence of dominant and antagonistic strains also depends on the functional traits of LAB species such as ability to synthesize and secrete bacteriocin, resistance to harsh fermentation environment (pH, competition and antagonistic metabolites) and ability to utilize available nutrients in the environment (De Vuyst and Vancanneyt, 2007).

1.8. Bacteriocins produced by LAB

Bacteriocins producing ability of LAB has attracted great interests in terms of food safety and increased shelf-life of fermented food. Salient features of bacteriocins include: (a) bacteriocins are ribosomally synthesized, (b) host cells or producer cells are immune to their action, (c) most of he bacteriocins act on bacterial membranes resulting in their permeabilization and (d) they have a narrow spectrum of activity and thus they are generally known to kill only closely related bacterial strains. Bacteriocin produced by LAB has conferred significant contribution as biopreservative in various fermentation processes (Abee *et al.,* 1995; De Vuyst and Leroy, 2007; Galvez, 2007; Ganzle, 2009; Rouse and van Sinderen, 2008; Settani and Corsetti, 2008). Apart from biopreservative applications in food, bacteriocins can find application as candidate drugs for specific pathogens without

harming the beneficial gut microflora. Thus bacteriocins can then reduce the use of antibiotics and with them the risk of developing antibiotic resistance. Besides, consumer awareness on the health risks associated with the use of chemical preservatives in food has enhanced the interest in bacteriocins. It is also noteworthy that bacteriocins are naturally produced, mostly proteinaceous and hence can be degraded under physiological conditions of the gut environment. This also leads to consumer acceptance. The following section deals with classification of bacteriocins produced by LAB.

1.9. Bacteriocin classification

Bacteriocins produced by lactic acid bacteria can be primarily divided into four major classes and Table 1.2 indicates the different classes of bacteriocin produced by LAB strains.

A. Class I:

Class I bacteriocin, also called lantibiotics, are small (<5 kDa) peptides synthesized as a pre-peptide molecule. During translocation and secretion, inactive pre-peptides are converted into active peptide after cleavage of the leader sequence. Lantibiotics contain unusual amino acids which are included in the peptide after post-translational modifications. These unusual amino acids are lanthionine, *β*-methyl-lanthionine and dehydrated serine and threonine amino acids. One of the most important members of this class of bacteriocin is nisin, which has been approved by FDA, USA as a food additive (Cotter and Hill, 2005; Stevens *et al.,* 1991).

B. Class II:

This class of bacteriocins molecules are generally small size (<10 kDa), heat-stable, non-lanthionine containing membrane-active peptides. Class II bacteriocin can be divided into three subclasses **(Table 1.2)**. Amongst these subclass IIa is the largest group with conserved amino-terminal sequence (YGNGVXC) and strong activity against *Listeria* (Ennahar *et al.,* 2000; Drider *et al.,* 2006; Jack *et al.,* 1995; Nes *et al.,* 1996). Subclass IIb includes bacteriocin with two peptides, whereas subclass IIc contains other non-lantibiotic bacteriocins or miscellaneous peptides. Class II bacteriocins have been of major interest as food additive after nisin, as they do not have limitation of narrow spectrum of antimicrobial activity and narrow range of pH dependent activity.

C. Class III:

Class III Bacteriocin are primarily large, more than 30 kDa heat-labile proteins, and may include bacteriolytic extracellular enzymes e.g. hemolysins and muramidases. The mode of action of these molecules involves the perturbation of the cell membrane integrity of target bacterial cells. Genus *Lactobacillus* is also known to produce class III group of bacteriocins molecules (Jack *et al.,* 1995, Nes *et al.,* 1996).

D. *Class IV:*

A fourth class of bacteriocin has also been defined, which contains bacteriocin composed of an undefined mixture of proteins, lipids, and carbohydrates. These are the complex cyclic antimicrobial molecules, which needs to be further characterized for its classification as a class of antimicrobial peptides. The existence of this class of bacteriocin was supported mainly by the observation that bacteriocin activities in cell-free supernatant of *Lactobacillus*

plantarum LPCO10 were completely abolished not only by protease treatments, but also by the treatment of glycolytic and lipolytic enzymes (Jimenez-Diaz *et al.,* 1993). However, such bacteriocins have been rarely characterized.

Table 1.2

Classification of bacteriocin produced by LAB

Class	Description	Molecular weight	Bacteriocin
Class I	Post-translationally modified bacteriocin called lantibiotics	< 5 kDa	Nisin A, Mutacin 1140, Lacticin 481, Lactocin S, Salivaricin A, Cytolysin
Class II	Small, heat-stable non-lantibiotics	< 10 kDa	
IIa	Anti-listerial with conserved KYYGNGVXC aa sequence at N terminus		Pediocin PA-1, Enterocin A Sakacin A, Mesentericin Y105
IIb	Two-peptide bacteriocins		Acidocin J1132, Lactacin F, Lactacin 3147, Plantaricin S
IIc	Other bacteriocins		Divergicin A, Lactococcin A
Class III	Large heat-labile bacteriocins	> 30 kDa	Caseicin, Helveticin J, Lactacin A
Class IV	Cyclic and complex bacteriocins with lipid and / or carbohydrates	< 10 kDa	Gassericin A, Reutericin 6

In the present thesis the major emphasis has been on the isolation of Class IIa bacteriocin producing LAB and characterization of the bacteriocin molecule. The subsequent sections briefly describe the operon structure of ClassIIa bacteriocins, their antimicrobial spectrum and their mode of action.

1.10. Operon structure of a typical Class IIa bacteriocin produced by LAB

In the present thesis the major emphasis has been on the isolation of Class IIa bacteriocin producing LAB and characterization of the bacteriocin molecule. Literature reports suggest

that for typical ClassIIa bacteriocin, the genes are organized in an operon like fashion and in the synthesis and export of class IIa bacteriocin, at least four genes are required (Ennahar *et al.,* 2000; Nes *et al.,* 1996). The genes responsible for synthesis and export of class IIa bacteriocins involves:

(i) The structural bacteriocins gene, encoding a prebacteriocin.

(ii) The immunity gene, encoding an immunity protein that protects the bacteriocin producer from its own bacteriocins.

(iii) A gene encoding an ABC (ATP-binding cassette) transporter necessary for secretion, processing and maturation of prebacteriocin molecule.

(iv) A gene encoding an accessory protein for the processing of prebacteriocin along with the ABC transporters.

Apart from translocation with dedicated transport system, few members of class IIa bacteriocin (bacteriocin 31, enterocin P, and listeriocin 431) are also secreted by the general sec-dependent export system (Herranz *et al.,* 2005). In other cases, the regulation of class IIa group of bacteriocin gene is governed by quorum sensing systems, which consists of three gene products and thus termed three-component regulatory systems (Diep *et al.,* 2000). The three components are (i) the inducer peptide (a peptide pheromone), (ii) the transmembrane histidine protein kinase (pheromone receptor), and (iii) the cytosolic response regulator. Growing awareness of the potential practical applications of bacteriocin has motivated renewed attempts to characterize the complete repertoire of genetic elements required for effective bacteriocins production. In depth understanding of the mechanisms involved in bacteriocin regulation, processing, translocation, and immunity should facilitate

attempts to optimize bacteriocins production and to venture in the field of *in vitro* modification of their antibacterial spectra.

1.11. Structure-function relationship and antimicrobial spectrum of Class IIa bacteriocin

Antimicrobial activity of bacteriocins depends on amino acid sequence and charge on the antimicrobial peptides; and the cell membrane composition of the target cells. Class IIa bacteriocin-induced cell death, has been shown to occur in concentration- and time-dependent manner and it is further influenced by other factors related either to the target cell or to the medium. It has been shown that the lipid composition of the target membrane is a determining factor in modulating the bactericidal activity of the bacteriocin pediocin PA-1 (Chen *et al.,* 1998). The structure-function relationship of class IIa bacteriocin molecules has been extensively reviewed by Ennahar *et al.* (2000), where the authors have discussed the role of the YGNGV motif, N-terminal hydrophilic/amphiphilic β-sheet, central hydrophilic/slightly amphiphilic α-helix, C-terminal hydrophobic/amphiphilic α-helix, role of disulfide bond, positively-charged amino acids and other amino acids on the potency of antimicrobial effect of bacteriocin molecule.

1.12. Mode of action of Class IIa bacteriocin

Class IIa bacteriocins are small heat-stable, non-lanthionine-containing membrane-active peptides. The class IIa bacteriocins defined under this group are listeria-active peptides with consensus sequence at N-terminus of –Tyr-Gly-Asn-Gly-Val- (YGNGV). This group has gained major importance because of its wide spectrum antimicrobial activity especially

against foodborne pathogen *Listeria monocytogenes* and its activity at broad range of pH. A representative member of this group of bacteriocins is pediocin PA-1. Mature pediocin PA-l is a highly hydrophobic, positively charged peptide consisting of 44 amino acids. Pediocin PA-1 was the first member of class IIa bacteriocins studied in detail for its structure-function relationship and molecular mechanism of its antimicrobial activity (Ennahar *et al.,* 2000; Drider *et al.,* 2006). Pediocin PA-l acts on the cytoplasmic membrane, thereby dissipating ion gradients and inhibiting transport of amino acids in sensitive cells. The protein contains two disulfide bonds of which, cysteine residues at positions 24 and 44 are essentially required for its activity. It is likely that pediocin PA-l, and probably other bacteriocins belonging to the group, form hydrophilic pores in the cytoplasmic membrane of target cells in a protein receptor-mediated, voltage-independent manner (Abee, 1995). The presence of amphiphilic segments of putative transmembrane helices in class IIa bacteriocins, their water solubility and membrane-binding ability suggests that they may form poration complexes following a barrel-stave model (Ennahar *et al.,* 2000). Lantibiotic antimicrobial peptides were also known to act following the same model during their antimicrobial activity (Montville and Chen, 1998). The class IIa group of antimicrobial peptides (AMPs) consists of mainly pediocin-like AMPs, which have been primarily studied to understand the mode of action of Listeria-active peptides. The study reports the importance of the C-terminal hairpin domain that interacts with the hydrophobic part of the cell membrane and determines the target-cell specificity (Fimland *et al.,* 2005). The members of class IIa group of bacteriocin can exert antimicrobial effect by affecting the proton motive force with perturbation of either transmembrane potential or pH difference across the target cell or both (Hechard and Sahl, 2002).

1.13. Methods to detect bacteriocin activity

Isolation and screening of bacteriocins producing lactic acid bacteria essentially depends on the various culture-dependent methods. A variety of techniques have been used to determine the potency of bacteriocin preparations. The methods like pour plating assay, agar well diffusion assay, spot on lawn assay has been developed to determine the inhibitory effect of bacteriocin producing LAB or its culture filtrate (Tagg and McGiven, 1971; Kekessy and Piguet, 1970). These have been based either upon broth dilution methods or the more frequently used plate assays. In the latter, the bacteriocin diffuses radially through an agar layer from circular cups cut into the gel or from containers or filter-paper discs placed on the surface of the medium seeded with the sensitive indicator strain. To compare quantitative production of nisin by natural isolates of *Lactococcus lactis* subsp. *lactis* and thus compare the variability in the production of nisin agar plate diffusion assay was applied (De Vuyst, 1994). Comparative analysis of bacteriocins assays was performed to evaluate the applicability of particular assay method (Parente *et al.,* 1995). Further, such assays were adopted and applied on routine basis to quantitate and compare the amount of bacteriocin production by natural antagonistic isolates of LAB.

Traditionally used plating-based microbiological assays on selective medium require long incubation time and provide limited information (Pucci *et al.,* 1988). With the increasing knowledge of chemistry of fluorophore, various fluorescence probe has been developed, which can rapidly assess the cell physiology and could easily differentiate between live and dead bacterial cells (Berney *et al.,* 2007; Bunthof *et al.,* 2001). Antimicrobial effect of pediocin on carboxyfluorescein diacetate (cFDA) labelled *Lactobacillus casei* NCFB 2714 has been studied by spectrofluorometer measurement of

effluxed carboxyfluorescein dye (Budde *et al.,* 2001). The method was found to be rapid and sensitive to quantitate the microbicidal activity of bacteriocin.

1.14. Food applications of bacteriocin producing LAB/bacteriocin molecule

Preservation of foods by fermentation is a well practiced technology since ancient times. Fermentation ensures not only increased shelf life and microbiological safety of a food but may also make some foods more digestible and reduces their toxicity to the consumer (Caplice *et al.,* 1999; Fleet *et al.,* 1999; Holzapfel *et al.,* 1995; Ross *et al.,* 2002). The antimicrobial activity of bacteriocinogenic LAB is essential in their applications in the food fermentation industry. Bacteriocin producing LAB strains have been widely reported as an inherent LAB population in milk, vegetable and meat products (Castellano *et al.,* 2008; Galvez *et al.,* 2007; Jones *et al.,* 2008).

In the past 20 years, numerous studies have focused on bacteriocins produced by lactic acid bacteria. This interest was inspired by the possibility of using bacteriocins as food preservatives. The review of Galvez *et al.* (2007) has discussed in detail the application of bacteriocins in food preservation. Bacteriocin preparation can be added to food as a concentrate additive or ingredient to extend the shelf-life of fermented food products or they can be produced *in situ* by bacteriocinogenic starters, adjunct or protective LAB cultures. The autochthonous or allochthonous bacteriocinogenic LAB or their secreted bacteriocins in fermented food samples are primarily involved as biopreservative to increase the shelf-life of cereal, vegetable, dairy and meat fermented or non-fermented products. The application of bacteriocins provides an alternative to satisfy the increasing consumer's demands for safe, fresh-tasting, ready-to-eat and minimally processed food.

Cereal grains constitute a major source of dietary nutrition with increased nutritional, sensorial and functional qualities after fermentation. In cereal based fermentations, like formation of sourdough bread, the autochthonous bacteriocinogenic LAB population plays crucial role in the increased shelf-life (Caplice *et al.,* 1999; Holzapfel *et al.,* 1995). The most important group of bacterial strains involved in sourdough fermentation includes strains from *Lactobacillus* genus. Bacteriocin produced by the starter culture as well was the adjuvant cultures have also played substantial role as a bio-preservative in vegetable fermentation (Settani and Corsetti, 2008). Bacteriocin-producing LAB have been also applied as starter culture or additives for the improvement and safety of dairy food products (O'Sullivan *et al.,* 2002; Jagannath *et al.,* 2001). Bacteriocins have been also applied as bio-protective agents in combination with hurdle technology to increase the shelf-life and quality of fermented sausages (Jones *et al.,* 2008; Tyopponen *et al.,* 2003). In addition to bacteriostatic or bactericidal effect conferred by bacteriocins produced by LAB, other metabolic products of LAB have been also studied for their antagonistic activity against various foodborne pathogens.

Although many fermentations are traditionally dependent on inoculation from a previous batch starter cultures known as backslopping. But, for large scale commercial application of the starter culture, strain identification and other technological and functional parameters of the starter strains need to be defined to ensure the increased shelf-life and organoleptic property of the fermented product (Coolbear *et al.,* 2008). Further, application of antagonistic LAB strains as a starter culture demands critical strain identification with molecular tools. The next section of this literature review highlights the approach and discusses the use of molecular tools for the identification of LAB strains.

1.15. Molecular detection of LAB

LAB drives food fermentation process and thus necessitates a routine basis of monitoring of fate of LAB strain during the course of fermentation along with other technological parameter of safety and quality control of fermented food products (Saarela *et al.,* 2000). In this context, reliable monitoring and identification of LAB remains a point of crucial concern. The 16S rRNA and 23S rRNA gene as well as an intergenic spacer (IGS) region gene have been the target of choice for molecular identification and genetic characterization of LAB strains (Acquilanti *et al.,* 2007; Chen *et al.,* 2008; Dolci *et al.,* 2008). The reasons justified for the selection of these targets in the molecular identification and taxonomic studies include universal abundance, evolutionary and phylogenetic properties, high discriminatory potential, multiple-copy nature and extensive availability of sequences in public databases (Juste *et al.,* 2008).

Over the past decade, the scientific community has paid special attention in the molecular identification of bacteria applied in health benefit and food industry. Molecular studies on LAB diversity and community analysis in various fermented product with culture-dependent and culture-independent molecular tools has been thoroughly studied (Delbes *et al.,* 2007; Miambi *et al.,* 2003; Temmerman *et al.,* 2004).

The demand of rapid identification technique has been contented with rapid, robust and culture-independent method without compromising their detection limits. Some of the molecular techniques used for LAB detection are discussed under the following sections:

1.16. Culture-dependent approach

Molecular identification of LAB strains carried out after enrichment in the culture medium is primarily defined as culture-dependent method. Culture-dependent based identification of LAB in various fermented food product and defined mixed culture have been carried out to study the applicability of various molecular identification methods (Temmerman *et al.,* 2004). Many genotypic methods are based on the principle of Polymerase Chain Reaction (PCR), which enables the selective amplification of specifically targeted DNA fragments through the use of oligonucleotide primers under controlled reaction conditions. On the basis of similar technique Pintado *et al.* (2003) quantified and profiled the diversity of a mixed culture of LAB strain by PCR-denaturing gradient gel electrophoresis (PCR-DGGE). Traditionally, the occurrence of microorganisms in a given environment or in an industrial process has been studied by culture-based methods. Nevertheless, it is well recognized that these methods often fail to select all type of LAB present in the food sample, due to their varying need of micronutrients and does not reflect the exact microbial diversity in food sample. Such limitations prompted the need to develop a culture-independent techniques based on polymerase chain reaction (PCR) amplification and detection of nucleic acids (Juste *et al.,* 2008).

1.17. Culture-independent approach

Molecular identification of LAB strains can be carried even without the enrichment process and this process encompasses the culture-independent method or direct-detection method. DNA-based culture-independent strategies have been applied for evaluating microbial communities in food-associated ecosystems (Giraffa and Neviani, 2001; Temmerman *et al.,*

2004). Real-time PCR based culture-independent method has been used to monitor and estimate the development of *Lactobacillus plantarum* in a complex microbial consortium of grass silages (Klocke *et al.,* 2006). LAB population of fermented milk samples were quantified by culture-independent method based on real-time PCR and the presence of LAB species, *S. thermophilus*, *L. delbrueckii*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. acidophilus* and *L. johnsonii* were detected in the fermented milk samples (Furet *et al.,* 2004). Recent advances in molecular techniques to study the microbial communities associated with food matrices and processes; and analysis of species level diversity of *Lactobacillus* have been extensively reviewed by (Juste *et al.,* 2008).

1.18. Probiotic attributes of LAB and their adhesion potential

LAB strains, apart from their antimicrobial attributes have been also reported as a major resident microflora of intestinal microbiota, with profound adhesion potential to epithelial cells (Ouwehand *et al.,* 2002; Saito, 2004; Vasiljevic and Shah, 2008). LAB are also in demand for their probiotic virtues as they can influence the activity and composition of the intestinal microbiota (Ouwehand *et al.,* 2002). A probiotic has been defined as "live microorganism which when administered in adequate amounts confers a health benefit on the host" (Salminen *et. al.,* 1999). Probiotic LAB are selected on the basis of their ability to adhere to the intestinal mucosa, acid and bile resistance, and antagonism against pathogenic bacteria. The ability to adhere to intestine is a key selection criterion, because it is considered to be paramount in persistence, enhanced healing of the damaged mucosa (Elliott *et al.,* 1998), antagonism against pathogens (Reid and Burton, 2002) and immunomodulation (Zhou and Gill, 2005).

One of the prerequisites of a good probiotic LAB strain is adhesion to mucus and epithelial cells. Adhesion to epithelial cells by LAB has been shown to be promoted by cell surface bound proteins. It is vital to establish *in vitro* adhesion potential of LAB strains before accrediting them as a probiotic strains. A number of *in vitro* models have been used to ascertain the adhesion of probiotic LAB to epithelial cells. Of these, the colon adenocarcinoma cells Caco-2 and HT-29 are the most ideal choice (Gopal *et al.,* 2001; Sambuy *et al.* 2005; Vesterlund *et al.,* 2005). Adhesion is strain-specific and *Lactobacillus rhamnosus* GG, one of the best studied probiotics, adheres at a relatively high level (9.7%) to Caco-2 cells (Tuomola and Salminen, 1998). The protective role of probiotic bacteria against gastrointestinal pathogens and the underlying mechanisms have received special attention. Consequently, a large number of research groups have demonstrated the potential of probiotic LAB in competitive exclusion and prevention of pathogen adhesion to model cell lines and mucus (Collado *et al.,* 2007; Gueimonde *et al.,* 2006; Sherman *et al.,* 2005). *In vitro* adhesion attributes of LAB have been widely elucidated by using radio-labelled probe, enzyme-linked immunosorbent assay, culture dependent plating techniques and fluorescence based techniques (Binachi *et al.,* 2004; Blay *et al.,* 2004; Gopal *et al.,* 2001; Guglielmotti *et al.,* 2007; Lee *et al.,* 2004; Matijašić *et al.,* 2003; Servin, 2004).

SCOPE AND SIGNIFICANCE OF THE PRESENT RESEARCH WORK

SCOPE AND SIGNIFICANCE OF THE PRESENT RESEARCH WORK

Based on the current knowledge on bacteriocin producing LAB and probiotic LAB research and the lacunae in the research area, the motivating factors for undertaking the research project outlined in this thesis were identified. The rationale of the present research work is explained in the following sections:

In recent years there has been an increasing consumer demand for minimally processed food devoid of chemical preservatives and enhanced shelf-life. Fermented food products constitute a considerable part of the diet and many of them are prepared using lactic acid bacteria (LAB). The primary role of LAB is to initiate fermentation, but it is desirable that the bacteria exhibits antimicrobial attributes. The antagonistic virtues of LAB are primarily due to high acid production and the ability to produce bacteriocin. Literature reports indicate that majority of the bacteriocins have a narrow spectrum of antimicrobial activity and there is need to screen for natural isolates of LAB that produce bacteriocins which act against a large number of pathogenic bacteria. This formed a strong basis of the present research work.

Literature reports suggest that in natural fermentation processes, LAB succession is a universal phenomenon and is a manifestation of microbial interaction, competition for nutrients and resistance to growth inhibitory environmental perturbations like high acidity and production of antimicrobials. Most of the studies on LAB succession reported the dominant population and do not reveal the antagonistic attributes, which contribute to their persistence. However, a realistic view of LAB succession in a natural fermentation process can be obtained from an experimental design, which is able to detect all the major groups of

interacting microorganisms, and relate the observed succession to their antagonistic properties such as bacteriocin production.

As far as the use of bacteriocin in food product is concerned, nisin has been in use for many years and has been approved by FDA. However, nisin is known to work only at acidic pH and is not readily soluble. Hence there is a need to probe for alternate bacteriocins from LAB that can act at neutral pH and are readily soluble. Thus the second endeavor of the research project was to isolate LAB that produce bacteriocins acting even at neutral pH.

A fundamental issue regarding the application of LAB strains in food fermentation process is to develop a robust method of strain detection, characterization and estimation of genetic diversity. A crucial factor in this regard is to extract good quality DNA from LAB strains prior to use of any molecular tool. Thus there is a need to develop a convenient nucleic acid extraction method from LAB strains and newer methods of strain typing that facilitate reliable identification of LAB strains as well as estimation of their genetic diversity. This formed one of the objectives of the present research work.

As far as bacteriocin from LAB is concerned, an important issue to be addressed is the mode of action of bacteriocin on target cells. This is crucial to get an insight of the probable mode of action which would eventually lead to a rational selection of the most efficient LAB or its bacteriocin extract to combat the challenge posed by foodborne pathogenic bacteria.

Literature reports indicate that LAB are known to influence the intestinal microbiota and exhibit significant probiotic attributes. Adhesion property of LAB to intestinal cells is one of the key selection criteria for probiotic use. It is thus imperative to establish *in vitro* cell culture models and assess adhesion potential of bacteriocin producing LAB. Quantitative

comparison of adhesion potential of bacteriocin producing LAB strains would assist rapid and rational selection of probiotic strains for commercial health applications.

Essentially it is envisaged that the present investigation would enable the development of a comprehensive protocol to rapidly screen potent bacteriocin producing LAB strains, estimate their genetic diversity and ascertain their *in vitro* adhesion potential to intestinal cells. This would in turn augment selection of appropriate LAB strains for future development of probiotic food formulations as well as identify potential vehicles for the delivery of mucosal vaccines.

SPECIFIC OBJECTIVES OF THE RESEARCH WORK

SPECIFIC OBJECTIVES OF THE RESEARCH WORK

In the background of the literature reviewed in Chapter 1 and by identifying the scope and significance of the present research work, the following specific objectives were defined:

1. Molecular detection of bacteriocin producing LAB during succession in salt- fermented cucumber.
2. Molecular fingerprinting of bacteriocin producing LAB using a robust PCR-compatible template DNA extraction method.
3. Isolation, molecular detection, bacteriocin potency and food application studies of anti-listerial bacteriocin producing LAB.
4. Estimation of the genetic diversity of anti-listerial bacteriocin producing LAB strains by a novel AP-PCR based fingerprinting method.
5. Strain identification of potent anti-listerial bacteriocin producer and studies on the functional characterization of anti-listerial bacteriocin produced by LAB strains.
6. Quantitative assessment of *in vitro* adhesion potential of potent anti-listerial bacteriocin producing LAB strains on cultured HT-29 cells.

CHAPTER 2

Molecular detection of bacteriocin producing lactic acid bacteria during succession in salt-fermented cucumber

ABSTRACT

The investigation outlined in this chapter describes the application of a PCR-based molecular method to detect the succession of major groups of lactic acid bacteria (LAB) and the simultaneous emergence of bacteriocin producing (Bac^{+}) LAB strains in salt-fermented cucumber. In a direct detection method (culture-independent) as well as a short enrichment process (culture-dependent), PCR enabled detection of *Leuconostoc* and *Lactobacillus* during early hours of fermentation. Subsequently, *Lactobacillus* and *Pediococcus* emerged as the dominant genera. Nucleic acid sequence of culture-independent clones confirmed the detection of *Pediococcus* as a dominant genera emerging during late stages of fermentation. PCR also revealed time-dependent emergence of mesentericin, pediocin and plantaricin A producers and accounted for the LAB succession in the fermenting samples. A total of 328 LAB isolates were obtained collectively from 30 cucumber samples, of which PCR could identify an overwhelming 186 *Lactobacillus* isolates followed by 113 *Pediococcus* and 29 *Leuconostoc* isolates respectively. Based on antimicrobial assay against target strain *Leuconostoc mesenteroides* NRRL B640, 28% of the LAB were bacteriocin producers, of which pediocin producers were substantial, followed by plantaricin A and mesentericin producers. The bacteriocins elaborated by the isolates were active against a large number of Gram-positive target LAB strains and pathogenic bacteria including *Bacillus cereus*, *Enterobacter aerogenes, Enterococcus faecalis, Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*.

2.1. Introduction

Lactic acid bacteria (LAB) belong to a versatile group of microorganisms, which enjoy a generally regarded as safe (GRAS) status and have profound applications in food fermentation processes. Apart from their beneficial attributes such as acid production, proteolytic activity, and aroma formation, which have direct relevance in food fermentation processes, they have an enormous potential to inhibit microorganisms owing to their capacity of producing bacteriocins (Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004; Drider *et al.,* 2006). Bacteriocins are essentially proteinaceous in nature and majority of the bacteriocins produced by LAB belong to the Class IIa category which are small heat-stable peptides with considerable sequence homology and strong antimicrobial activity against closely related Gram-positive bacteria (Ennahar *et al.,* 2000; Drider *et al.,* 2006). Class IIa bacteriocins like pediocin are particularly known for their strong anti-listerial activity, making them attractive candidates for application in food systems (Jagannath *et al.,* 2001; Rodriguez *et al.,* 2002). Owing to an impending threat from foodborne pathogens, the discovery of new food processes and the consumer demand for minimally processed food products, LAB have come to the forefront as biopreservative agents and substantial research has been focused in recent times to isolate bacteriocin producing LAB from natural sources and exploit them for production and preservation of fermented product.

LAB are generally associated with nutrient rich habitats such as milk, meat, vegetables, but some are also known to exist as members of the normal flora of oral and intestinal tract. These environments are essentially conducive to the growth of other microorganisms and thus LAB have developed strategies to efficiently compete and survive in such habitats. Further different members of LAB genera (*Lactobacillus*, *Lactococcus*, *Leuconostoc* and

Pediococcus) are known to coexist in such nutrient rich habitat. Conceivably, the ability to produce acid and antagonistic attributes such as bacteriocin production has evolved in LAB as a means to outcompete closely related members. In natural fermentation processes, LAB succession is a universal phenomenon and is often a reflection of ensuing microbial interaction, competition for intrinsic growth factors such as nutrients and resistance to growth inhibitory environmental perturbations like high acidity and production of antimicrobials. Consequently, robust LAB populations that enjoy a selective advantage owing to either acid resistance or antagonistic traits such as the ability to produce bacteriocins emerge as the dominant population at the end of fermentation. Knowledge of such interactions amongst LAB in a natural fermentation process not only provides a fundamental understanding of the process parameters, but can also form the basis of designing a strategy of isolating antagonistic cultures from a natural source.

Investigations concerning microbial dynamics in food system have adopted a PCR-based approach to study development of community structure and the fate of interacting microorganisms in defined fermented products (Ercolini, 2004; Randazzo *et al.,* 2006; Rantsiou and Cocolin, 2006; Delbe's *et al.,* 2007). However, these studies largely reported the succession of a dominant population and do not reveal the antagonistic attributes, which contribute to their persistence. A holistic view of the ensuing LAB succession in a natural fermentation process can be rationally obtained from an experimental design, which is able to detect all the major groups of interacting microorganisms, and relate the observed succession to their antagonistic properties. The present investigation is primarily based on the aforesaid concept and is aimed to analyze the succession of LAB in salt-fermented cucumber and concomitantly detect the emergence of bacteriocin producers as fermentation proceeds.

Cucumber was selected as a model system based on a previous investigation, which demonstrated its promise as a source for isolating pediocin-like bacteriocin producers (Halami *et al.,* 2005). In this chapter a molecular approach based on PCR was employed to ascertain the succession of LAB existing as inherent microflora in fermented cucumber samples and correlate the succession with the emergence of bacteriocin producing strains.

2.2. Materials and Methods

2.2.1. Reference bacterial strains and culture conditions

The reference LAB and pathogenic bacterial strains used in the investigation outlined in this chapter are shown in Table 2.1. All LAB strains were maintained as frozen stocks in skim milk and glycerol (10% each) at –20°C. The frozen stock cultures of LAB strains were prepared as follows: Reference LAB strains were grown in de Man, Rogosa and Sharpe (MRS) broth at 37°C for 16 h in a temperature controlled static incubator (LabTech, LMI, South Korea). LAB cells were harvested from a 1.0 ml aliquot of the culture broth by centrifugation at 5687 x g for 5 min at 4°C in a table top centrifuge (Sigma, 3K-30, USA). The cell pellet was washed twice with 1.0 ml phosphate buffered saline (PBS, pH 7.2) and finally resuspended in a mixture of 50 µl of 10% sterile skim milk and 50 µl of 10% sterile glycerol and the resuspended cells were stored at -20°C. LAB strains were subcultured every fortnight by inoculation of 0.1 ml stock culture to 5.0 ml of MRS broth and the seeded culture was grown in static incubator at 37°C for 12-16 h.

The preparation and propagation of stock cultures of non-LAB reference strains were accomplished as follows: All stock cultures of non-LAB reference strains were maintained in 15% sterile glycerol solution. Strains of *Escherichia coli*, *Listeria monocytogenes*,

Staphylococcus aureus and *Enterococcus faecalis* were propagated in brain-heart infusion (BHI) broth whereas *Bacillus cereus* and *Enterobacter aerogenes* were grown in nutrient broth (NB). All non-LAB reference strains were grown at 180 rpm in a shaker incubator at 37°C for 12-16 h in respective medium. Stocks were subcultured once in a month on a routine basis by inoculating 0.1 ml of stock culture in fresh broth medium and growing the cultures at 180 rpm in a shaker incubator at 37°C for 12-16 h. All dehydrated NB, BHI and MRS medium were purchased from HiMedia, Mumbai, India.

Table 2.1

Reference strains of lactic acid bacteria (LAB) and pathogenic bacteria used in the present investigation

Organism	Strain
Lactobacillus acidophilus	MTCC 447
Lactobacillus casei subsp. *casei*	NRRL B1922
Lactobacillus delbrueckii subsp. *bulgaricus*	NRRL B548
Lactobacillus delbrueckii subsp. *lactis*	MTCC 911
Lactobacillus fermentum	MTCC 903
Lactobacillus johnsonii	NRRL B2178
Lactobacillus plantarum	MTCC 1325
Lactobacillus rhamnosus	MTCC 1408
Lactobacillus sakei	NRRL B1917
Lactococcus lactis subsp. *cremoris*	MTCC 1484
Lactococcus lactis subsp. *lactis*	MTCC 3041
Leuconostoc mesenteroides	NRRL B640
Leuconostoc mesenteroides subsp. *mesenteroides*	MTCC 107
Pediococcus acidilactici	NRRL B14009, CFR K7[a]
Escherichia coli	MTCC 443
Bacillus cereus	MTCC 1305
Listeria monocytogenes	Scott A[a]
Staphylococcus aureus	MTCC 96
Enterobacter aerogenes	MTCC 2822
Enterococcus faecalis	MTCC 439

NRRL: Northern Regional Research Laboratory, Peoria, IL, USA; MTCC: Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India.
[a] Culture provided by Dr. Prakash Halami, Central Food Technological Research Institute (CFTRI), Mysore, India

2.2.2. Molecular detection of LAB succession in fermented cucumber

A total of 30 cucumber samples randomly collected from various vegetable markets of Guwahati city were chosen for this study. Cucumber samples were gently washed with sterile distilled water to remove soil particles, sliced and incubated at 35°C in autoclaved 4% NaCl solution taken in sterile screw capped glass bottles. Samples were periodically withdrawn aseptically at intervals of 6, 12, 18, 24, 36, 48 and 72 h after the onset of fermentation. In the culture-independent method, 1.0 ml aliquot of the samples were centrifuged briefly at 5687 x g for 5 min at 4°C to pellet cells and washed thrice with PBS followed by single wash with autoclaved ultrafiltered water. Washed cells were resuspended in 100 µl of sterile water and 400 µl of 1% Triton X-100. For DNA extraction, resuspended cell were incubated in boiling water for 5 min and snap cooled on ice. In the culture-dependent method, 1.0 ml aliquots of the sample were subjected to short enrichment in MRS broth for 6 h at 37°C. Cells were separated from a 1.0 ml aliquot of enriched culture broth by centrifugation, washed thrice with PBS and DNA was extracted using 1% Triton X-100 as mentioned earlier. A 2.0 µl aliquot of extracted DNA from both the methods was used in PCR in conjunction with genus-specific 16S rRNA gene and bacteriocin gene specific primers. A schematic depiction of the steps followed for molecular detection of LAB succession in fermented cucumber samples is indicated in Figure 2.1.

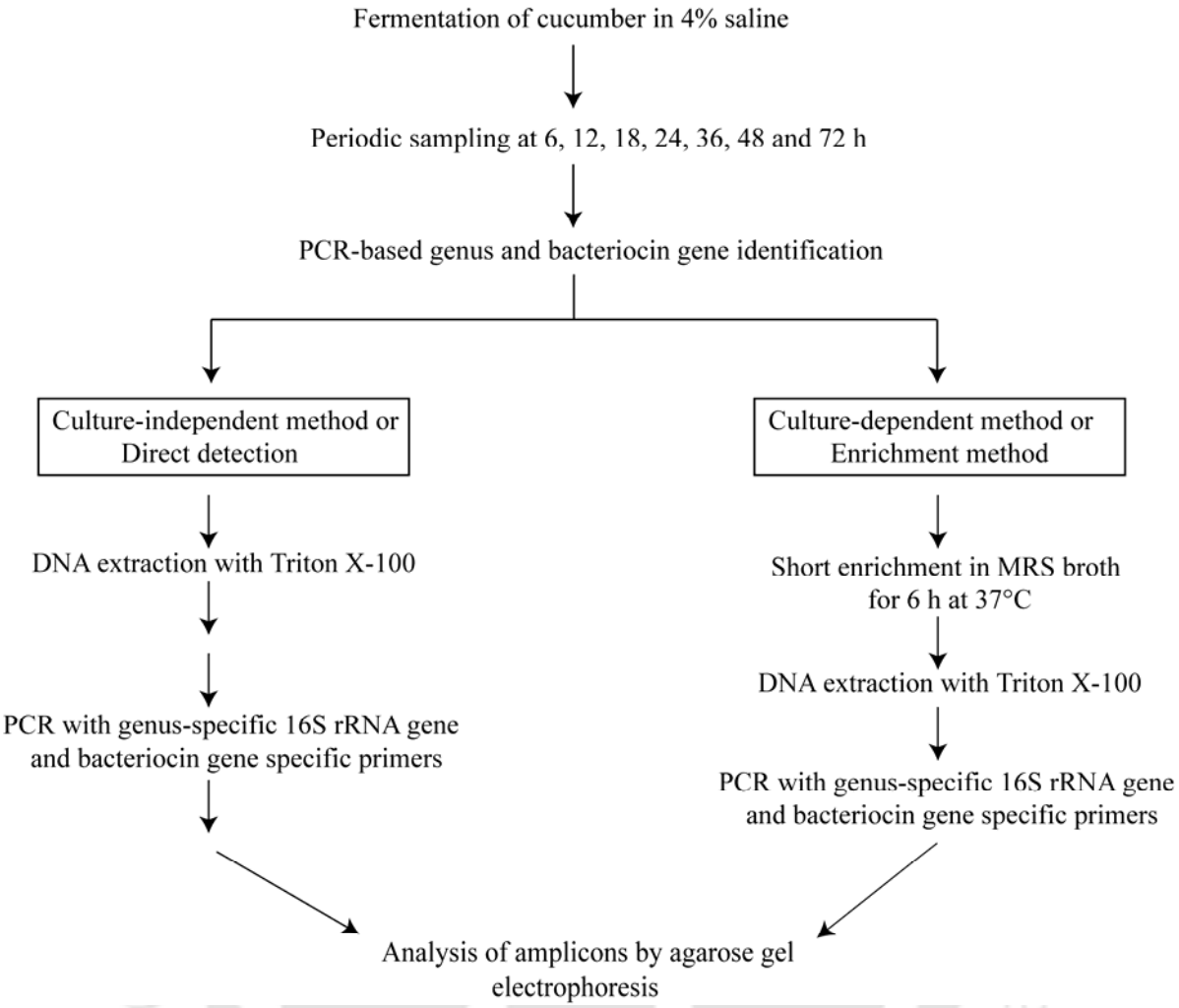

Figure 2.1 Schematic representation of the molecular method used for the identification of antagonistic LAB from salt-fermented cucumber.

2.2.3. Nucleic acid sequence

The 16S rRNA gene sequence of three uncultured clones obtained by culture-independent method from the fermented cucumber samples was determined. These culture-independent clones responded positively in PCR with genus-specific primers for *Pediococcus*. The amplicons obtained from these clones were directly sequenced in the National Facility for Automated DNA Sequencing, Department of Biochemistry, University of Delhi, South Campus (UDSC). Homology search for the sequences was performed by BLAST analysis

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the sequences were deposited in GenBank database with accession numbers EF636660, EF636661 and EF636662. The size of the sequenced amplicons was 656, 689 and 696 bp. The 16S rRNA gene sequences of the three uncultured clones of *Pediococcus* were also subjected to sequence-based phylogenetic analysis by using a web-based program (http://www.phylogeny.fr).

2.2.4. Genus-specific and bacteriocin gene specific primers

Nucleotide sequence of the 16S rRNA gene of lactic acid bacteria comprising of strains belonging to genera *Lactobacilli*, *Pediococci*, *Lactococci* and *Leuconostoc* (28 sequences) and select non-lactic acid bacteria were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov). The sequences were aligned using the web-based multiple alignment program DIALIGN (http://www.genomatix.de/cgi-bin/dialign/dialign.pl). Based on the alignment, 16S rRNA gene sequences unique to the selected LAB genera were identified and primers specific to the genera *Lactobacilli*, *Pediococci*, *Lactococci* and *Leuconostoc* were designed as shown in Table 2.2. The sequences of the primers were subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine their specificity. The specificity of the genus-specific primers was also validated by PCR using reference strains of LAB. Primers were also designed for the following bacteriocins- pediocin, mesentericin, enterocin A, nisin and plantaricin A using the program Primer 3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of bacteriocin gene specific primers are indicated in Table 2.2.

Table 2.2

16S rRNA gene and bacteriocin gene specific primers used in the present investigation.

Primer Name	Nucleotide sequence (5'-3')	Primer Annealing Position (5'- 3')	Reference Gene Accession Number	PCR Target
1F	AGAAGAGGACAGTGGAAC	677-694	AB104855	*Lactobacillus*
1R	TTACAAACTCTCATGGTGTG	1424-1443		
2F	TAAAGCGAGCGCAGGTGG	572-589	AF515226	*Lactococcus*
2R	GGTTACCTTGTTACGACTT	1492-1510		
3F	CTGAATGAGATTTTAACACG	95-114	AF515229	*Pediococcus*
3R	GGTTTTAAGAGATTAGCT	1307-1324		
4F	AGAGATGGATCCGCGGTGCA	232-251	M23035	*Leuconostoc*
4R	TTACAAACTCCCATGGTGTG	1416-1435		
IF	TGGCCAATATCATTGGTGGT	37-56	AY083244	Pediocin
IR	GCATTTATGATTACCTTGATGTCC	165 -188		
IIF	ACCAAAATCCATTTCCACCA	383-402	AY286003	Mesentericin
IIR	TCTGTGGAAGCATATCAGCAA	531-551		
IIIF	CAGACACAACTTATCTATGGGGGTA	40-64	AF240561	Enterocin A
IIIR	CCTGGAATTGCTCCACCTAA	178-197		
IVF	CTTGGATTTGGTATCTGTTTCG	21-42	AY526091	Nisin
IVR	TTGCTTACGTGAATACTACAATGACA	145-170		
VF	GAAAATTCAAATTAAAGGTATGAAGCA	553-579	X75323	Plantaricin A
VR	ATTGCAGTTGCCCCCATCT	639-657		

2.2.5. PCR conditions

Amplification of bacterial DNA was performed in a total reaction volume of 25 µl, which contained 2 µl of DNA samples comprising either genomic DNA from reference strains and natural isolates or DNA extracted from fermented cucumber samples in case of culture-independent method of detection. Separate PCR reactions were performed to detect a particular genus of LAB or a bacteriocin gene. The reaction mixture consisted of 10 times diluted PCR buffer, 200 µM of each deoxynucleoside triphosphate (dNTP), 1U of *Taq* DNA polymerase (Sibenzyme, Novosibirsk, Russia) and 50 pmol each of forward and reverse primer. Template DNA was initially denatured at 94°C for 2 min. Subsequently, a total of 35 amplification cycles were carried out in a programmable thermal cycler (Gene Amp Gold PCR System, Applied Biosystems, USA). Each cycle consisted of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C and extension for 1 min at 72°C. The last cycle was followed by a final extension at 72°C for 10 min. Amplification of bacteriocin genes was carried out under similar conditions except for an annealing temperature of 58°C. PCR products were analyzed by agarose (1%) gel electrophoresis (Sambrook and Russell, 2001).

2.2.6. Enumeration and detection of antagonistic LAB in salt-fermented cucumber

The total LAB count in the fermented cucumber samples was determined by conventional microbiological plating technique. As mentioned previously in section 2.2.2, samples were collected periodically and the total LAB count for every time period was determined by pour plating in MRS-agar media (HiMedia, India). Estimation of acid production resulting from the growth of LAB was performed by centrifuging aliquots of fermented cucumber samples briefly and measuring the pH of the supernatant.

Antagonistic or bacteriocin producing (Bac^+) LAB isolates obtained at each time period from the fermented cucumber sample were identified by a colony overlay assay as described by Halami *et al.* (2005). Essentially the LAB colonies obtained on MRS agar plates at different time periods from the cucumber samples were overlaid with 10^6 cells of freshly grown target strain of *Leuconostoc mesenteroides* NRRL B640 seeded in MRS soft agar (0.8% agar). Following 24 h incubation of the overlaid samples at 37°C, bacteriocin producing (Bac^+) LAB strains were scored by observing zone of growth inhibition of the target strain around the colonies (Halami *et al.*, 2005). Select colonies of LAB consisting of bacteriocin producers (Bac^+) and non-producers (Bac^-) obtained collectively from 30 fermented cucumber samples were maintained as frozen stocks in milk and glycerol (10% each) at –20°C as described in section 2.2.1. The isolates were propagated overnight in MRS medium at 37°C under static condition, prior to subsequent experiments. The genomic DNA from the isolates was extracted using the Triton-X 100 method as mentioned before in section 2.2.2 and the genera of the LAB isolates and the class of bacteriocin produced were identified by PCR using genus-specific 16S rRNA gene and bacteriocin gene specific primers (Table 2.2).

2.2.7. Characterization of bacteriocin-like inhibitory substance (BLIS) produced by antagonistic LAB isolates

LAB isolates exhibiting a zone of inhibition against *Leuconostoc mesenteroides* NRRL B640 in the colony overlay assay were selected to characterize the antimicrobial activity of the culture supernatant. The isolates were grown in 10 ml MRS broth for 36 h and the culture filtrate was recovered by centrifugation at 8832 x g for 10 min at 4°C. The pH of the culture

filtrate was adjusted to 7.0 with 2N NaOH followed by filter sterilization using a 0.2 µm membrane filter (Millipore, India). The resulting solution, referred as bacteriocin-like inhibitory substance (BLIS) was dialyzed extensively against de-ionized water using a 1.0 kDa molecular weight cut-off (MWCO) dialysis bag (Sigma, USA), freeze dried in a lyophilizer (ALPHA 1-4 LD, CHRIST, Germany) and reconstituted in 1.0 ml of 10mM phosphate buffer (pH 7.0). A 100 µl aliquot of the BLIS sample was treated with 1.0 mg/ml of trypsin and catalase (Sigma-Aldrich, USA) separately in 1.0 ml reaction volume for 1 h at 37^{o}C in a circulating water bath incubator (Julabo F12-MP, Germany). Heat inactivated enzyme solution alone was used as control to detect any inhibition caused by the enzyme. In case of treated BLIS samples, aliquots were heat-inactivated prior to testing bacteriocin activity. For testing the activity at different pH, aliquots of the dialyzed and lyophilized sample were adjusted to pH 3.5, 5.5 and 7.0. Buffer adjusted to the same pH served as control. The thermal stability of BLIS was tested by subjecting aliquots of the sample to 121°C for 20 min (autoclave) and 100 °C for 30 min. Culture filtrate from antagonistic LAB isolates was also subjected to extensive dialysis against de-ionized water using 1.0 kDa and 12.0 kDa dialysis bags (Sigma Aldrich, USA) and bacteriocin activity was measured in the dialysate. Bacteriocin activity in all the aforesaid treated samples was determined by an agar well diffusion assay using *Leuconostoc mesenteroides* NRRL B640 as an indicator strain. The agar well diffusion assay is described in the subsequent section 2.2.8.

2.2.8. Antimicrobial spectrum of bacteriocin producing (Bac^{+}) LAB isolates

The bacteriocin producing (Bac^{+}) LAB isolates obtained from fermented cucumber samples were grown in MRS broth at 37°C for 18 h. Cells were separated by centrifugation at 8832 x g

for 10 min at 4°C and the pH of the culture filtrate (CF) was adjusted to 7.0 with 2 M NaOH, filtered through a 0.2 μm membrane filter (Millipore, India) and stored at -20°C till further use. Prior to assay, the samples were incubated in boiling water for 20 min, cooled to room temperature and then used against indicator strains consisting of LAB strains or pathogenic bacteria (Table 2.1). The antimicrobial activity of the samples was ascertained by an agar well diffusion assay.

2.2.9. Agar well diffusion assay

Agar well diffusion assay was conducted to ascertain the bacteriocin activity and antimicrobial spectrum of the antagonistic LAB isolates. In case of assay against LAB indicator strains, the assay plates had a bottom layer of MRS agar (1.5% agar). It was overlaid with MRS soft agar (0.8% agar) seeded with 10^6 cells of freshly grown target strain of LAB. In case of non-LAB pathogenic strains, the assay plates had a bottom layer of NB or BHI agar (1.5% agar). Subsequently the target strains of pathogenic bacteria seeded in NB or BHI-soft agar (0.8% agar) were overlaid on the bottom layer. Requisite number of holes of 6 mm diameter was punched in all the assay plates. To each well, 50 μl of test sample was added. The plates were preincubated at 4°C for 3 h to facilitate diffusion of the sample followed by incubation at 37°C for 24 h. The antimicrobial activity of the samples was determined by observing the zone of inhibition produced around the wells.

2.3. Results and Discussion

2.3.1. Genus-specific primers

Multiple alignment of the 16S rRNA gene sequence of LAB enabled the selection of candidate regions for designing genus-specific primers. The expected size of amplicons for *Lactobacilli, Lactococci, Pediococci* and *Leuconostoc* were approximately 800, 960, 1200, and 1100 bp, respectively. The primers designed for specific detection of LAB genera were validated by performing PCR with DNA isolated from standard LAB strains indicated in Table 2.1. Amplicons obtained from two strains each belonging to the four major genera of LAB is indicated in Figure 2.2.

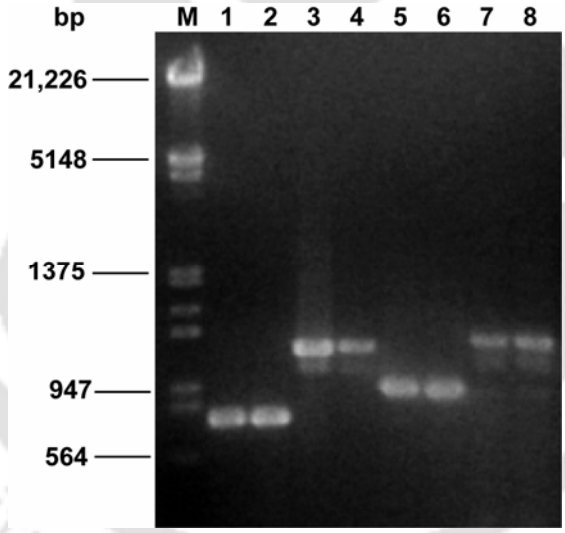

Figure 2.2 Amplicons obtained from reference strains of lactic acid bacteria using genus-specific primers designed for 16S rRNA gene. Lanes: M: λ DNA *Eco*RI/*Hin*dIII double digest size marker; 1: *Lactobacillus casei* subsp. *casei* NRRL B1922; 2: *Lactobacillus plantarum* MTCC 1325; 3 & 4: *Pediococcus acidilactici* CFR K7 and NRRL B14009; 5: *Lactococcus lactis* subsp. *cremoris* MTCC 1484; 6: *Lactococcus lactis* subsp. *lactis* MTCC 3041; 7: *Leuconostoc mesenteroides* subsp. *mesenteroides* MTCC 107; 8: *Leuconostoc mesenteroides* NRRL B640.

It is evident from the figure that the size of the amplicons coincided with the expected size. The specificity of the designed primers was further confirmed by the lack of cross-reactivity of the primers amongst the LAB genera and the non-LAB pathogens listed in Table 2.1. This was critical in the context of the present study, which involved simultaneous detection of mixed LAB population likely to be present as a natural microflora in cucumber. Previous studies have focused on the application of 16S rRNA primers to detect a specific LAB genus or species using conventional and multiplex PCR approach (Dubernet *et al.,* 2002; Settanni *et al.,* 2005). Substantial research efforts have also been directed on the design of 16S rRNA primers and its application to monitor LAB dynamics and community development in food system (Randazzo *et al.,* 2006; Delbe's *et al.,* 2007). In the present investigation, efforts were concerted towards the design of 16S rRNA gene specific primers that enabled simultaneous detection and prediction of interactions between the major LAB groups in a natural fermentation process.

2.3.2. PCR-based detection of dominant LAB and bacteriocin producers in fermented cucumber

PCR was employed to salt-fermented cucumber samples to detect the succession of major LAB groups existing as inherent microflora and correlate the succession with the emergence of bacteriocin producing LAB. The cucumber samples were collected from various vegetables markets in and around Guwahati city to account for any sample variations. The method of analysis comprised time-dependent sampling during fermentation with an aim to understand microbial dynamics, and included a culture-independent and culture-dependent approach to decipher any bias introduced due to selection pressure exerted by the growth medium.

Table 2.3 indicates the results obtained from 30 samples using a culture-independent (direct detection) and culture-dependent (enrichment) technique.

In the direct detection method, PCR enabled the detection of *Lactobacillus* and *Leuconostoc* as early as 12 h of fermentation and revealed their coexistence upto 18 h in 11 samples, whereas in the remaining 19 samples only *Lactobacillus* could be detected. The trend for the enriched samples was similar wherein *Lactobacillus* and *Leuconostoc* was observed in 5 samples and *Lactobacillus* alone in the remaining 25 samples after 18 h of fermentation. From Table 2.3 it is also evident that as the fermentation progressed, *Leuconostoc* could be detected in only 6 samples by 36 h in the direct detection method. Concomitantly, the emergence of *Pediococcus* was observed in the samples. The persistence of *Lactobacillus* was evident in the samples during this time period, as it co-evolved either with *Pediococcus* and *Leuconostoc* (6 samples) or with *Pediococcus* alone (24 samples). It was noteworthy that in case of enriched samples, only *Lactobacillus* could be detected by 36 h indicating a selective growth of *Lactobacillus* during the enrichment process. In the latter part of fermentation (48 and 72 h), the persistence of *Lactobacillus* and *Pediococcus* was clearly established as they were detected in all 30 samples in the direct detection as well as enrichment method. Amplicons obtained from fermenting cucumber samples with genus-specific primers in both the methods (direct detection and enrichment) clearly demonstrate the prevalence of *Lactobacillus* throughout the fermentation as well as the emergence of *Pediococcus* in the late stages of fermentation (Figure 2.3).

Table 2.3

Molecular detection of LAB succession and bacteriocin producers in salt-fermented cucumber.

Fermentation time (h)	Direct detection		Enrichment	
	Genus[a]	Bacteriocin[b]	Genus[a]	Bacteriocin[b]
6	Nil	Nil	Nil	Nil
12	*Lb.*, *Leu.* (11) *Lb.* (19)	Pediocin (08) Mesentericin (04)	*Lb.*, *Leu.* (08) *Lb.* (22)	Pediocin (05) Mesentericin (02)
18	*Lb.*, *Leu.* (11) *Lb.* (19)	Pediocin (14) Mesentericin (04)	*Lb.*, *Leu.* (05) *Lb.* (25)	Pediocin (07) Mesentericin (02)
24	*Lb.*, *Leu.* (08) *Lb.* (22)	Pediocin (20) Mesentericin (02)	*Lb.*, *Leu.* (03) *Lb.* (27)	Pediocin (11)
36	*Lb.*, *Ped.*, *Leu.* (06) *Lb.*, *Ped.* (24)	Pediocin (24) Mesentericin (02) Plantaricin A (04)	*Lb.* (30)	Pediocin (19)
48	*Lb.*, *Ped.* (30)	Pediocin (26) Plantaricin A (10)	*Lb.*, *Ped.* (30)	Pediocin (22) Plantaricin A (06)
72	*Lb.*, *Ped.* (30)	Pediocin (27) Plantaricin A (11)	*Lb.*, *Ped.* (30)	Pediocin (23) Plantaricin A (06)

[a] Genus-specific 16S rRNA primers were used for *Lactobacillus*: *Lb.*, *Pediococcus*: *Ped.*, *Lactococcus*: *Lc.*, *Leuconostoc*: *Leu.* Numbers in parenthesis imply the number of samples in which the indicated LAB genera were obtained.
[b] Bacteriocin gene specific primers were used for Pediocin, Mesentericin, Enterocin A, Nisin and Plantaricin A. Numbers in parenthesis indicate the number of samples in which bacteriocin-encoding genes were detected at specific time periods of fermentation.

The genus *Lactococcus* could not be detected in any of the samples by the PCR method. This could possibly be attributed to the presence of extremely low numbers of *Lactococcus* in cucumber samples in comparison to other dominant genus. Consequently, in presence of overwhelming competing DNA, amplicons specific for the genus *Lactococcus* could not be detected by PCR.

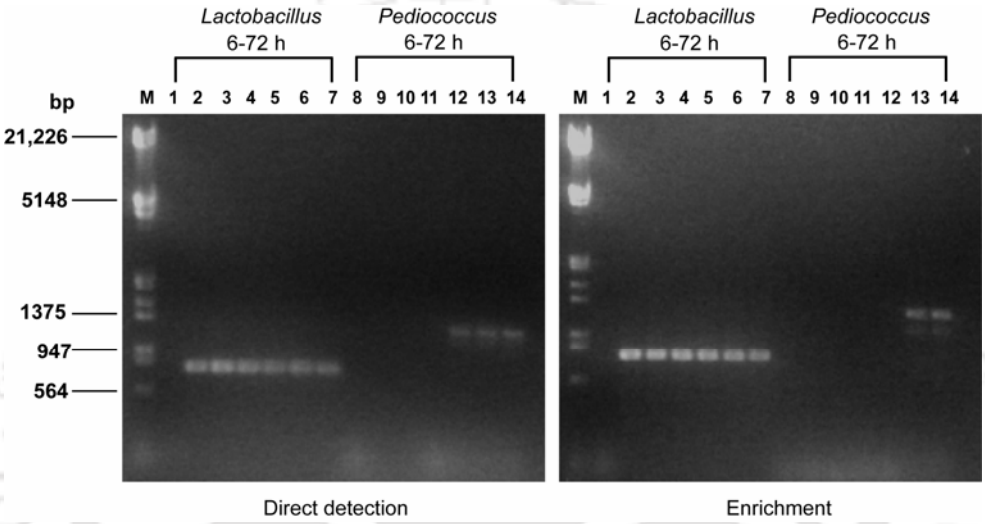

Figure 2.3 PCR-based detection of LAB succession by direct detection and enrichment method in salt-fermented cucumber using genus-specific 16S rRNA primers for *Lactobacillus (Lb.)* and *Pediococcus (Ped.)*. Lane M: λ DNA *Eco*RI/*Hin*dIII double digest size marker.

In the present investigation, submersion of sliced cucumber samples in salt solution and their tight packing in screw-cap bottles resulted in an anaerobic environment. Consequently, the growth of aerobic non-LAB was suppressed and lactic acid fermentation was initiated by facultative anaerobes. Besides, salt favors the development of anaerobic conditions in the fermentation vessel and exerts a selective effect on LAB naturally present on cucumber. Although the population of lactic acid bacteria present in plant material is presumably small, nonetheless it is assumed that plants are a natural habitat for many LAB species (Daeschel *et al.,* 1987). *Leuconostocs* are facultative anaerobes, present on plant materials, and they are

known to initiate fermentation in vegetable products. The initial loads of population, growth rate, salt and acid tolerance, as well as bacteriocin producing attributes are apparently involved in the succession of various lactic acid bacteria in vegetable fermentations. *Pediococcus pentosaceus* and *Lactobacillus plantarum* are more acid tolerant and are generally present during the later stages of fermentation as the dominant species (Daeschel *et al.,* 1987; Sandhu and Shukla, 1996; Harris, 1998). The initial load of LAB in cucumber samples is known to be low at the onset of fermentation and the inability of conventional microbiological techniques to enumerate and identify specific LAB genera is a bottleneck to study the succession of LAB in the samples. However, a simple detergent based DNA extraction process providing PCR-compatible templates, in conjunction with the designed genus-specific primers adopted in the present study could overcome this limitation and detect *Lactobacillus and Leuconostoc* in the samples during early hours of fermentation. The inability of the PCR method to detect *Lactococcus* and *Pediococcus* during initial stages of fermentation is possibly a reflection of the limit of detection of the PCR method, owing to the presence of copious amount of competing DNA. Select amplicons obtained in the culture-independent method using genus-specific primers for *Pediococcus* were subjected to nucleic acid sequencing. The sequences were deposited in GenBank with accession numbers EF636660, EF636661 and EF636662. Homology search of the sequence of the culture independent clones by BLAST analysis revealed that the clones (Cu1, Cu2 and Cu3) exhibited very high similarity (99%) to strains of *Pediococcus pentosaceus*.

Phylogenetic tree constructed by neighbour-joining method for partial 16S rRNA gene sequence of uncultured *Pediococcus* clones revealed close affiliation with standard strains of *Pediococcus* sp. (Figure 2.4). Thus, the identity of the dominant strain evolving during the course of cucumber fermentation was established.

PCR was also used to detect the succession of bacteriocin producers. It is evident from Table 2.3 that pediocin and mesentericin producers could be detected in the samples by 12 h of fermentation. After 24 h of fermentation, pediocin producers could be detected in majority of the samples by the direct detection method (20 samples) whereas the number of mesentericin producers dropped drastically as they were detected in only 2 samples. In contrast, only pediocin producers could be detected in the enriched samples (11 nos.) by 24 h of fermentation. The ability to produce bacteriocin is an inherent attribute in LAB. The significance of bacteriocin producing LAB is even more in the context of microbial interaction observed in a natural fermentation process, wherein antagonistic cultures are expected to enjoy a distinct selective advantage as fermentation proceeds. In the present study, it was observed that *Leuconostocs* thrive during the early stages of fermentation. This could possibly account for the detection of mesentericin producers in the samples at early stage of fermentation. It was also observed that *Lactobacillus* could be detected in the initial stages of fermentation. It is quite likely that some strains of *Lactobacillus* were presumably able to produce pediocin. Heterologous production of pediocin by *Lactobacillus* has been reported earlier (Ennahar *et al.,* 1996). In an earlier study a pediocin specific probe was used in colony hybridization experiments for isolating pediocin producers from fermented cucumber (Halami *et al.,* 2005). However, the study was not conducted on a time-dependent basis to evaluate the succession of LAB strains in the samples.

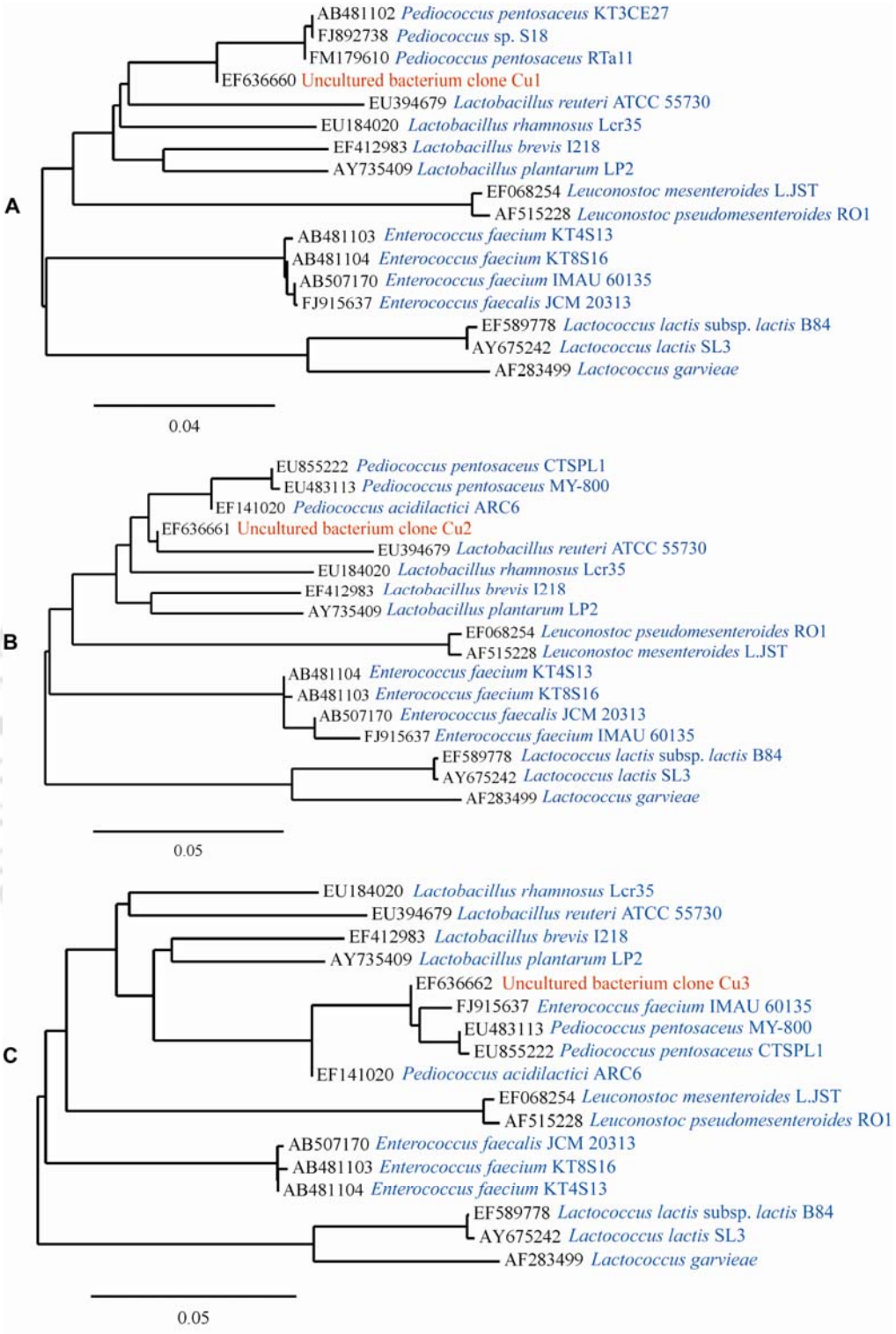

Figure 2.4 16S rRNA gene sequence based phylogenetic tree constructed for uncultured *Pediococcus* clones (Cu1, Cu2 and Cu3) detected in fermented cucumber samples by PCR in the direct detection method. Phylogenetic tree was constructed by using neighbour-joining algorithm (www.phylogeny.fr).

As fermentation progressed (36 h), the number of pediocin producers enhanced and they could be detected in 24 samples by the direct detection method as depicted in Table 2.3. However, the number of mesentericin producers still remained very low (only 2 samples) whereas plantaricin A producers started to emerge and were detected in 4 samples. For the enriched samples, only pediocin producers could be detected in 19 samples after 36 h of fermentation. The elimination of mesentericin producers and the steady increase in the number of pediocin producers in the samples corroborates well with earlier results which reveal the emergence of *Lactobacillus* and *Pediococcus* as the dominant genera after 36 h of fermentation. Subsequently, pediocin and plantaricin A producers persisted and could be detected after 48 and 72 h of fermentation in both the direct and enrichment method. The detection of plantaricin A producers can be accounted by the emergence of *Lactobacillus* as the dominant genus during the later stages of fermentation. It is evident from Table 2.3 that there is a qualitative differences observed in the number of pediocin producers in the direct and enrichment method. It is also observed that although there is a general increase of *Lactobacillus* due to enrichment, the proportion of samples indicating the presence of pediocin producers were relatively low compared to the direct detection method. The population of *Lactobacillus* obtained from fermented cucumber samples comprises of either pediocin producers or pediocin resistant population of *Lactobacillus* which may not be pediocin producers themselves. Presumably the enrichment process favors the selective growth of the pediocin resistant population of *Lactobacillus* resulting in the observation that a steady increase in the detection of *Lactobacillus* is not accompanied by a proportionate increase in the number of samples having pediocin producers. Enterocin and nisin producers could not be detected in any of the samples by the PCR method. A representative agarose gel

indicated in Figure 2.5 demonstrates the presence of amplicons obtained using bacteriocin gene specific primers and reflects the time-dependent emergence of bacteriocin producers in the direct detection method adopted in the present investigation.

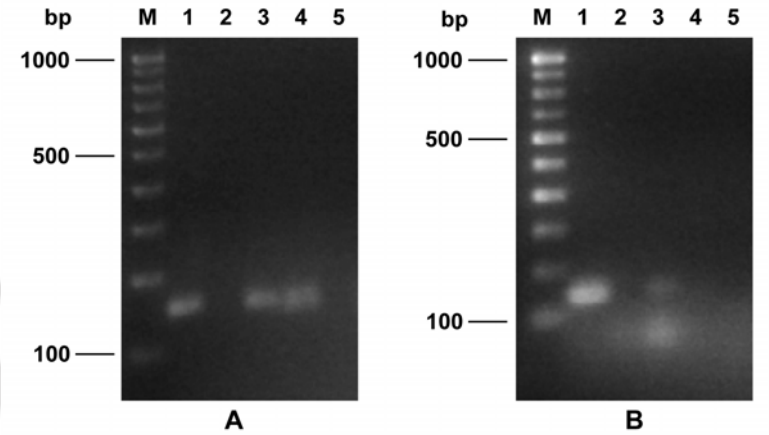

Figure 2.5 PCR-based direct detection of bacteriocin encoding genes of LAB in fermented cucumber sample after **(A)** 36 h and **(B)** 72 h of fermentation. Lanes: M: 100 bp DNA ladder; 1: Pediocin; 2: Enterocin A; 3: Plantaricin A; 4: Mesentericin; 5: Nisin.

Considerable research has been carried out to demonstrate LAB dynamics in fermented food samples. A common strategy in majority of the investigations is the use of molecular techniques such as PCR-DGGE to ascertain microbial dynamics and community development in food systems (Fontana *et al.,* 2005; Randazzo *et al.,* 2006). Some studies have also been conducted on community structure and detection of native LAB in traditional fermented vegetable products (Kim and Chun, 2005; Tamang *et al.,* 2005). These methods essentially identify the dominant LAB species, but do not reveal their antagonistic attributes that are consequential in conferring selective advantage to the dominant population. In the present study, for the first time, a combined PCR-based holistic approach to detect the major LAB genera as well as characteristic Class IIa bacteriocin producers addresses the aforementioned phenomenon and provides a pragmatic picture of LAB succession in fermented cucumber

samples. The presence of different LAB genera and Class IIa bacteriocin producers in cucumber samples provides the possibility of rapid large scale screening of samples through the application of multiplex PCR which is amicable to automation. The use of automated PCR techniques to analyze LAB succession and dynamics has been reported earlier (Lazzi *et al.*, 2004; Brusetti *et al.*, 2006).

2.3.3. Growth profile of LAB, isolation and classification of bacteriocin producing LAB in fermented cucumber

The growth profile of LAB and acid production in salt-fermented cucumber is shown in Figure 2.6A as an average of 30 samples. Analyses of samples at the initial stages of fermentation revealed the presence of approximately 5.5 $\log_{10}$ cfu/ml. However, from Table 2.3 it can be seen that PCR failed to detect any LAB genera and bacteriocin producers at 6h of fermentation. The population of LAB obtained after 6 h of fermentation indicates the total LAB count and comprises of all the genera. The proportion of individual LAB genus present in early stages of fermentation is presumably low. Thus the sensitivity of PCR-based detection is compromised due to the presence of miniscule amount of target DNA in comparison to competing DNA from other genera. It is also evident from Figure 2.6A that there is a rapid increase in growth in the first 18 h of fermentation wherein the average cell number amounted to approximately 8.0 $\log_{10}$ cfu/ml. Subsequently, the LAB population followed a steady decline to reach approximately 7.0 $\log_{10}$ cfu/ml after 72 h of fermentation. The increase in LAB cell number was accompanied by a concomitant decline of pH from 6.7 to 3.25, indicating growth-associated acid production.

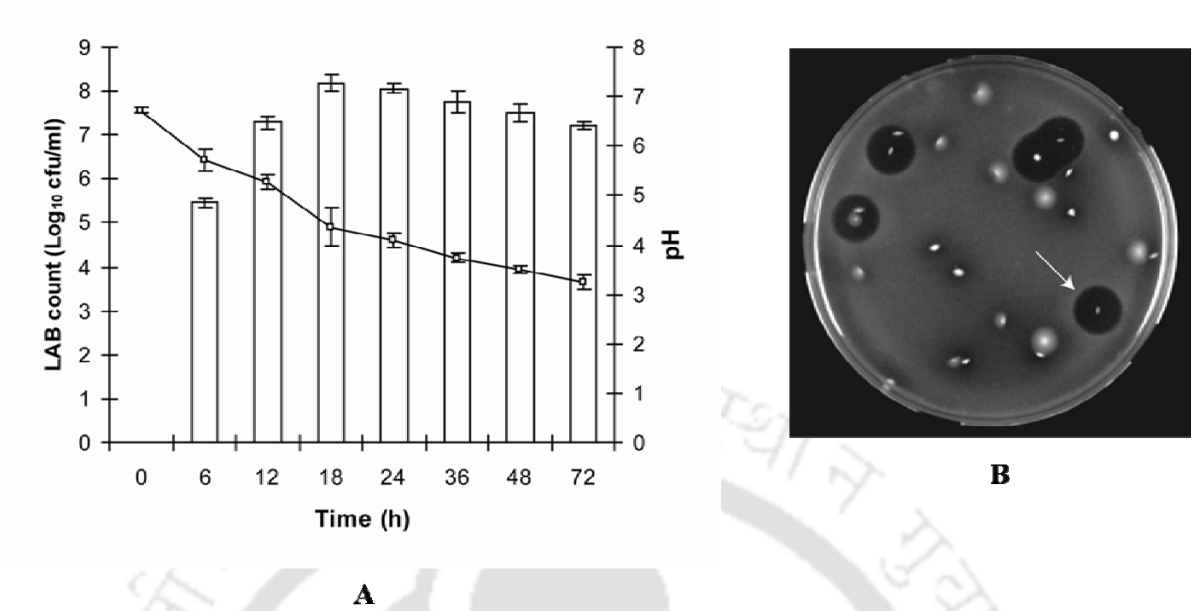

Figure 2.6 (A) Kinetics of growth of LAB (□) and acidification (○) in fermented cucumber samples. Results are demonstrated as average of 30 samples. **(B)** Colonies of lactic acid bacteria obtained from salt-fermented cucumber. Arrow indicates a bacteriocin producing colony with zone of inhibition produced on a lawn of the target strain *Leuconostoc mesenteroides* NRRL B640.

Since fermentation mainly occurs in the liquid phase, rapid release of nutrients from sliced cucumber samples results in accelerated growth and high acid production. The rapid drop in pH observed in the present study is a combined effect of high acid production and inadequate buffering capacity of the fermenting cucumber samples. The variations observed for growth kinetics and acid production in the samples analyzed is essentially a function of the type, availability and concentration of fermenting substrate, buffering capacity, competing microorganisms and antagonists inherently present in cucumber.

A total of 328 LAB isolates obtained from 30 cucumber samples at different time periods of fermentation were subjected to PCR-based genus identification. It is clear from Table 2.4 that *Lactobacillus* comprised a significant proportion of the population, amounting to nearly 56% (186 nos.) of the isolates followed by *Pediococcus*, which accounted for approximately 34% (118 nos.) of the isolates.

Table 2.4

PCR-based identification of LAB and bacteriocin producers obtained at different time periods from fermented cucumber samples[a].

	Fermentation time period							Total Count
	6h	12h	18h	24h	36h	48h	72h	
LAB genera[b]								
Lactobacillus	03	10	17	23	31	48	54	186
Pediococcus	02	07	15	19	20	22	28	113
Leuconostoc	03	14	08	04	Nil	Nil	Nil	29
Lactococcus	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Total LAB number								328
Bacteriocin producers[c]								
Pediocin	Nil	04	04	06	09	13	21	57
Plantaricin A	Nil	Nil	Nil	Nil	Nil	07	09	16
Mesentericin	Nil	04	03	Nil	Nil	Nil	Nil	07
Enterocin A	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Nisin	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Unidentified bacteriocin producers								12
Total No. of bacteriocin producers								92

[a] Data shown as aggregate of 30 cucumber samples.
[b] LAB genera was determined by PCR using genus-specific primers for 16S rRNA gene.
[c] Bacteriocin producing colonies were selected by colony overlay assay using *Leuconostoc mesenteroides* NRRL B640 as target strain; Bacteriocin encoding genes were detected in the isolates by PCR using gene specific primers for pediocin, plantaricin A, mesentericin, enterocin A and nisin.

A small fraction of the isolates belong to genus *Leuconostoc* (29 nos.). The overwhelming presence of *Lactobacillus* and *Pediococcus* agree well with earlier results shown in Table 2.3 wherein they could be detected in large number of samples in both the direct and enriched method. Low numbers of *Leuconostoc* are expected considering that they were obtained from samples taken during early hours of fermentation. Subsequently, due to acid production and copious numbers of bacteriocin producers the *Leuconostoc* population was eliminated.

Bacteriocin producing attribute of the LAB isolates was ascertained by overlaying the colonies with a lawn of the sensitive strain *Leuconostoc mesenteroides* NRRL B640. A representative assay plate indicating the presence of bacteriocin producing colonies of LAB is shown in Figure 2.6B. It is clear from the figure that bacteriocin producing colonies were clearly discernible from non-producers as they produce a large zone of inhibition on a lawn of the indicator strain. The total number of bacteriocin producing LAB colonies obtained collectively from 30 samples was 92, which correspond to approximately 28% of the total LAB colonies obtained. The class of bacteriocin produced by the antagonistic isolates was assessed by PCR using primers specific for the bacteriocins. The quantitative comparison of the class of bacteriocin producers obtained from the cucumber samples is depicted in Table 2.4. A total number of 7 mesentericin producers were obtained after 12-18 h of fermentation. The number of pediocin producers encompassed a vast majority of the bacteriocin producers (57 nos.). Presumably, strains belonging to genus *Lactobacillus* and *Pediococcus* together account for the large number of pediocin producers observed. Out of a total of 57 pediocin producers, the highest numbers (21 nos.) were obtained after 72 h of fermentation. Likewise, a total of 16 plantaricin A producers emerged late following 48 and 72 h of fermentation. Thus different classes of bacteriocin producers evolve at defined time-

periods and this can form a strategic basis for isolating potent bacteriocin producing strains from a natural source. Amongst the bacteriocin producers, 12 additional LAB isolates were established as bacteriocin producers by colony overlay assay. However, no specific amplicon could be detected using the bacteriocin gene specific primers. Evidently, the bacteriocin produced by these isolates are different from the ones tested by the PCR method in the present investigation and hence could not be identified.

2.3.4. Characterization of antimicrobial compound

Bacteriocin-like inhibitory substance (BLIS) present in the culture filtrate of 92 antagonistic LAB isolates was characterized. A representative result for one of the LAB isolates is shown in Figure 2.7. The inhibitory molecule in culture filtrate was confirmed to be proteinaceous as the culture filtrate treated with trypsin lost its activity against the target strain of *Leuconostoc mesenteroides* NRRL B640. The culture filtrate treated with heat inactivated trypsin demonstrated bacteriocin activity reiterating the proteinaceous nature of BLIS. Heat inactivated trypsin alone did not reveal any inhibition in agar well diffusion assay (Figure 2.7). The antimicrobial activity of BLIS was not affected by catalase treatment. Hence, the role of hydrogen peroxide as one of the components of BLIS was negated. It is also clear from Figure 2.7 that the antimicrobial activity was retained in a 1.0 kDa dialysis bag, but lost when a 12.0 kDa dialysis bag was used, indicating the small molecular size of the inhibitory molecule. Additional experiments clearly established the heat stability of BLIS (Figure 2.7, well nos. 7 and 8). It was also observed that BLIS displayed antimicrobial activity against the target strain at pH 3.5, whereas buffer adjusted to the same pH could not demonstrate any activity and antagonism due to acidity was negated (Figure 2.7, well nos. 9 and 10).

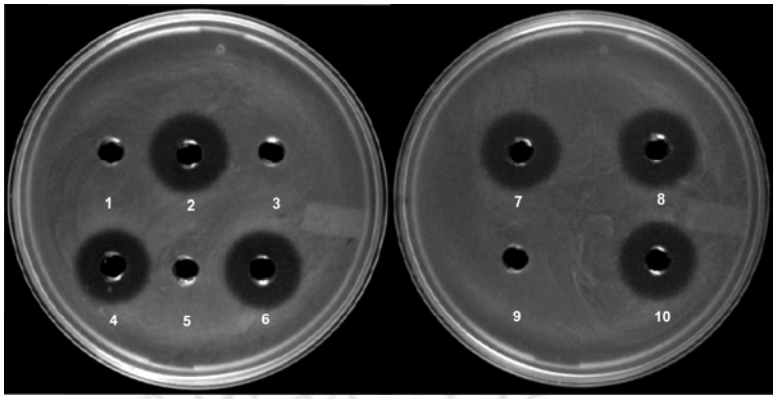

Figure 2.7 Effect of trypsin, dialysis, temperature and pH on bacteriocin-like inhibitory substance (BLIS) of antagonistic LAB isolated from fermented cucumber. Well nos. **1:** BLIS treated with trypsin; **2:** BLIS treated with heat inactivated trypsin; **3:** Heat inactivated trypsin solution alone; **4:** BLIS treated with catalase; **5:** BLIS dialyzed with 12.0 kDa MWCO bag; **6:** BLIS dialyzed with 1.0 kDa MWCO bag; **7:** BLIS treated at 120°C/20 min; **8:** BLIS at 100°C/30 min; **9:** Buffer, pH 3.5; **10:** BLIS pH 3.5. Antimicrobial activity of BLIS was tested by agar well diffusion assay on a lawn of the target strain *Leuconostoc mesenteroides* NRRL B640.

All the 92 antagonistic LAB isolates, which were found to produce BLIS, gave similar results in the characterization test. Collectively these results provide strong evidence for bacteriocin production by the antagonistic LAB isolates obtained from salt-fermented cucumber and supports earlier research work, which report similar salient features of bacteriocin (Halami *et al.*, 2005; Todorov and Dicks, 2006).

2.3.5. Antimicrobial spectrum of bacteriocin producing LAB

The neutralized and filter-sterilized culture filtrate of all the 92 bacteriocin producing LAB were tested by agar well diffusion assay against a range of target LAB strains and pathogenic bacteria indicated in Table 2.5. It is evident from the table that a large number of bacteriocin producers exhibited activity against the chosen LAB target strains. The highest numbers of antagonistic isolates were obtained against *Leuconostoc mesenteroides* NRRL B640, which was originally used as a target strain to isolate the bacteriocin producers from fermented

cucumber. The neutralized culture filtrate of a significant number of isolates (87 nos.) also demonstrated inhibitory activity against *Lactobacillus casei* subsp. *casei* NRRL B1922, *Lactobacillus fermentum* MTCC 903, *Lactobacillus sakei* NRRL B1917 and *Pediococcus acidilactici* NRRL B14009. Amongst the LAB used as target strain, *Lactobacillus johnsonii* NRRL B2178 and *Lactobacillus rhamnosus* MTCC 1408 were resistant. Majority of the bacteriocins detected in the natural isolates (mesentericin, pediocin and plantaricin A) belong to the Class IIa category, which is known to act on closely related LAB strains (Ennahar *et al.*, 2000). It is also evident from the table that the culture filtrate of the isolates was inhibitory to pathogenic bacterial strains.

Table 2.5

Antimicrobial spectrum of bacteriocinogenic LAB isolates (Bac^+) obtained from salt-fermented cucumber samples against target LAB and pathogenic bacterial strains

Target bacterial strain	No. of Bac^+ LAB isolates
Lactobacillus acidophilus MTCC 447	48
Lactobacillus casei subsp. *casei* NRRL B1922	87
Lactobacillus delbrueckii subsp. *bulgaricus* NRRL B548	33
Lactobacillus delbrueckii subsp. *lactis* MTCC 911	28
Lactobacillus fermentum MTCC 903	72
Lactobacillus johnsonii NRRL B2178	Nil
Lactobacillus plantarum MTCC 1325	14
Lactobacillus rhamnosus MTCC 1408	Nil
Lactobacillus sakei NRRL B1917	68
Lactococcus lactis subsp. *cremoris* MTCC 1484	42
Lactococcus lactis subsp. *lactis* MTCC 3041	11
Leuconostoc mesenteroides NRRL B640	92
Leuconostoc mesenteroides subsp. *mesenteroides* MTCC 107	51
Pediococcus acidilactici NRRL B14009	65
Escherichia coli MTCC 443	05
Bacillus cereus MTCC 1305	17
Listeria monocytogenes Scott A	48
Staphylococcus aureus MTCC 96	14
Enterobacter aerogenes MTCC 2822	04
Enterococcus faecalis MTCC 439	06

NRRL: Northern Regional Research Laboratory, Peoria, IL, USA; MTCC: Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India.

A large number of isolates (48 nos.) could readily inhibit the growth of the foodborne pathogen *Listeria monocytogenes* Scott A. Class IIa bacteriocin-like pediocin is known to have strong anti-listerial activity (Jagannath *et al.,* 2001; Rodriguez *et al.,* 2002). The antimicrobial activity of the culture filtrates of selected isolates on a lawn of *L. monocytogenes* Scott A is also depicted in Figure 2.8D, wherein zone of growth inhibition around the well is conspicuous. Some isolates were antagonistic to Gram-positive pathogens such as *Bacillus cereus* MTCC 1305 and *Staphylococcus aureus* MTCC 96 (Table 2.5 and Figure 2.8B and C). Interestingly, bacteriocin present in the culture filtrate of few isolates could also hinder the growth of Gram-negative bacteria such as *Escherichia coli* MTCC 443. Bacteriocins working against Gram-positive pathogenic bacteria have been reported earlier (Albano *et al.,* 2007a). In the present study bacteriocin producing LAB strains, which were antagonistic to even Gram-negative pathogen like *Escherichia coli* were also obtained. The broad spectrum bacteriocin producing LAB cultures obtained in this investigation can find useful applications in food fermentation and preservation processes.

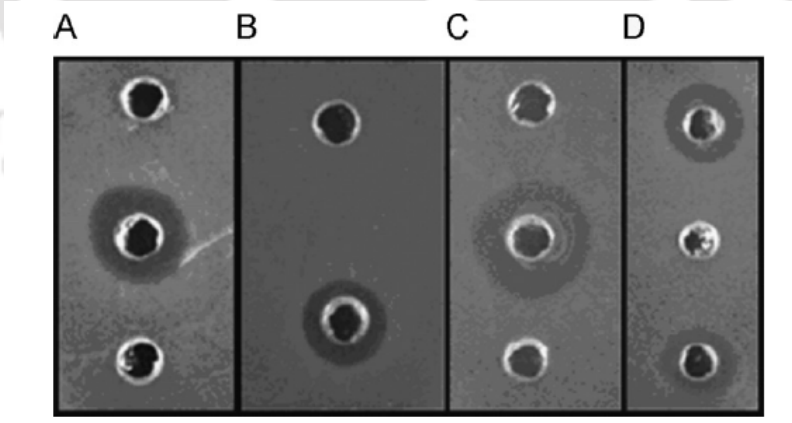

Figure 2.8 Antimicrobial activity of the culture filtrate of select LAB isolates obtained from fermented cucumber against pathogenic bacteria. **(A)** *Escherichia coli* MTCC 443; **(B)** *Bacillus cereus* MTCC 1305; **(C)** *Staphylococcus aureus* MTCC 96 and **(D)** *Listeria monocytogenes* Scott A. Bacteriocin activity is revealed as a zone of growth inhibition around the well.

2.4. Conclusion

The investigation outlined in this chapter clearly demonstrated the strength of the PCR-based approach in simultaneously detecting major LAB groups in fermenting cucumber. The salient achievements of the investigation are as follows:

1. Judicious design of genus-specific and bacteriocin gene specific primers in conjunction with a simple DNA extraction method enabled PCR-based detection of all four major LAB genera as well as multiple bacteriocin producers in fermented cucumber samples.

2. Time-dependent succession of LAB genera in the fermenting samples was monitored and the succession of the LAB population could be rationally linked to the emergence of bacteriocin producing strains. A schematic representation of PCR-based direct detection of LAB succession and emergence of bacteriocin producers in fermenting cucumber samples is depicted in Figure 2.9.

3. The culture-independent and culture-dependent methods were highly sensitive and specially proved useful to detect small numbers of the initial LAB population present in cucumber and also demonstrate their bacteriocin producing ability.

4. Quantitative differences could be observed in the culture-independent and culture-dependent methods thus accounting for the biased selection usually associated with enrichment techniques.

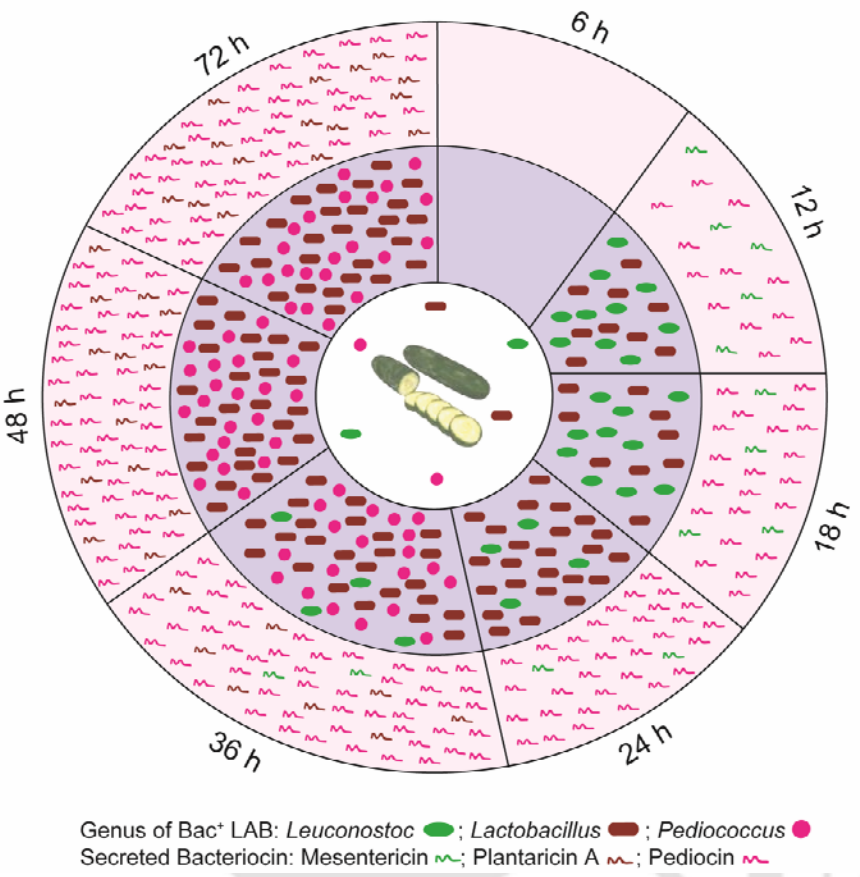

Figure 2.9 A schematic representation of PCR-based direct detection of LAB succession and emergence of bacteriocin producing (Bac^{+}) LAB in salt-fermented cucumber samples.

Interestingly, in the present investigation, a large number of antagonistic LAB were obtained with potent activity against pathogenic bacteria. On further characterization, these natural isolates can either be used as starter cultures or their antagonistic attributes can be genetically transferred to construct competitive LAB starters and achieve superior shelf-life of fermented food products. Hence, the next aim was to ascertain the genetic diversity of the antagonistic bacteriocin producing LAB isolates obtained from fermented cucumber samples. The detailed investigation in this regard is highlighted in the next chapter.

CHAPTER 3

Molecular fingerprinting of bacteriocin producing lactic acid bacteria using a robust PCR-compatible template DNA extraction method

ABSTRACT

The objective of the research work carried out in this part of thesis was to develop a convenient and reliable method of generating template DNA for routine PCR-based detection and molecular fingerprinting of lactic acid bacteria (LAB). Template DNA extracted from *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* using a combination of urea, SDS and NaOH yielded amplicons of expected size in PCR with genus-specific primers. Apart from LAB, the proposed method could also be adopted to generate PCR-compatible template DNA from a number of Gram-positive and Gram-negative bacterial strains. DNA template prepared by the proposed method from various standard strains of *Lactobacillus* sp. also generated discriminating fingerprints with BOXA1R primer in rep-PCR. A significant finding of the investigation was that a comparable banding profile of LAB strains was obtained in rep-PCR using template DNA prepared by urea-SDS-NaOH method and a commercially available DNA isolation kit. This was further evidenced by high Dice coefficient values obtained in the range of 81.8-96.7 when cluster analysis was performed by UPGAMA method. The application potential of this DNA extraction method for PCR-based direct detection of LAB in fermented food samples such as *dahi*, *idli* batter and salt-fermented cucumber was validated by detecting specific amplicons of LAB genera in the fermented samples. The applicability of the proposed template DNA extraction method was further substantiated when 29 bacteriocinogenic LAB strains (Bac^{+}) previously detected in salt-fermented cucumber by PCR generated differentiating fingerprints in BOX element based rep-PCR and formed clusters with reference LAB strains.

3.1. Introduction

The starter culture and flavor forming traits of LAB have been recognized as cardinal features, which have significantly contributed to their wide spread use in food fermentation processes. (Di Cagno *et al.,* 2008; Coolbear *et al.,* 2008; Leroy and De Vuyst, 2004; van Hylckama Vlieg and Hugenholtz, 2007). LAB are also known for probiotic attributes and can influence the activity and composition of the intestinal microbiota (Ouwehand *et al.,* 2002; Saito, 2004). Apart from the starter culture and probiotic features, the capacity of LAB to produce antimicrobial agents such as bacteriocin and inhibit food-borne pathogens has reiterated their promise and strengthened their application potential in food fermentation processes and biopreservation (Castellano *et al.,* 2008; Drider *et al.,* 2006 ; Jagannath *et al.,* 2001; Jones *et al.,* 2008 ; Settani and Corsetti, 2008).

In the previous chapter, bacteriocin producing LAB strains were isolated from salt-fermented cucumber. Interestingly, a number of native LAB strains were antagonistic to pathogenic bacteria, indicating the possible application of these strains in food fermentation processes and as bioprotective cultures. The choice of an appropriate LAB strain for food fermentation demands critical attention to strain identification. Besides, the need to characterize LAB strains is justified since estimating the genetic diversity of strains and appraising the fate of LAB starter cultures are vital in the context of defining technological parameters for food fermentation processes. The classical approaches based on morphological and biochemical identification of LAB strains are arduous and error-prone. Alternative DNA based techniques include 16S rRNA probes, plasmid profiles, and pulsed-field gel electrophoresis (Heilig *et al.,* 2002; Cusick and O'Sullivan, 2000; Ventura and Zink, 2002). However, these methods are cumbersome for routine large scale application. Polymerase

chain reaction (PCR)-based methods offer rapid and convenient options for LAB strain identification, monitoring of starter cultures, detection of antagonistic LAB and molecular typing (Coudeyras *et al.,* 2008; Nielsen *et al.,* 2007; Settani *et al.,* 2005; Yost and Nattress, 2002).

The successful application of PCR-based techniques for LAB detection and typing essentially depends on the quality of the template DNA. Hence, efficient extraction of template DNA from LAB strains is a decisive factor to confer reliability to PCR-based analysis. The crux of the problem is that LAB being gram positive possess a recalcitrant cell wall, necessitating the use of cell lytic enzymes like lysozyme (Chagnaud *et al.,* 2001). In other instances, protocols for efficient DNA isolation from LAB report the use of chelex resins, detergents like CTAB, nuclease inhibitors like proteinase K and organic solvents like phenol-chloroform (Dubernet *et al.,* 2002; Giraffa *et al.,* 2000). However, the aforementioned interventions make the entire process laborious and cost intensive. Besides, the presence of even trace amounts of these reagents as contaminants in the template DNA sample is of great concern and could significantly hamper subsequent PCR-based analysis. Another cardinal issue is the fact that LAB strains are known to be diverse and it is quite likely that a variety of native LAB isolates with different cell wall characteristics could be encountered in food samples. Hence, the DNA extraction method should be able to address this issue and readily provide PCR-compatible templates from a wide range of native LAB strains. It is also envisaged that the DNA extraction method should have wide scale adaptability in detecting LAB present in food samples by generating inhibitor free PCR-compatible templates. Lastly, exploitation of the DNA extraction method to generate templates that facilitate PCR-based fingerprinting of native LAB strains will also be an added advantage in estimating the genetic

diversity of the strains. In the present investigation the endeavor was to develop a robust method of DNA extraction which generates PCR-compatible templates from various LAB strains, with minimal sample manipulation steps. A combination of urea, SDS and NaOH could rapidly generate PCR-compatible templates and facilitate detection and molecular typing of various LAB strains. Interestingly, the proposed method of DNA extraction was able to generate PCR-compatible template DNA from wide range of Gram-positive and Gram-negative bacteria. The reliability of the method was tested by comparing the fingerprinting patterns obtained from the templates from select LAB strains to that obtained using a commercially available DNA isolation kit. The application potential of this method was tested in PCR-based detection of LAB strains from fermented food samples and in estimating the genetic diversity of bacteriocin producing (Bac^+) native LAB isolates which were obtained in the previous investigation (Chapter 2) from salt-fermented cucumber.

3.2. Materials and Methods

3.2.1. Bacterial strains and growth conditions

The reference strains of LAB and other Gram-positive and Gram-negative bacteria used in the present investigation are shown in Table 3.1. All LAB strains were maintained as frozen stocks in milk and glycerol (10% each) at -20°C and propagated in MRS medium as mentioned in section 2.2.1 of Chapter 2. Strains of *Enterococcus faecalis, Listeria monocytogenes*, *Staphylococcus aureus* and *Yersinia enterocolitica* were propagated aerobically at 37°C in brain-heart infusion (BHI) broth (HiMedia, India) whereas *Bacillus cereus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Escherichia coli*, *Micrococcus luteus and Pseudomonas aeruginosa* were grown aerobically at 37°C in nutrient broth (NB) medium (HiMedia, India).

Table 3.1

Reference bacterial strains used in the present investigation.

Bacteria	Strain
Lactobacillus acidophilus	MTCC 447
Lactobacillus brevis	NRRL B4527, NCIM 2090
Lactobacillus casei	NCIM 2151, MTCC 1423
Lactobacillus casei subsp. *casei*	NRRL B1922
Lactobacillus delbrueckii subsp. *delbrueckii*	NCIM 2025
Lactobacillus delbrueckii subsp. *bulgaricus*	NRRL B548
Lactobacillus delbrueckii subsp. *lactis*	MTCC 911
Lactobacillus fermentum	MTCC 903
Lactobacillus gasseri	NRRL B4240, NRRL B14168
Lactobacillus helveticus	NCIM 2126
Lactobacillus johnsonii	NRRL B2178
Lactobacillus lactis	NCIM 2368
Lactobacillus paracasei subsp. *paracasei*	NRRL B4564
Lactobacillus plantarum	MTCC 1325, MTCC 1746, MTCC 1407, MTCC 2083, NCIM 2592
Lactobacillus reuteri	NRRL B14171
Lactobacillus rhamnosus	MTCC 1408, NRRL B442
Lactobacillus sakei	NRRL B1917
Lactobacillus salivarius subsp. *salivarius*	NRRL B1949
Lactococcus lactis subsp. *chacetylactis*	MTCC 3042
Lactococcus lactis subsp. *lactis*	MTCC 440, MTCC 3041, MTCC 3038
Leuconostoc mesenteroides	NRRL B640
Leuconostoc mesenteroides subsp. *mesenteroides*	MTCC 107
Leuconostoc oenos	NCIM 2219
Pediococcus acidilactici	CFR K7[a], NRRL B14009, NRRL B14958, NRRL B1153
Pediococcus pentosaceus	NCIM 2295
Bacillus cereus	MTCC 1305
Bacillus subtilis	MTCC 441
Enterococcus faecalis	MTCC 439
Listeria monocytogenes	Scott A[a]
Micrococcus luteus	NCIM 2704
Staphylococcus aureus	MTCC 96
Enterobacter aerogenes	MTCC 2822
Escherichia coli	MTCC 118, MTCC 443
Pseudomonas aeruginosa	MTCC 2488
Yersinia enterocolitica	MTCC 859

NRRL: Northern Regional Research Laboratory, Peoria, IL, USA; MTCC: Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India; NCIM: National Collection of Industrial Microorganism, National Chemical Laboratory (NCL), Pune, India.

[a] Culture provided by Dr. Prakash Halami, Central Food Technological Research Institute (CFTRI), Mysore, India.

3.2.2. Isolation of template DNA from bacterial strains

DNA was isolated from LAB and other bacterial strains listed in Table 3.1. The bacterial strains were grown for 6h under the specified growth conditions mentioned before, prior to isolation of DNA. The template DNA isolation protocol is outlined in Figure 3.1. The purity of the isolated template DNA was ascertained for select strains of LAB by measuring A_{260}/A_{280} (Sambrook and Russell, 2001).

3.2.3. Evaluation of urea-SDS-NaOH method of template DNA isolation for PCR-based detection and molecular fingerprinting of LAB

The ability of the urea-SDS-NaOH method of DNA isolation to generate PCR-compatible templates from select strains of LAB was evaluated by using 2.0 µl of the extracted DNA sample in conventional PCR along with genus-specific primers for LAB. The universality of the DNA isolation method was ascertained by extracting DNA from LAB strains as well as various Gram-positive and Gram-negative bacterial strains (Table 3.1) and performing PCR using universal primers for 16S rRNA gene. To ascertain the sensitivity of PCR-based LAB detection template DNA was extracted by urea-SDS-NaOH method from varying cell number (10^6-10^1 cfu/ml) of standard LAB strains belonging to genus *Lactobacillus*, *Pediococcus, Lactococcus and Leuconostoc*. PCR was then performed in conjunction with genus-specific 16S rRNA primers. The amplicon intensity was quantified by a plot profile analysis with NIH ImageJ software (http://rsb.info.nih.gov/ij).

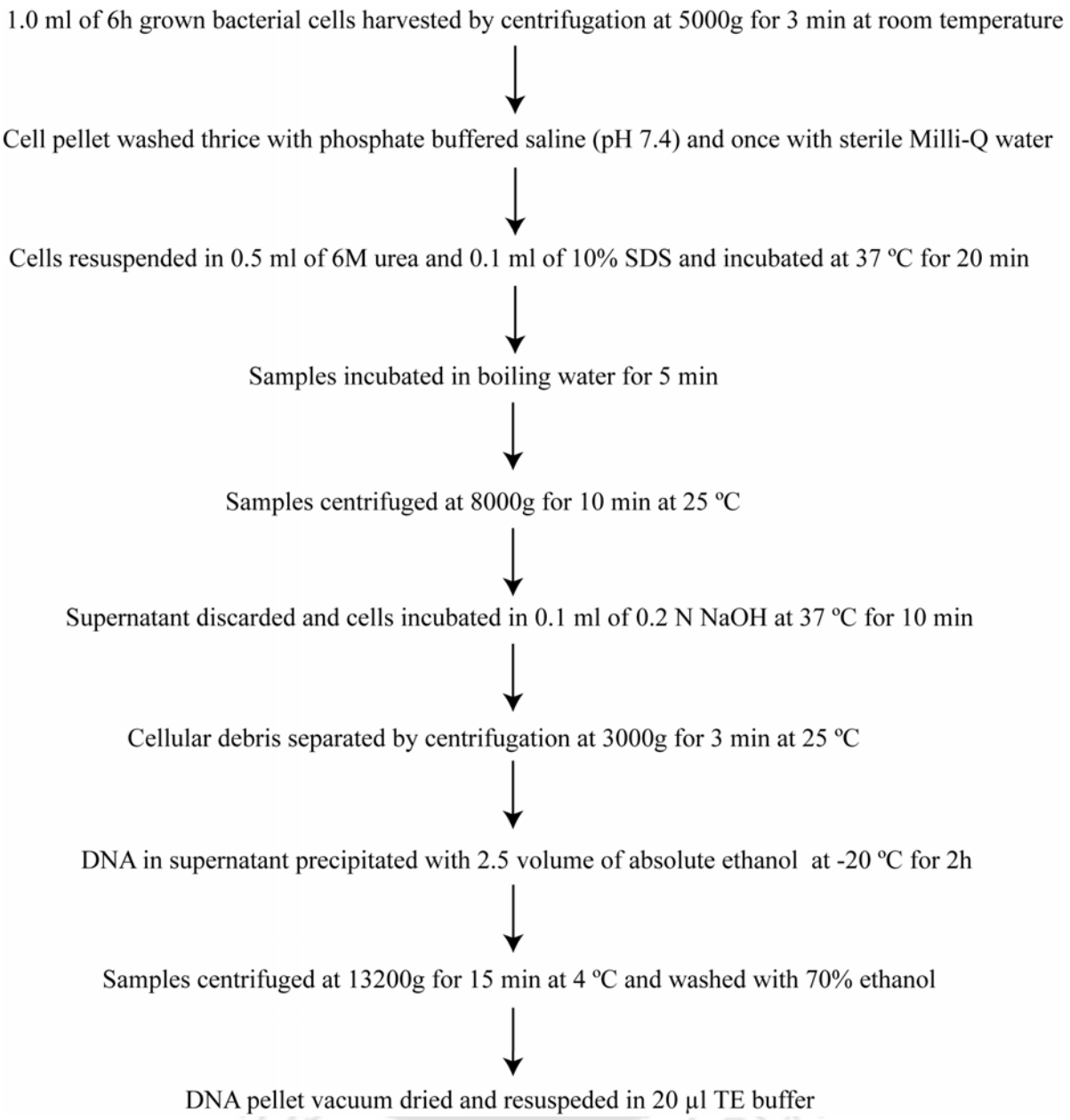

Figure 3.1 Scheme of template DNA extraction from LAB and non-LAB bacterial strains

The applicability of the urea-SDS-NaOH method in generating PCR-compatible templates for fingerprinting of LAB strains was determined by extracting template DNA from 25 standard strains of *Lactobacillus* and performing rep-PCR with BOX element specific

primer. The reproducibility of the fingerprint profiles was also evaluated by extracting DNA separately from triplicate samples and performing PCR.

3.2.4. Comparative analysis of PCR-based detection and molecular fingerprinting of LAB with extracted DNA templates

DNA was isolated from select LAB strains using urea-SDS-NaOH method as well as GenElute bacterial genomic DNA isolation kit (Sigma, USA). A conventional PCR using universal primers for 16S rRNA gene and rep-PCR with BOX element primer was performed with the templates generated by both the methods. The quality of amplicons and fingerprinting profiles obtained from LAB strains were compared.

3.2.5. PCR conditions and fingerprinting profiles

PCR amplification was performed in a total reaction volume of 25.0 µl. The proportions of PCR components and cycle parameters were essentially same as mentioned before in section 2.2.4 of Chapter 2. The sequence of universal primer for bacteria, genus-specific primers for *Lactobacilli*, *Lactococci, Pediococci* and *Leuconostoc* and conserved BOX element specific primer are indicated in Table 3.2. A total of 35 amplification cycles were performed using a programmable thermal cycler (Gene Amp Gold PCR System, Applied Biosystems, USA). Primer annealing temperatures were set at 55°C for 1 min for genus-specific and universal primers and 50°C for 1 min in case of BOX element based primer. The PCR products were analyzed by agarose (0.8%) gel electrophoresis (Sambrook and Russell, 2001). Analysis of the fingerprinting profiles obtained in rep-PCR was accomplished by using Quantity One software, version 4.5 (Bio-Rad, USA). Similarity of the profiles was evaluated by obtaining

Dice coefficient values and the phylogenetic tree was drawn on the basis of unweighted pair group method using arithmetic averages (UPGAMA). The mathematical equation for computing Dice coefficient value is described in section 1 of Appendix.

Table 3.2

Primers used in the present investigation.

Primer	Nucleotide sequence (5'-3')	PCR target	Reference
U1F U1R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	Universal-16S rRNA	Weisburg *et al.*, (1991)
1F 1R	AGAAGAGGACAGTGGAAC TTACAAACTCTCATGGTGTG	*Lactobacillus*-16S rRNA	Present work
2F 2R	TAAAGCGAGCGCAGGTGG GGTTACCTTGTTACGACTT	*Lactococcus*-16S rRNA	Present work
3F 3R	CTGAATGAGATTTTAACACG GGTTTTAAGAGATTAGCT	*Pediococcus*-16S rRNA	Present work
4F 4R	AGAGATGGATCCGCGGTGCA TTACAAACTCCCATGGTGTG	*Leuconostoc*-16S rRNA	Present work
BOXA1R	CTACGGCAAGGCGACGCTGACG	Interspersed repetitive DNA element	Versalovic *et al.*, (1994)

3.2.6. Application of urea-SDS-NaOH method of DNA extraction for molecular detection of LAB in fermented samples

Five samples each of *dahi* (an indigenous lactic cultured milk product), *idli* batter (fermented batter produced from rice powder and dehulled black gram, which is steamed to prepare *idli*) and cucumber samples fermented in 4% saline solution for 72 h were chosen as model system for these experiments. The following steps were adopted for direct extraction of DNA from the samples. Aliquots of each sample (0.3 ml of *dahi* and salt-fermented cucumber and 0.3 g

in case of *idli* batter) were taken in separate tubes and mixed with 0.7 ml of phosphate buffered saline containing 0.5% Tween 20 (PBS-T). The samples were thoroughly vortexed for 10 minutes and centrifuged at low speed of 450 x g for 2 minutes at room temperature to separate the food matrix. The supernatant containing bacterial cells was collected and centrifuged at 8000 x g for 5 minutes at room temperature and the cell pellet was processed for template DNA extraction using the method outlined in Figure 3.1. The extracted template DNA was subjected to PCR with *Lactobacillus, Lactococcus, Pediococcus* and *Leuconostoc* genus-specific primers.

3.2.7. Molecular fingerprinting of bacteriocin producing (Bac^+) LAB isolated from fermented cucumber using template DNA extracted by urea-SDS-NaOH method

The application potential of the template DNA extraction method for molecular typing analysis was also tested for 29 bacteriocin producing (Bac^+) LAB strains obtained earlier from salt-fermented cucumber (Chapter 2). DNA was extracted from Bac^+ LAB by urea-SDS-NaOH method and rep-PCR was performed with BOX element primer. Standard strains of *Lactobacillus* and *Pediococcus* were included as reference strains to compare the fingerprinting profiles. Cluster analysis and construction of phylogenetic tree was accomplished as stated before in section 3.2.5.

3.3. Results and Discussion

3.3.1. Isolation of template DNA

The main endeavor of the present investigation was to establish a convenient DNA extraction method for LAB strains using readily available and cost effective reagents and generate PCR-compatible template DNA that facilitate LAB detection and typing analysis. A major concern was to ensure that the method is rapid, involving minimal sample manipulation steps and applicable to large number of bacterial strains with varying cell wall composition. The scheme of urea-SDS-NaOH method of template DNA preparation used in the present investigation is shown in Figure 3.1. DNA was isolated from 1.0 ml of 6h grown culture (~10^6 cfu) of various LAB strains as well as other Gram-positive and Gram-negative bacteria (Table 1). The primary challenge in bacterial DNA extraction is effective cell lysis. Based on an earlier study where urea was employed for DNA extraction from milk samples (Ramesh *et al.*, 2002), urea was selected in the present investigation as a chaotropic agent to induce cell lysis. It is known that urea can promote denaturation of proteins by destabilizing protein-water interactions. Hydrophobic effects are the main determinants of urea-induced protein denaturation and urea can preferentially solvate non-polar and aromatic amino acid residues, as well as the peptide backbone in proteins (Stumpe and Grubmuller, 2007). The overall weakening of the protein-water interactions by urea may possibly render denaturation of bacterial membrane proteins by exposure of the hydrophobic core and dissociation of secondary structure elements of membrane proteins. The destabilization of the bacterial cell membrane was further propagated by inclusion of a strong anionic detergent such as SDS. As a consequence of exposure to urea and SDS, and the subsequent boiling step, the bacterial cell membrane was weakened considerably. The fragile cells were then readily lysed on exposure

to 0.2N NaOH resulting in effective release of DNA from the bacterial strain. The denatured proteins and other cellular debris were removed by centrifugation. The extracted DNA was then precipitated in presence of absolute alcohol. $A_{260/280}$ measurements for the extracted template DNA from select LAB were in the range of 1.87-1.92 indicating lack of protein contamination in the samples.

3.3.2. PCR-based detection of LAB and other bacterial strains

Template DNA was isolated from reference LAB strains using the urea-SDS-NaOH method. The PCR-compatibility of the extracted template DNA was assessed by performing a conventional PCR with genus-specific primers for *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*. Figure 3.2 depicts the amplicons obtained from PCR reactions. It is evident that distinct amplicons were obtained from the LAB strains.

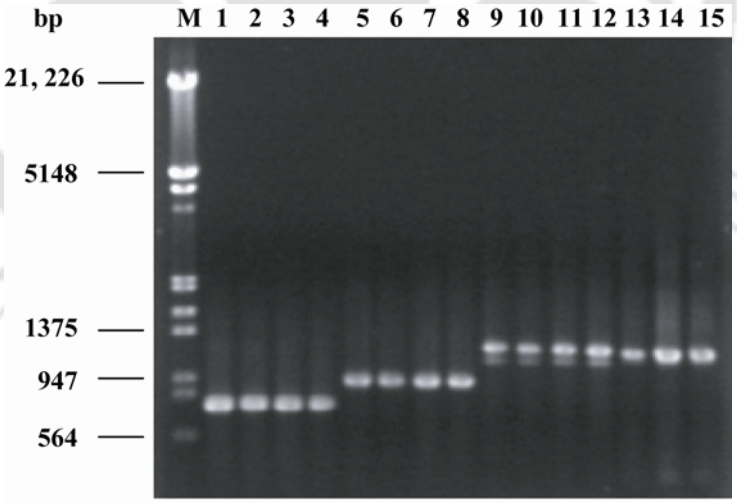

Figure 3.2 Agarose gel electrophoresis of PCR amplicons obtained with genus-specific primers targeting 16S rRNA gene of LAB strains. Lanes M: λ DNA *Eco*RI/*Hin*dIII double digest size marker; 1: *Lactobacillus acidophilus* MTCC 447; 2: *Lactobacillus casei* NCIM 2151; 3: *Lactobacillus helveticus* NCIM 2126; 4: *Lactobacillus gasseri* NRRL B4240; 5, 6 and 7: *Lactococcus lactis* subsp. *lactis* MTCC 440, MTCC 3041 and MTCC 3038; 8: *Lactococcus lactis* subsp. *chacetylactis* MTCC 3042; 9, 10 and 11: *Pediococcus acidilactici* CFR K7, NRRL B1153 and NRRL B14958; 12: *Pediococcus pentosaceus* NCIM 2295; 13: *Leuconostoc oenos* NCIM 2219; 14: *Leuconostoc mesenteroides* NRRL B640; 15: *Leuconostoc mesenteroides* subsp. *mesenteroides* MTCC 107.

The size of the amplicons were around 800, 960, 1200 and 1100 bp, which coincided with the expected amplicon size of the LAB strains as reported in Chapter 2. A positive PCR reaction demonstrated that the urea-SDS-NaOH method was effective in generating PCR-compatible templates from LAB strains. Previous reports on PCR based LAB detection describe the application of specialized resins to extract bacterial DNA such as chelex resin (Giraffa *et al.,* 2000) or the use of strong detergents such as CTAB (Dubernet *et al.,* 2002; Elegado *et al.,* 2004), zirconium beads (Delbes *et al.,* 2007) and cell lytic enzymes such as lysozyme (Chagnaud *et al.,* 2001; Dubernet *et al.,* 2002). The use of nuclease inhibitors like proteinase K and deproteinizing agents such as phenol-chloroform have also been reported (Dubernet *et al.,* 2002; Sharma and Singh, 2005). In contrast, the DNA extraction method from LAB described in this investigation is relatively simple and avoids the use of strong organic solvents such as phenol-chloroform. Organic solvents are potential inhibitors of the PCR reaction and even trace amounts are sufficient to completely preclude the amplification process. In the present method, this possibility was eliminated by avoiding the use of organic solvents. Additionally, a distinct advantage of this method is that the reagents used are readily available and inexpensive. Further, the method is quite rapid considering the relatively short time taken to prepare the template DNA. In the present study, the observation that amplification could be achieved from DNA templates prepared from LAB strains belonging to various genera indicated that the DNA isolation method was not hampered by the choice of the strains.

The versatility of the template DNA preparation method was substantiated when positive amplification was obtained from selected LAB and non-LAB bacterial strains with

universal primers for 16S rRNA gene. Amplicons of expected size (~1485 bp) were obtained from various LAB as well as non-LAB strains (Figure 3.3).

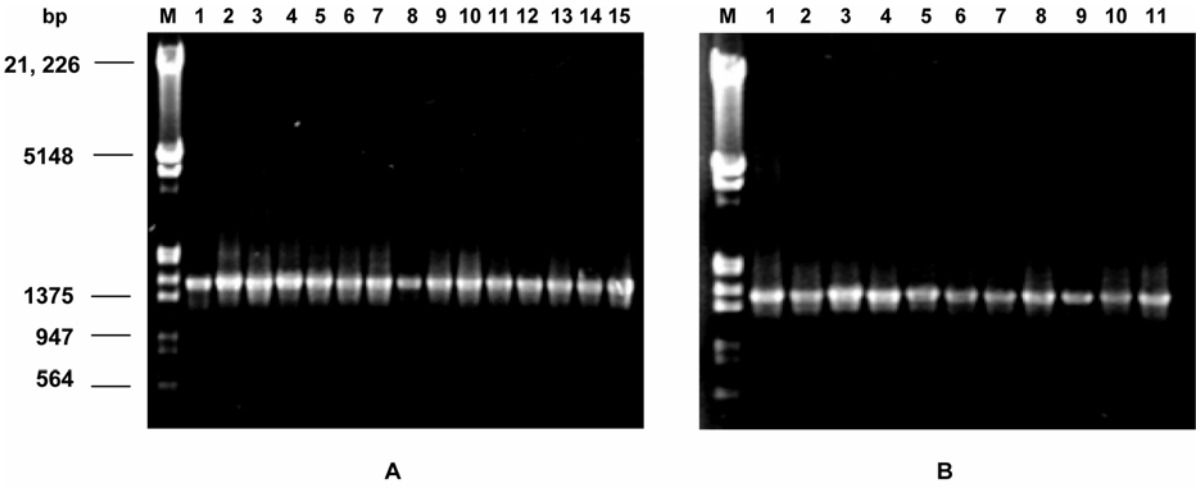

Figure 3.3 Amplicons obtained from LAB and non-LAB strains using universal primers for 16S rRNA gene. Lane M: λ DNA EcoRI/HindIII double digest size marker. **3A** LAB strains, Lane 1: *Lactobacillus gasseri* NRRL B4240; 2: *Lactobacillus plantarum* MTCC 1325; 3: *Lactobacillus delbrueckii* subsp. *delbrueckii* NCIM 2025; 4: *Lactobacillus brevis* NRRL B4527; 5, 6 and 7: *Lactococcus lactis* subsp. *lactis* MTCC 3041, MTCC 3038 and MTCC 440; 8: *Lactococcus lactis* subsp. *chacetylactis* MTCC 3042; 9, 10 and 11: *Pediococcus acidilactici* CFR K7, NRRL B14009 and NRRL B14958; 12: *Pediococcus pentosaceus* NCIM 2295; 13: *Leuconostoc mesenteroides* subsp. *mesenteroides* MTCC 107; 14: *Leuconostoc mesenteroides* NRRL B640; 15: *Leuconostoc oenos* NCIM 2219. **3B** Non-LAB strains, Lane 1: *Staphylococcus aureus* MTCC 96; 2: *Enterobacter aerogenes* MTCC 2822; 3: *Listeria monocytogenes* Scott A; 4: *Enterococcus faecalis* MTCC 439; 5: *Micrococcus luteus* NCIM 2704; 6: *Bacillus cereus* MTCC 1305; 7: *Bacillus subtilis* MTCC 441; 8 and 9: *Escherichia coli* MTCC 118 and MTCC 443; 10: *Yersinia enterocolitica* MTCC 859; 11: *Pseudomonas aeruginosa* MTCC 2488.

It may be mentioned here that the selection of these bacterial strains encompassed a combination of Gram-positive and Gram-negative bacteria, with evidently varying cell wall composition. The rationale of the selection of non-LAB strains was to demonstrate the potential of the DNA extraction method in providing PCR-compatible templates from these strains. Foodborne pathogenic bacterial strains such as *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis,* and *Yersinia enterocolitica* are often present in fermented food products and hence need to be detected to ensure food safety and

quality control. In this context, the present method of DNA extraction is indeed handy in readily providing PCR-compatible templates and is thus a promising tool in molecular detection of LAB as well as foodborne pathogenic strains. Interestingly, PCR-compatible template DNA could be readily extracted even from bacterial strains such as *Staphylococcus aureus* MTCC 96, which is known to possess a recalcitrant cell wall and thus cell lytic enzymes such as lysostaphin are often used in the DNA isolation protocol (Hein *et al.,* 2005; Padmapriya *et al.,* 2003).

3.3.3. Sensitivity of LAB detection

Template DNA extracted by urea-SDS-NaOH method from varying cell number of LAB strains belonging to genus *Lactobacillus*, *Pediococcus*, *Lactococcus* and *Leuconostoc* was used in PCR to establish the sensitivity of detection. A representative agarose gel picture shown in Figure 3.4A depicts the amplicons obtained from 10^6 - 10^1 cfu/ml of *Lactobacillus casei* subsp. *casei* NRRL B1922 and *Pediococcus acidilactici* NRRL B14009. It is quite evident from the Figure that there was a decrease in the amplicon intensity which was proportional to the cell numbers. Quantitative analysis of the amplicons in Figure 3.4B reflects higher band intensity for *Lactobacillus casei* subsp. *casei* NRRL B1922 in comparison to *Pediococcus acidilactici* NRRL B14009. The limit of detection for all the LAB strains tested was 10^2 cfu/ml. The achieved level of detection has significant implications for routine PCR-based detection of LAB in fermented food samples.

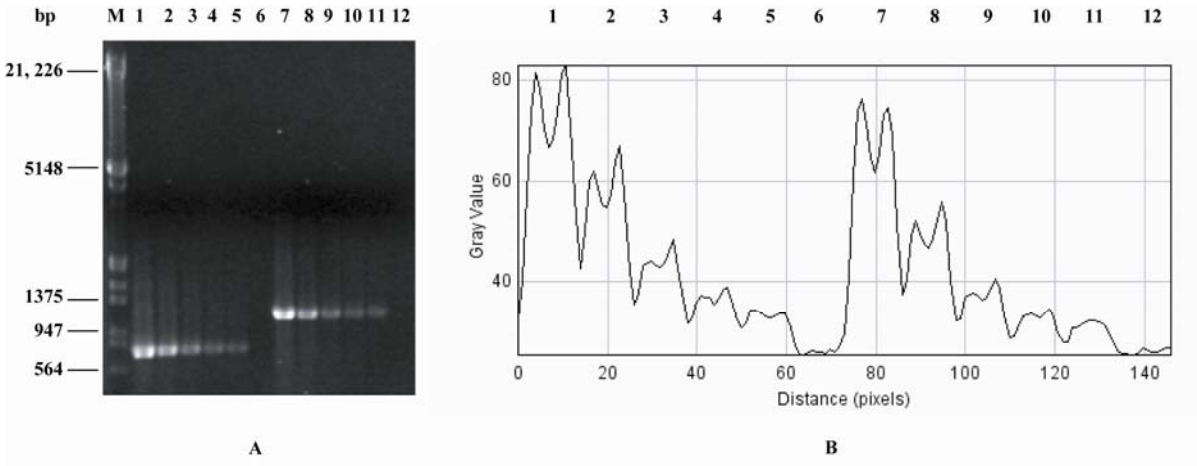

Figure 3.4 Sensitivity of PCR-based detection of LAB **(A)** Amplicons obtained from *Lactobacillus casei* subsp. *casei* NRRL B1922 (Lanes 1-6: 10^6-10^1 cfu/ml) and *Pediococcus acidilactici* NRRL B14009 (Lanes 7-12: 10^6-10^1 cfu/ml). **(B)** Quantification of amplicons with NIH ImageJ software.

3.3.4. Fingerprinting profiles of Lactobacillus strains

Template DNA was extracted from 25 reference strains of *Lactobacillus* using the urea-SDS-NaOH method and subjected to rep-PCR with primers for BOX elements. The fingerprinting profiles of LAB strains consisted of approximately 3-7 DNA bands as visualized in Figure 3.5. For example, the number of prominent bands obtained was 3 in case of *L. plantarum* MTCC 1325, *L. brevis* NCIM 2090, *and L. acidophilus* MTCC 447 and *L. helveticus* NCIM 2126. However, for some strains such as *L. sakei* NRRL B1917 and *L. lactis* NCIM 2368 the number of bands obtained was 5 and 7, respectively. The banding pattern also revealed that the approximate size of the obtained fragments were in the range of 0.6-5.0 kb. It is also evident from Figure 3.5 that discrete clusters based on Dice coefficient values could be obtained for various standard *Lactobacillus* strains. At around 0.77 Dice coefficient value, various species of *Lactobacillus* could be discriminated. The similarity index for strains of *L. gasseri* (NRRL B14168 and NRRL B4240) was considerably high based on Dice coefficient values (0.85) whereas for strains of *L. plantarum* (MTCC 1746, NCIM 2592, MTCC 1325

and NCIM 2083), Dice coefficient values were even greater (0.92). Consequently, they were clustered in close vicinity. rep-PCR based fingerprinting of LAB strains using BOXA1R primer has been reported earlier (De Angelis *et al.,* 2007; Terzic-Vidojevic *et al.,* 2007).

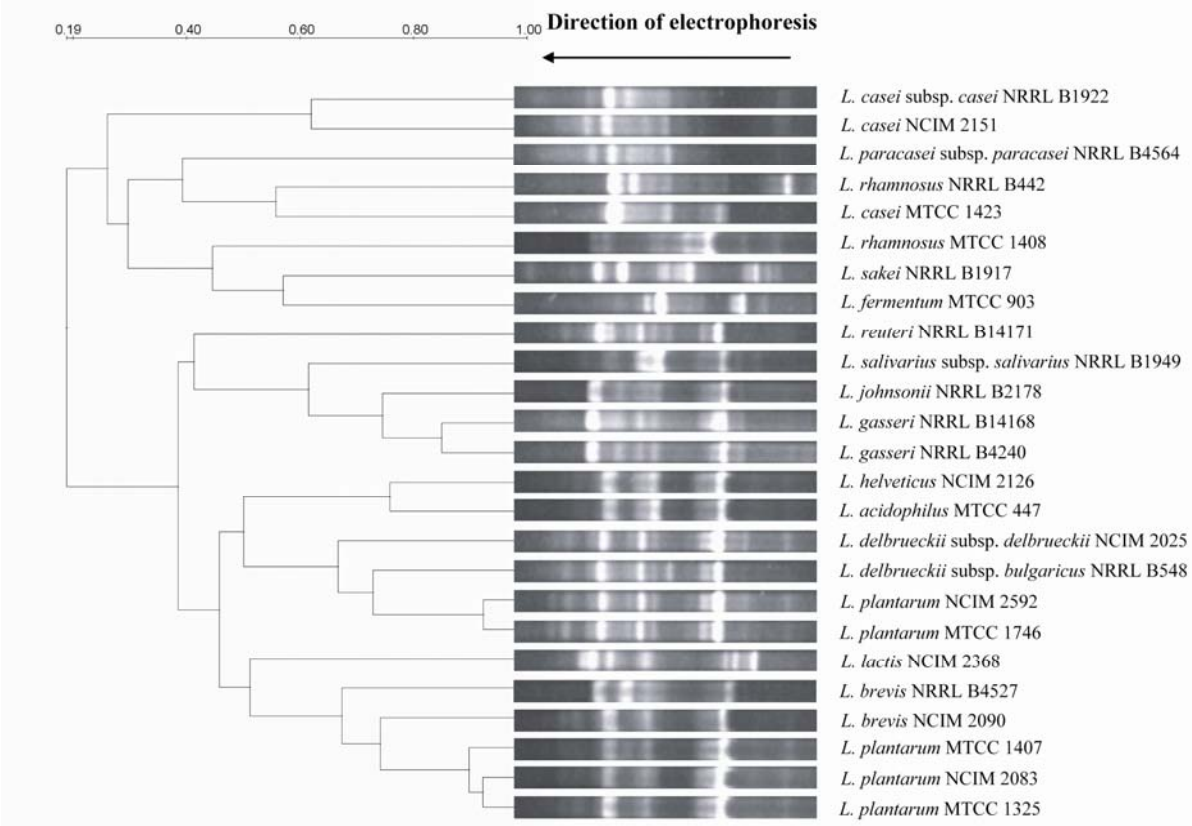

Figure 3.5 UPGAMA based cluster analysis for standard strains of *Lactobacillus* on the basis of Dice coefficient values of banding pattern generated in rep-PCR with BOXA1R element based primer.

In the present investigation, the overall clustering of reference strains of *Lactobacillus* is in conformity with the expected pattern based on genetic relatedness amongst the various species of *Lactobacillus*. For instance, it is known that the *L. casei* group comprises of strains belonging to *L. casei*, *L. paracasei* and *L. rhamnosus* (Song *et al.,* 2000; Ryu *et al.,* 2001). As evident from Figure 3.5, *L. casei* NRRL B1922 and NCIM 2151 share a reasonably high similarity (Dice coefficient value of 0.62), whereas *L. rhamnosus* NRRL B442 and *L. casei* MTCC 1423 exhibit a Dice coefficient value of 0.56 and are clustered in close proximity.

Further, *L. johnsonii* NRRL B2178 revealed a high Dice coefficient value of 0.75 with strains of *L. gasseri* and clustered in close vicinity. It can be mentioned that *L. johnsonii* and *L. gasseri* are genetically close and known to belong to the *L. acidophilus* group (Ryu *et al.,* 2001). Similarly, strains of *L. brevis* were clustered along with strains of *L. plantarum* which is agreement with the taxonomic proximity of these strains on the basis of their genetic relatedness (Makarova and Koonin, 2007). The unusual grouping observed in some cases wherein strains belonging to a given species did not cluster together (for example some strains of *L. plantarum*) indicates heterogeneity amongst these strains. Similar findings have also been reported earlier (De Angelis *et al.,* 2001).

The reproducibility of the banding pattern obtained with rep-PCR using BOXA1R primer was also assessed for select *Lactobacillus* strains. Triplicate samples of template DNA isolated separately from each LAB strain was subjected to rep-PCR analysis. There was no qualitative difference in banding patterns and the subsequent cluster analysis was not affected (gel picture not shown). Thus the present method of DNA extraction was consistent in generating PCR-compatible template that facilitated reproducible molecular typing analysis of LAB.

3.3.5. Comparative analysis of PCR-based detection and molecular fingerprinting of LAB with extracted DNA templates.

The PCR compatibility of the template DNA prepared by the urea-SDS-NaOH method was compared with template DNA extracted using a commercially available bacterial genomic DNA isolation kit. Template DNA prepared from select standard strains of *Lactobacillus* and *Pediococcus* using the urea-SDS-NaOH method as well as the kit were subjected to PCR in

conjunction with universal primers for bacterial 16S rRNA gene. Amplicons of equivalent size (~1485 bp) and comparable intensity were obtained when template DNA prepared by the urea-SDS-NaOH method and the kit were used in PCR (Figure 3.6). These results indicated that the quality of template DNA prepared by urea-SDS-NaOH method was comparable to that obtained using the kit. This renders the present DNA extraction method potentially applicable for routine PCR-based screening of LAB isolates circumventing the need of expensive kit-based DNA isolation.

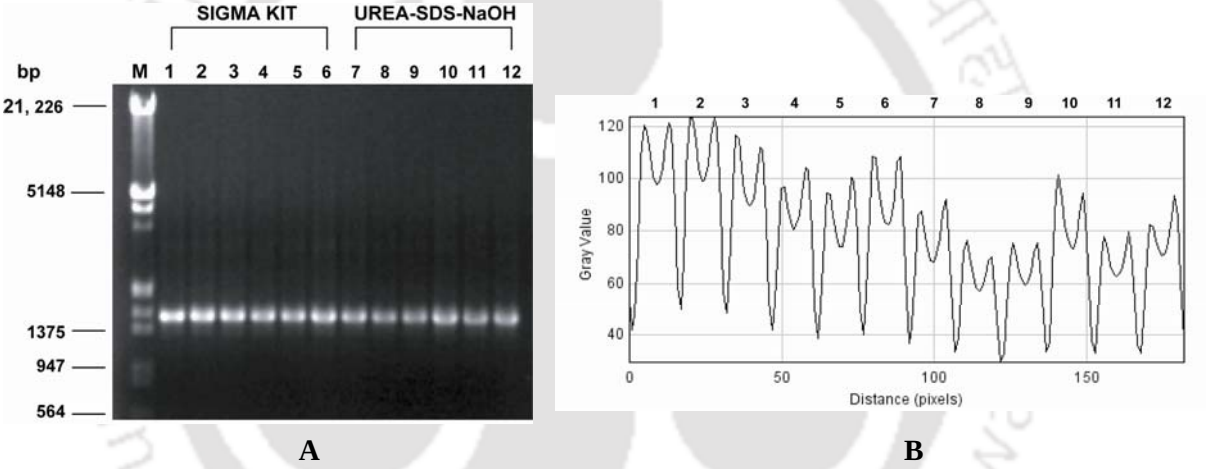

Figure 3.6 (A) PCR amplicons of select LAB strains obtained with universal primer for 16S rRNA gene using template DNA extracted by GenElute bacterial genomic DNA isolation kit (Sigma, USA) and urea-SDS-NaOH method. Lane M: λ DNA *Eco*RI/*Hin*dIII double digest size marker; 1 and 7: *Lactobacillus plantarum* MTCC 1325; 2 and 8: *Lactobacillus acidophilus* MTCC 447; 3 and 9: *Lactobacillus brevis* NRRL B4527; 4 and 10: *Pediococcus acidilactici* CFR K7; 5 and 11: *Pediococcus acidilactici* NRRL B14009; 6 and 12: *Pediococcus pentosaceus* NCIM 2295. **(B)** Quantification of band intensity with NIH ImageJ software.

Table 3.3

Dice coefficient based similarity analysis of fingerprint profiles[a] from DNA templates of LAB strains.

LAB strain	No. of bands from template A[b]	No. of bands from template B[c]	Dice coefficient value[d]
Leuconostoc oenos NCIM 2219	6.0	5.0	81.8
Leuconostoc mesenteroides NRRL B640	4.0	4.0	95.0
Lactobacillus casei subsp. *casei* NRRL B1922	5.0	5.0	94.0
Lactobacillus gasseri NRRL B4240	7.0	7.0	96.5
Lactobacillus helveticus NCIM 2126	6.0	7.0	88.4
Lactobacillus plantarum MTCC 1325	7.0	7.0	85.2
Lactobacillus reuteri NRRL B14171	6.0	5.0	85.7
Lactobacillus salivarius subsp. *salivarius* NRRL B1949	4.0	4.0	90.2
Lactococcus lactis subsp. *chacetylactis* MTCC 3042	5.0	5.0	96.7
Pediococcus acidilactici CFR K7	4.0	4.0	92.3
Pediococcus acidilactici NRRL B14958	3.0	3.0	79.4

[a] Fingerprinting profile obtained by rep-PCR with BOXA1R primer.
[b] Template A prepared by GenElute bacterial genomic DNA isolation kit (Sigma, USA).
[c] Template B prepared by urea-SDS-NaOH method.
[d] Dice coefficient values for matched banding patterns obtained with template A and B from LAB strain.

Template DNA extraction from various LAB strains was also accomplished using the proposed method and a bacterial genomic DNA isolation kit as mentioned before. rep-PCR fingerprinting analysis with BOXA1R primer was performed to compare the banding patterns produced from the templates and ascertain the authenticity of fingerprinting profiles. DNA fingerprints obtained from select LAB strains were compared based on Dice coefficient values. It is quite clear from Table 3.3 that nine standard LAB strains out of a total of eleven clustered together with high Dice coefficient values (>85.0). The highest Dice coefficient value of 96.7 was obtained for *L. lactis* subsp. *chacetylactis* MTCC 3042 followed by *L. gasseri* NRRL B4240 for which the Dice coefficient value was 96.5. For some strains such as *L. oenos* NCIM 2219, *L. helveticus* NCIM 2126 and *L. reuteri* NRRL B14171, the number of bands obtained from the two templates differed. However, the fingerprinting profiles for these strains were still comparable as evident from Dice coefficient values which were considerably high in the range of 81.8-88.4, whereas *P. acidilactici* NRRL B14958 revealed lowest Dice coefficient value of 79.4. Overall, the results depicted in Table 3.3 reflect significantly high Dice coefficient values and thus strengthens the possibility of using the urea-SDS-NaOH method of template DNA preparation for consistent and reliable PCR-based fingerprinting of LAB.

3.3.6. Application potential of urea-SDS-NaOH template DNA extraction method

(A) Direct detection of LAB in fermented samples

Based on the results obtained for pure cultures of various standard LAB strains the next aim was to apply the DNA extraction method to fermented samples and ascertain the applicability

of the method in facilitating PCR-based direct detection of LAB in such samples. Template DNA was extracted from *dahi, idli* batter and salt-fermented cucumber samples as mentioned in section 3.2.6. A representative agarose gel picture which shows the amplicons obtained in PCR using template DNA extracted from the fermented samples is shown in Figure 3.7. It is evident that *Lactobacillus* and *Lactococcus* could be detected in *dahi* samples whereas in *idli* batter *Lactobacillus, Pediococcus* and *Leuconostoc* could be detected. *Dahi* is an indigenous lactic cultured milk product and the presence of *Lactobacillus* and *Lactococcus* has been reported earlier in *dahi* samples (Varadaraj *et al.,* 1993). The prevalence of *Lactobacillus, Pediococcus* and *Leuconostoc* in *idli* batter is in agreement with the inherent LAB profile reported earlier for such samples (Blandino *et al.,* 2003).

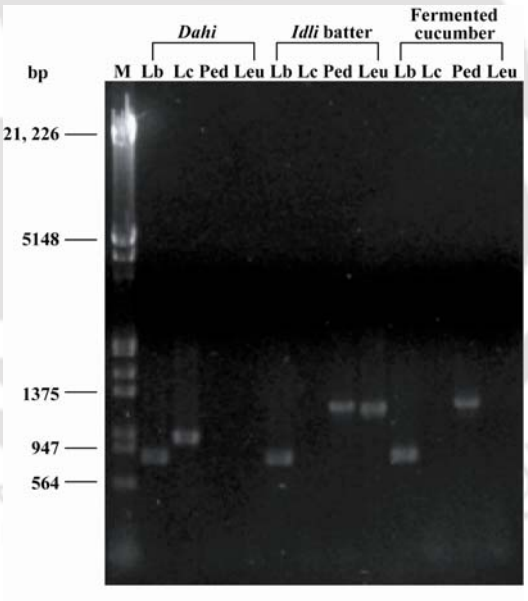

Figure 3.7 Agarose gel electrophoresis of amplicons obtained using template DNA extracted directly from fermented samples by urea-SDS-NaOH method. *Lactobacillus* (Lb), *Lactococcus* (Lc), *Pediococcus* (Ped) and *Leuconostoc* (Leu) genus-specific primers for 16S rRNA gene were used in PCR. Lane M indicates λ DNA *Eco*RI/*Hin*dIII double digest size marker.

In case of fermented cucumber samples, direct DNA extraction by the urea-SDS-NaOH method followed by PCR enabled the detection of *Lactobacillus* and *Pediococcus*. This finding corresponds to earlier results outlined in Chapter 2 wherein PCR-based detection of *Lactobacillus* and *Pediococcus* was demonstrated for samples of salt-fermented cucumber. Similar trend of LAB genus detection was observed for all five samples of *dahi*, *idli* batter and fermented cucumber. The level of detection in the fermented samples corresponded to a cell number which is either equivalent or greater than the limit of detection (10^2 cfu/ml), established in an earlier experiment (Figure 3.4). This level of detection is comparable with an earlier investigation wherein LAB could be quantified by PCR in fermented milk product (Furet *et al.,* 2004). The absence of certain LAB genus in the fermented samples could be attributed to either very low initial load of the cells or an overwhelming presence of competing DNA obtained from bacterial cells of other genus. Sequestration of bacterial cells from food matrix is a crucial prerequisite that can influence the outcome of PCR-based culture-independent detection. In the present study, a short pre-treatment of the fermented samples with PBS-T (PBS and Tween-20) was included. It is plausible that the presence of Tween-20 promotes efficient cell separation from the food matrix. PCR results obtained in the present experiments also point out that the template DNA extracted directly from fermented samples was either free from PCR inhibitors or the concentration of inhibitors if any, was lower than the levels which can inhibit the amplification reaction. Significantly, the template DNA prepared from fermented samples was also amicable for multiple LAB genus detection in PCR, emphasizing the analytical value of the urea-SDS-NaOH method for multiplex LAB detection in food samples.

Further, it was important to investigate whether the PCR results obtained with template DNA isolated by the urea-SDS-NaOH method was comparable to the results obtained when alternate DNA isolation protocols were adopted. Suspensions of food samples (*dahi*, *idli* batter and cucumber) were prepared in PBS-T as mentioned before and DNA was extracted from the samples using the methods reported by Chagnaud *et al.,* (2001) and Dubernet *et al.,* (2002). PCR with these templates failed to yield any amplicons with genus-specific primers.

(B) Molecular fingerprinting of bacteriocin producing (Bac$^+$) LAB obtained from fermented cucumber samples

It is evident from the results obtained in the previous section that the application potential of the proposed template DNA extraction method was validated by PCR-based detection of multiple LAB strains in liquid broth and fermented samples. Further, the same templates could also generate reliable fingerprinting profiles for LAB strains. As an extension of this application, the next important aim was to assess the utility of the DNA extraction method in generating PCR-compatible templates from select bacteriocin producing (Bac$^+$) LAB strains and determine their genetic diversity by rep-PCR. This study assumes significance in the context of assigning taxonomic affiliation and identifying signature patterns for various native Bac$^+$ LAB strains.

The antagonistic LAB strains were previously isolated from salt-fermented cucumber and identified as *Lactobacillus* sp. and *Pediococcus* sp. by PCR as reported in Chapter 2. DNA was extracted from 29 Bac$^+$ isolates as well as reference LAB strains of *Lactobacillus* and *Pediococcus* genus by urea-SDS-NaOH method. PCR amplification was carried out with BOXA1R primer. The DNA fingerprints of native Bac$^+$ isolates were compared with the

reference strains to calculate the Dice coefficient values and generate the clusters (Figure 3.8). It is evident that the bacteriocin producing LAB strains yielded profiles which resulted in the generation of discrete clusters.

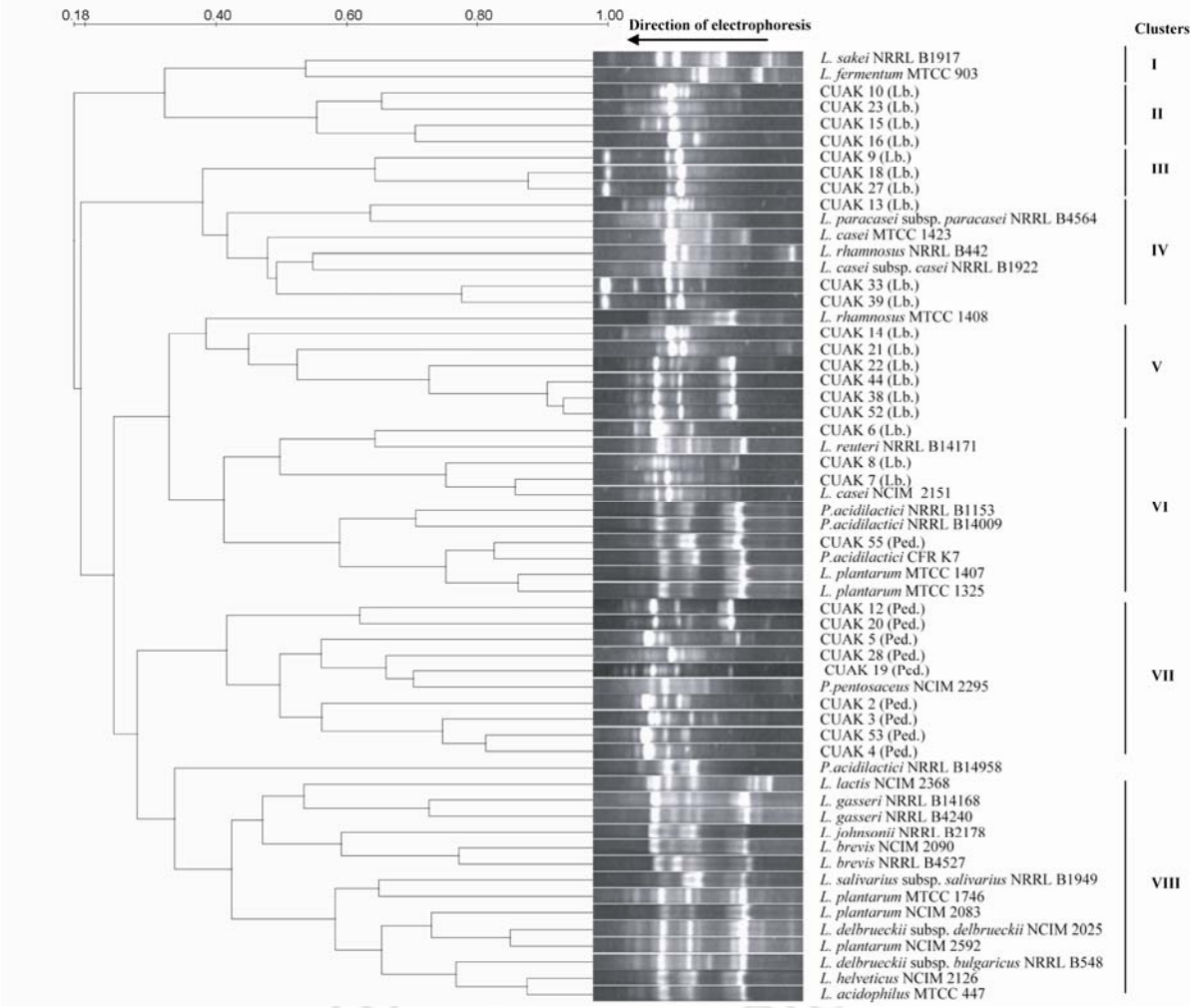

Figure 3.8 UPGAMA based cluster analysis of bacteriocin producing (Bac^+) *Lactobacillus* sp. and *Pediococcus* sp. isolates obtained from salt-fermented cucumber. Clusters were generated on the basis of Dice coefficient values of banding pattern obtained in rep-PCR with BOXA1R element based primer. Genus identity of Bac^+ native LAB isolates is indicated in parenthesis (*Lactobacillus*-Lb; *Pediococcus*- Ped).

The number of bands obtained from the Bac^+ LAB isolates and standard LAB strains varied from 3-7. At 0.40 Dice coefficient value eight distinct clusters could be delineated. The

native antagonistic isolates obtained from fermented cucumber were distributed in cluster II-VII. Cluster II, III and V solely consisted of Bac^+ isolates which were identified as *Lactobacillus* sp. using genus-specific primers for 16S rRNA gene as reported in Chapter 2. Cluster IV consisted of 7 LAB strains of which 3 were Bac^+ isolates. These isolates clustered with standard *Lactobacillus* strains (Figure 3.8). In this cluster isolate CUAK 13 revealed high similarity with *L. paracasei* subsp. *paracasei* NRRL B4564 (Dice coefficient value of 0.64). Cluster VI consisted of 11 LAB strains of which 4 included Bac^+ isolates (CUAK 6, 8, 7 and 55). It can be observed from Figure 3.8 that isolate CUAK 6 revealed high similarity with *L. reuteri* NRRL B14171 (Dice coefficient value of 0.64) whereas isolate CUAK 55 clustered with *P. acidilactici* CFR K7 (Dice coefficient value of 0.83). Bac^+ LAB strains which formed cluster VII consisted of *Pediococcus* sp. (9 isolates). One of the isolates CUAK 19 revealed high similarity with *P. pentosaceus* NCIM 2295 (Dice coefficient value of 0.70). Cluster VIII consisted of 14 *Lactobacillus* standard LAB strains belonging to the *Lactobacillus acidophilus* group. The clusters observed for standard LAB strains did not exactly match with the earlier profile shown in Figure 3.5. The plausible explanation for this deviation is that a greater genotypic discrimination is likely to be observed due to higher number of strains used for profile comparison. This observation has also been reported earlier (De Angelis *et al.,* 2001). The application of BOXA1R primers in rep-PCR for typing of LAB strains has been demonstrated earlier in case of differentiation of *L. sanfranciscensis* isolates (De Angelis *et al.,* 2007) and for molecular typing of probiotic *L. rhamnosus* 35 (Coudeyras *et al.,* 2008). The overall clustering observed for the native Bac^+ LAB isolates in the present

investigation conformed to their expected taxonomic units. Thus the utility of the urea-SDS-NaOH template DNA extraction method in estimating genetic diversity of native Bac^+ strains was clearly demonstrated as adequate discriminating fingerprinting patterns were obtained for the strains in rep-PCR.

3.4. Conclusion

The investigation outlined in this chapter clearly demonstrated the utility of the urea-SDS-NaOH DNA extraction method in generating PCR-compatible templates and augment PCR-based detection and fingerprinting of LAB strains. The salient achievements of the investigation are as follows:

1. In the present investigation, a simple and effective method of generating PCR-compatible template DNA from various LAB as well as other bacterial strains was developed. The method is rapid, cost-effective and obviates the need of commonly used cell lytic enzymes and strong organic solvents.

2. The reliability of the DNA extraction method was validated as templates prepared from LAB strains by urea-SDS-NaOH method and a commercially available kit yielded comparable results in conventional PCR as well as rep-PCR based fingerprinting analysis.

3. A major highlight of the investigation was the successful implementation of the template DNA extraction method in facilitating PCR-based direct detection of LAB in fermented samples.

4. Another important application of the template DNA extraction method was emphasized when PCR-compatible DNA was extracted from bacteriocin producing native LAB strains their genetic diversity was assessed by rep-PCR.

5. The DNA extraction method outlined in the present investigation can be adopted on a routine basis in food microbiology laboratories for PCR-based detection of LAB in various indigenous fermented foods as well as in studies that aim to establish the genetic diversity of native LAB strains by PCR-based fingerprinting.

To continue the work on bacteriocin producing LAB, the next aim was to explore the possibility of screening indigenous fermented samples for the presence of anti-listerial bacteriocin producing LAB. *Listeria monocytogenes* is a psychrotrophic foodborne pathogen and is known to be resistant to acid stress, a condition which is commonly encountered in fermented samples. Literature reports suggest strong anti-listerial activity of ClassIIa bacteriocins produced by LAB. In the next chapter, isolation of anti-listerial bacteriocin producing LAB from indigenous samples, partial characterization of the bacteriocins, assessment of their potency and food application potential is described.

CHAPTER 4a

Isolation, molecular detection and characterization of anti-listerial bacteriocin producing lactic acid bacteria from indigenous source

ABSTRACT

The aim of the research work carried out in this part of thesis was to isolate anti-listerial bacteriocin producing lactic acid bacteria from indigenous samples such as *dahi*, dried fish and salt-fermented cucumber and ascertain their potency. A total of 231 LAB isolates were obtained from the samples of which 51 isolates displayed anti-listerial activity. The anti-listerial LAB were identified by PCR as *Lactobacillus* sp., *Pediococcus* sp. and *Lactococcus* sp. PCR also enabled the detection of Class IIa bacteriocin encoding genes such as enterocin, pediocin and plantaricin A in some of the LAB isolates. The culture filtrate from anti-listerial LAB isolates demonstrated antimicrobial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli* and *Enterobacter aerogenes*. Partial characterization of the inhibitory substance and partial gene sequence analysis confirmed bacteriocin production by the LAB isolates. Anti-listerial activity of the bacteriocin produced by select LAB isolates was clearly revealed by growth inhibition studies, fluorescent dye-leakage assay and epifluorescence microscopy. The food application potential of plantaricin A produced by a native isolate *Lactobacillus* sp. CRA52 was evidenced as the bacteriocin suppressed the growth of *Listeria monocytogenes* Scott A inoculated in paneer samples stored at 8^{o}C for 5 days.

4a.1. Introduction

Lactic acid bacteria (LAB) play a critical role in food processing and spontaneous fermentation, and are used in a wide range of fermented food. LAB contribute significantly to flavor, aroma, and texture development (Coolbear *et al.,* 2008; Leroy and De Vuyst, 2004), have tremendous potential in improving the shelf-life of food products and ensure food safety by producing bacteriocins (Castelano *et al.,* 2008; Jones *et al.,* 2008; Settani and Corsetti, 2008). Bacteriocins are essentially ribosomally synthesized antimicrobial peptides which are considered to be safe natural biopreservatives, and are effective in controlling foodborne pathogens (Cleveland *et al.,* 2001). Amongst the foodborne pathogens, *Listeria monocytogenes* has been a serious cause of concern as it is ubiquitous and can contaminate food at pre-and post-harvest stages of production. Food safety issues are compounded as *Listeria* is psychrotrophic and tolerant to stresses caused by low pH and high acid content (Le Marc *et al.,* 2002). The use of bacteriocin producing (Bac^{+}) LAB to combat *Listeria* is especially attractive as reports suggest that bacteriocins from LAB such as nisin and pediocin-like Class IIa bacteriocins exhibit significant anti-listerial activity (Drider *et al.,* 2006; Montville and Chen, 1998). A large number of reports have demonstrated the application potential of anti-listerial LAB or the bacteriocin produced by such strains to inhibit the growth of *Listeria* in fermented food samples (Albano *et al.,* 2009; Allende, *et al.,* 2007; Jagannath *et al.,* 2001).

In recent years, research efforts have been focused towards isolation of anti-listerial LAB from various food samples including traditionally fermented food (Albano *et al.,* 2007; Belgacem *et al.,* 2008). It may be envisaged that indigenous fermented food are likely to constitute a unique ecological niche to screen bacteriocin producing LAB strains. This

formed the basis of the present investigation wherein anti-listerial bacteriocin producing LAB was isolated from samples of *dahi*, dried fish and fermented cucumber. Characterization and comparative analysis of the potency of bacteriocin produced by select LAB isolates are reported. The efficacy of an anti-listerial bacteriocin to inhibit the growth of *Listeria monocytogenes* in paneer (soft cheese) samples which is a widely consumed perishable dairy product in India is demonstrated.

4a.2. Materials and Methods

4a.2.1. Bacterial strain maintenance and growth conditions

All LAB and non-LAB strains were propagated and maintained under the conditions mentioned in section 2.2.1 of Chapter 2 and section 3.2.1 of Chapter 3.

4a.2.2. Isolation of anti-listerial LAB

Anti-listerial LAB were isolated from *dahi* (a lactic cultured milk product obtained from domestic source), dried fish and salt-fermented cucumber. Dried fish sample consisted of freshwater fish *Corica soborna* also known as the Ganges river sprat. The commercial designation of the fish is 'Keski' and it is sold as sun-dried whole fish. A total of 15 samples were analyzed from each source. *Dahi* and dried fish samples were added to MRS broth at 5% (w/v) level and enriched for 48 h at 37^{o}C. The enriched samples were pour plated on MRS agar medium at various dilutions and incubated at 37°C for 24-48 h to obtain presumptive LAB colonies. In case of salt-fermented cucumber samples enriched in MRS, isolation of presumptive LAB was performed as described in section 2.2.2 of Chapter 2. All putative LAB colonies were subjected to microscopic observation to determine cell morphology and

Gram-staining. LAB isolates identified as Gram-positive rods or cocci were further tested for catalase activity. Anti-listerial LAB isolates were identified by a colony overlay assay, wherein the LAB colonies were overlaid with BHI-soft agar medium (0.85% agar) seeded with *Listeria monocytogenes* Scott A and observed for zone of inhibition around the colonies.

4a.2.3. PCR-based genus and bacteriocin gene identification

DNA was isolated from the anti-listerial LAB isolates using the urea-SDS-NaOH method as described in Figure 3.1 of chapter 3. Genus identification of anti-listerial LAB isolates and the presence of bacteriocin structural gene were ascertained by PCR. Amplification conditions, sequence of genus-specific primers (*Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*), bacteriocin gene specific primers (plantaricin A, pediocin, enterocin A and nisin) and analysis of PCR products by agarose gel electrophoresis were as reported in the chapter 2 section 2.2.4.

4a.2.4. Nucleic acid sequence of bacteriocin gene

Bacteriocin gene amplicons from isolate DF14, CRA21 and CRA51 were sequenced in the National Facility for Automated DNA Sequencing, Department of Biochemistry, University of Delhi, South Campus (UDSC). Homology search for the sequences was performed by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The accession numbers for the bacteriocin gene sequences from isolate DF14, CRA21 and CRA51 were FJ424062, FJ424063 and EU616745, respectively.

4a.2.5. Antimicrobial spectrum

LAB isolates exhibiting a zone of inhibition of ≥10 mm against *L. monocytogenes* Scott A in the colony overlay assay were selected to determine their antimicrobial spectrum. The isolates (27 nos.) were grown in MRS broth at 37°C for 18 h and the culture filtrate was recovered by centrifugation at 8832 x g for 10 min at 4°C. The pH of the culture filtrate was adjusted to 7.0 with 2N NaOH followed by filter sterilization using a 0.2 μm membrane filter (Millipore, India). The resulting solution was referred to as bacteriocin-like inhibitory substance (BLIS). Antimicrobial activity of BLIS against pathogenic bacterial strains was assessed by agar well diffusion assay (section 2.2.9 of Chapter 2) in three independent experiments. Activity against each indicator strain was compared by analysis of variance (ANOVA) test. Instat Plus version 3.36 (http://www.reading.ac.uk/ssc/software /instat/instat.html) was used to perform ANOVA test.

4a.2.6. Preliminary phenotypic and biochemical tests for potent anti-listerial LAB

Potent anti-listerial LAB isolates were characterized by Gram-staining followed by microscopic examination and by conventional biochemical and physiological tests. The cultures were examined for CO_2 production from glucose, growth at different temperatures (10 and 45°C) and pH (4.4 and 9.6), as well as the ability to grow in MRS broth added with different concentrations of NaCl (6.5 and 18%). For CO_2 production from glucose, the anti-listerial LAB isolates were grown in MRS broth tube containing an inverted Durham tube. Following 24 h of growth in static condition at 37^{o}C, the tubes were observed for the presence gas bubble in the Durham tube as an indicator of CO_2 production from glucose. For catalase test a loopful of overnight grown culture in MRS broth was taken on a clean glass

slide, to which a drop of 3% H_2O_2 was added. The presence of effervescence (bubble formation) was recorded as catalase positive and absence as catalase-negative isolate.

4a.2.7. Partial characterization of BLIS

BLIS from select anti-listerial LAB isolates (10 nos.) was dialyzed extensively against de-ionized water using a 1.0 KDa cut-off dialysis bag (Sigma, USA), freeze dried in a lyophilizer (ALPHA 1-4 LD, CHRIST, Germany) and reconstituted in 10mM phosphate buffer (pH 7.0). An aliquot of the sample was treated with trypsin (1.0 mg/ml; Sigma-Aldrich, USA) and catalase (1.0 mg/ml; Sigma-Aldrich, USA) for 1 h at 37^{o}C in a circulating water bath incubator (Julabo F12-MP, Germany). Heat inactivated enzyme solutions alone were used as control to detect any inhibition caused by the inactive enzymes. In case of treated samples, the enzymes were heat-inactivated prior to testing bacteriocin activity. For testing the activity at different pH, aliquots of the dialyzed and lyophilized sample were adjusted to pH 3.5, 5.5 and 7.0. Buffer adjusted to the same pH served as control. The thermal stability of BLIS was tested by subjecting aliquots of the sample to 121°C for 20 min (autoclave) and 100 °C for 30 min. Culture filtrate from antagonistic LAB isolates was also subjected to extensive dialysis against de-ionized water using 1.0 kDa and 12.0 kDa dialysis bags (Sigma Aldrich, USA) and bacteriocin activity was measured in the dialysate. Bacteriocin activity in all the aforesaid treated samples was determined by an agar well diffusion assay using *Listeria monocytogenes* ScottA as target as mentioned in section 2.2.9 of Chapter 2.

4a.2.8. Determination of bacteriocin titre and activity by spot-on-lawn assay

Bacteriocin titre and activity of the dialyzed and lyophilized BLIS sample from select LAB isolates was also ascertained by spot-on-lawn assay as described by Pucci *et al.* (1988). Serial two fold dilutions of the reconstituted BLIS samples were made in 10 mM phosphate buffer, pH 7.0. A 5.0 µl aliquot of each dilution was spotted on BHI soft agar overlay seeded with 10^6 cells of freshly grown *Listeria monocytogenes* Scott A. The assay plates were incubated at 37°C for 24 h and observed for zone of inhibition. One arbitrary unit (AU) of bacteriocin was defined as the highest dilution yielding a definite zone of growth inhibition of 2 mm on the lawn of target strain. The titre was expressed as the reciprocal of the highest dilution showing growth inhibition. Bacteriocin activity was calculated as titre x dilution factor (1000 µl/ 5.0 µl = 200 on per ml basis). The bacteriocin activity was finally expressed as arbitrary units per ml (AU/ml).

4a.2.9. Studies on anti-listerial activity of bacteriocin

4a.2.9.1 Growth inhibition studies

A concentrated plantaricin A sample from isolate CRA52 (dialyzed and lyophilized culture filtrate) was reconstituted in 10mM phosphate buffer (pH 7.0). Bacteriocin activity in the reconstituted plantaricin A sample was ascertained against *L. monocytogenes* Scott A by a spot-on-lawn assay as described in section 4a.2.8. For the growth inhibition experiments, target cells of *L. monocytogenes* Scott A were inoculated into 25 ml of BHI broth and grown at 37°C in an incubator shaker. The reconstituted plantaricin A sample was added to the target cells in the order of 3200 AU/ml at different time periods of growth (4, 8, 12 h). The effect of

plantaricin A on *Listeria monocytogenes* Scott A was determined by observing viable cell count (log_{10} cfu/ml) on BHI-agar medium. Control sample consisted of target cell grown in the absence of bacteriocin.

4a.2.9.2. Fluorescence based dye leakage assay and microscopy

Listeria monocytogenes Scott A cells were labeled with fluorophore 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE), procured from Sigma-Aldrich, USA as per the method described by Lee *et al.* (2004). In short overnight grown cells of *L. monocytogenes* Scott A were harvested by centrifugation at 3,000 x g for 10 min. The cell pellet was washed twice in sterile phosphate buffer and labeled with cFDA-SE (final concentration of 50 µM) at 37°C for 20 min. The labeling reaction was terminated by pelleting and washing of bacterial cells twice with phosphate buffer to remove excess cFDA-SE molecules. Finally the labeled target cells were resuspended in 1.0 ml sterile phosphate buffer and 10^6 cfu/ml (or 6.0 log_{10} cfu/ml) of labeled cells were used to study the effect of bacteriocin. Dialyzed and lyophilized bacteriocin samples obtained from isolates CRA18, CRA52, CDRA57, CDRA60 and DF3 were added at varying concentrations (400, 800, 1600, 3200 and 6400 AU/ml) separately to 6.0 log_{10} cfu/ml cFDA-SE labeled *L. monocytogenes* cells resuspended in phosphate buffer and incubated at 37°C for 6 h in a water bath (Amersham, USA). A control sample without bacteriocin was incubated in phosphate buffer under the same conditions. Following 6 h of incubation, all the samples were centrifuged at 8832 x g for 5 min. Leakage of carboxyfluorescein from cells was determined by measuring fluorescence of the cell free supernatant (λ_{ex}488 nm and λ_{em}518 nm) in a fluorescence spectrophotometer (FluoroMax-3, HORIBA). The fluorescence of control samples was

subtracted from each measurement of treated samples and results were expressed as mean values of three independent readings. For fluorescence microscopic studies, cFDA-SE labeled *L. monocytogenes* cells (6.0 $\log_{10}$ cfu/ml) were treated with plantaricin A from isolate CRA52 (3200 AU/ml) for 6 h at 37°C. Control samples of labeled target cells alone were incubated in phosphate buffer. Treated and control target cells were fixed in 2.5% glutaraldehyde, washed twice with phosphate buffer and a 10 µl aliquot of the sample was spotted on a clean glass slide, air dried and observed under fluorescence microscope (Axioskop2MAT, Carl Zeiss, Oberkochen, Germany).

4a.2.9.3. Anti-listerial activity of plantaricin A in paneer samples

Paneer was chosen as a model food sample to study the effect of plantaricin A obtained from strain CRA52 on the growth of *Listeria monocytogenes* Scott A inoculated in the product. Paneer is an Indian heat-acid coagulated product of milk similar to tofu. Essentially, *chhana* is used as a base material for the production of paneer. For preparation of *chhana*, milk is heated to near boiling temperature, followed by cooling and coagulation by using 1-2% citric acid or sour whey. The resulting whey is drained off and the *channa* is pressed into blocks, which are then immersed in chilled water for 2-3 h to finally obtain the product.

The scheme of the experiment to study the effect of plantaricin A from isolate CRA52 on the growth of *L. monocytogenes* Scott A inoculated in paneer samples is shown in Figure 4a.1. Fresh paneer samples were purchased from commercial outlet, aseptically cut into cubes of approximate dimensions 2 x 2 x 1 cm (L x B x H) using a sterile knife and portions of 10 g were placed into several pre-sterilized 250 ml conical flasks and covered with sterile cotton plug. Paneer samples (10 g each) were inoculated by adding 100 ml cell

suspensions of *L. monocytogenes* Scott A prepared in sterile saline from overnight grown cultures. The concentration of *L. monocytogenes* in 100 ml saline suspension was appropriately adjusted to achieve a final cell concentration of approximately 6.3 and 4.3 $\log_{10}$ cfu/g of paneer samples. The inoculated samples were held in the respective saline suspensions for a period of 1 h at 8^{o}C and then the suspensions were decanted. The bacteriocin sample used in the experiments consisted of dialyzed and lyophilized bacteriocin extract obtained from strain CRA52. A 100 ml aliquot of bacteriocin reconstituted in 10 mM phosphate buffer (pH 7.0) was added to two sets of inoculated paneer samples (6.3 and 4.3 $\log_{10}$ cfu/g of *L. monocytogenes*) to achieve a final bacteriocin concentration of 2560 and 1280 AU/g of paneer sample, respectively. Appropriate bacteriocin negative control samples were prepared by adding 100 ml of 10mM phosphate buffer (pH 7.0) to the inoculated paneer samples. The bacteriocin added samples and the bacteriocin negative control were incubated at 8^{o}C for 1 h followed by decanting of the solutions. The paneer samples were subsequently incubated for 5 days at 8^{o}C to simulate refrigerated conditions of storage and analyzed for the growth of *L. monocytogenes* Scott A at designated time intervals. For enumeration of *L. monocytogenes* Scott A paneer samples (10 g) were added to 90 ml of sterile saline. The samples were then agitated in an orbital shaker for 1 h and 0.1 ml aliquots of appropriate dilutions were surface plated in triplicate on Listeria-Oxford media (HiMedia, Mumbai). The plates were incubated for 48 h at 37^{o}C and characteristic colonies of *Listeria* were counted and expressed as average $\log_{10}$ cfu/g of paneer.

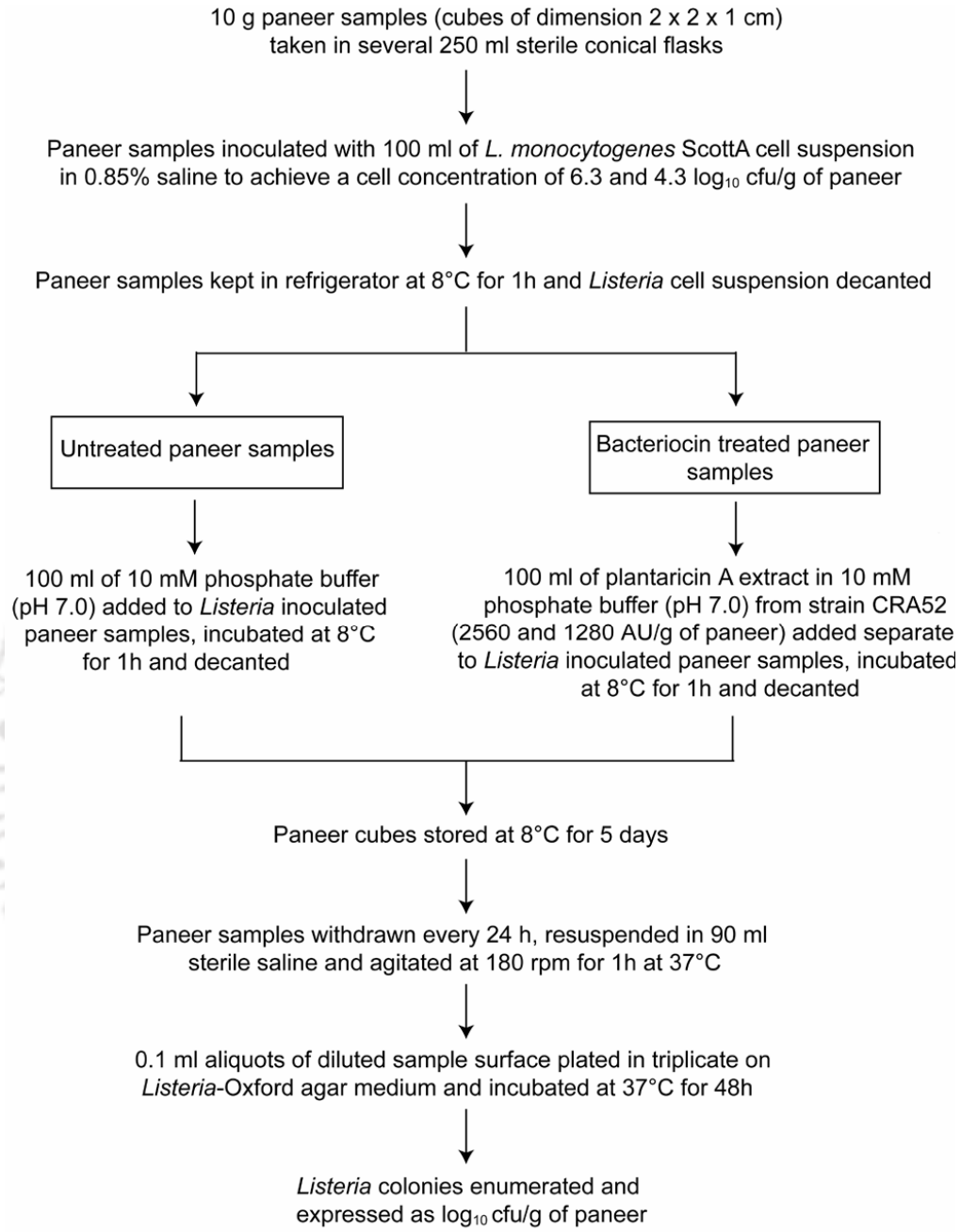

Figure 4a.1 Scheme of experiment to study the effect of plantaricin A produced by strain CRA52 on the growth of *Listeria monocytogenes* Scott A inoculated in paneer samples.

4a.3. Results and Discussion

4a.3.1. Screening of anti-listerial LAB isolates

The main goal of this study was to isolate and characterize potent anti-listerial bacteriocin producing LAB from indigenous samples (*dahi*, dried fish and fermented cucumber). For isolation of LAB, an enrichment process was adopted to ensure sufficient proliferation of the inherent LAB population and maximize the chances of their recovery from the chosen complex samples. A total of 231 isolates were obtained cumulatively from enriched indigenous samples, which encompassed 112 isolates from fermented cucumber, 75 isolates from *dahi* and 44 isolates from dried fish. The pH of the MRS medium at the end of enrichment step varied from 3.4-3.8 for the samples, indicating the presence of acid producing microorganisms. All the isolates obtained from the samples were either rod- or cocci-shaped and were attributed to LAB based on the premise that they were Gram-positive and catalase-negative (results for select isolates are indicated in Table 4a.4 of section 4a.3.4). The prevalence of LAB isolates in *dahi* and fermented cucumber agrees with previous results shown in Chapter 2 and Chapter 3. Interestingly, LAB was also present, albeit in lesser numbers in dried fish samples. The presence of LAB in freshwater fish, in the intestinal contents of fish and traditional fish products has been reported earlier (Bucio *et al.,* 2006; Gonzalez *et al.,* 2000; Thapa *et al.,* 2006).

In the colony overlay assay, 51 colonies revealed anti-listerial activity (Table 4a.1). A representative result obtained from a dried fish sample depicted in Figure 4a.2 indicated that some LAB colonies such as colony A revealed considerable anti-listerial activity, producing a large zone of inhibition of around 12 mm diameter whereas in some cases a smaller zone of inhibition was observed such as colony B (around 8 mm). The maximum number of anti-

listerial LAB isolates was obtained from fermented cucumber (26 nos.) followed by *dahi* (14 nos.) and dried fish samples (11 nos.).

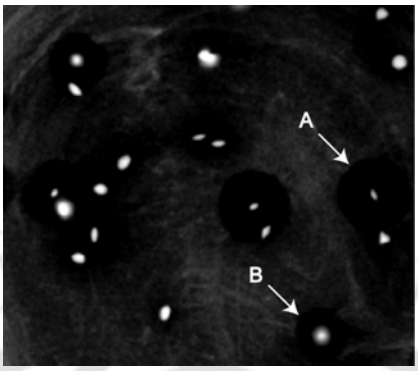

Figure 4a.2 LAB colonies obtained from dried fish sample screened for bacteriocin activity on a lawn of *Listeria monocytogenes* Scott A. Arrow A and B indicates varying size of zone of growth inhibition produced by bacteriocin producing LAB colonies.

The presence of anti-listerial bacteriocin producing LAB in fermented cucumber samples corroborates with previous results shown in Chapter 2. LAB isolates that exhibit anti-listerial activity were also obtained from *dahi* samples, supporting earlier reports (Varadaraj *et al.,* 1993). Some of the LAB isolates from dried-fish samples, has displayed considerable anti-listerial activity. This finding is significant in the light of only a few reports that indicate the presence of antagonistic LAB in fish samples (Thapa *et al.,* 2006; Tome *et al.*, 2006).

4a.3.2. PCR-based identification of anti-listerial LAB isolates and bacteriocin gene

PCR facilitated rapid genus identification of the anti-listerial LAB isolates. It is evident from Table 4a.1 that PCR experiments indicated the presence of a vast majority of *Lactobacillus* sp. (11 nos.) in *dahi* samples, whereas 3 isolates were recognized as *Lactococcus* sp. The presence of *Lactobacillus* and *Lactococcus* in *dahi* samples as evident from PCR experiments supports earlier results obtained in Chapter 3.

Table 4a.1

PCR based genus identification and detection of bacteriocin gene in anti-listerial LAB isolates.

Source [a]	Isolates [b]	Bacteriocin [d]
Dahi (14)	*Lactobacillus* sp. CDRA10	nd
	Lactobacillus sp. CDRA17	nd
	Lactobacillus sp. CDRA19	nd
	Lactobacillus sp. CDRA27	Plantaricin A
	Lactobacillus sp. CDRA31	nd
	Lactobacillus sp. CDRA39	nd
	Lactobacillus sp. CDRA45	nd
	Lactobacillus sp. CDRA49	nd
	Lactobacillus sp. CDRA57	Plantaricin A
	Lactobacillus sp. CDRA60	nd
	Lactobacillus sp. CDRA66	nd
	Lactococcus sp. CDRA13	nd
	Lactococcus sp. CDRA15	nd
	Lactococcus sp. CDRA 71	nd
Dried fish (11)	*Lactobacillus* sp. DF3	nd
	Lactobacillus sp. DF9	Plantaricin A
	Lactobacillus sp. DF13	nd
	Lactobacillus sp. DF27	nd
	Lactobacillus sp. DF32	nd
	Lactobacillus sp. DF35	nd
	Lactobacillus sp. DF39	nd
	Lactobacillus sp. DF45	nd
	Lactobacillus sp. DF59	nd
	Lactobacillus sp. DF62	nd
	DF14[c]	Enterocin A
Fermented cucumber (26)	*Lactobacillus* sp. CRA1	nd
	Lactobacillus sp. CRA3	nd
	Lactobacillus sp. CRA5	nd
	Lactobacillus sp. CRA6	Pediocin
	Lactobacillus sp. CRA13	nd
	Lactobacillus sp. CRA14	nd
	Lactobacillus sp. CRA16	nd
	Lactobacillus sp. CRA21	Plantaricin A
	Lactobacillus sp. CRA23	nd
	Lactobacillus sp. CRA38	Plantaricin A
	Lactobacillus sp. CRA39	Pediocin
	Lactobacillus sp. CRA49	Plantaricin A
	Lactobacillus sp. CRA52	Plantaricin A
	Lactobacillus sp. CRA55	nd
	Lactobacillus sp. CRA57	nd
	Lactobacillus sp. CRA58	nd
	Lactobacillus sp. CRA61	Plantaricin A
	Pediococcus sp. CRA9	Pediocin
	Pediococcus sp. CRA11	Pediocin
	Pediococcus sp. CRA18	Pediocin
	Pediococcus sp. CRA20	Pediocin
	Pediococcus sp. CRA28	Pediocin
	Pediococcus sp. CRA50	Pediocin
	Pediococcus sp. CRA51	Pediocin
	Pediococcus sp. CRA53	Pediocin
	Pediococcus sp. CRA59	Pediocin

[a] The number of anti-listerial isolates from each source are indicated in parenthesis; [b]Isolates were identified with 16S rRNA primers specific for genus *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*. [c]Isolate DF14 could not be identified with genus-specific primers; [d] Bacteriocin gene was detected by PCR using primers specific for plantaricin A, nisin, pediocin and enterocin A. nd: bacteriocin gene could not be detected by PCR.

In dried-fish samples, PCR enabled detection of *Lactobacillus* and vindicated earlier reports (Bucio *et al.,* 2006; Gonzalez *et al.,* 2000; Thapa *et al.,* 2004). The predominance of *Lactobacillus* and *Pediococcus* in fermented cucumber samples was also evident and corroborated with previous results obtained in Chapter 2. In dried fish samples, PCR revealed the predominance of *Lactobacillus* sp. (10 nos.), whereas isolate DF14 could not be identified. In fermented cucumber samples, PCR revealed the presence of *Lactobacillus* sp. (17 nos.) and *Pediococcus* sp. (9 nos.). A representative figure of an agarose gel demonstrating the presence of specific amplicons corresponding to various LAB genus is shown in Figure 4a.3.

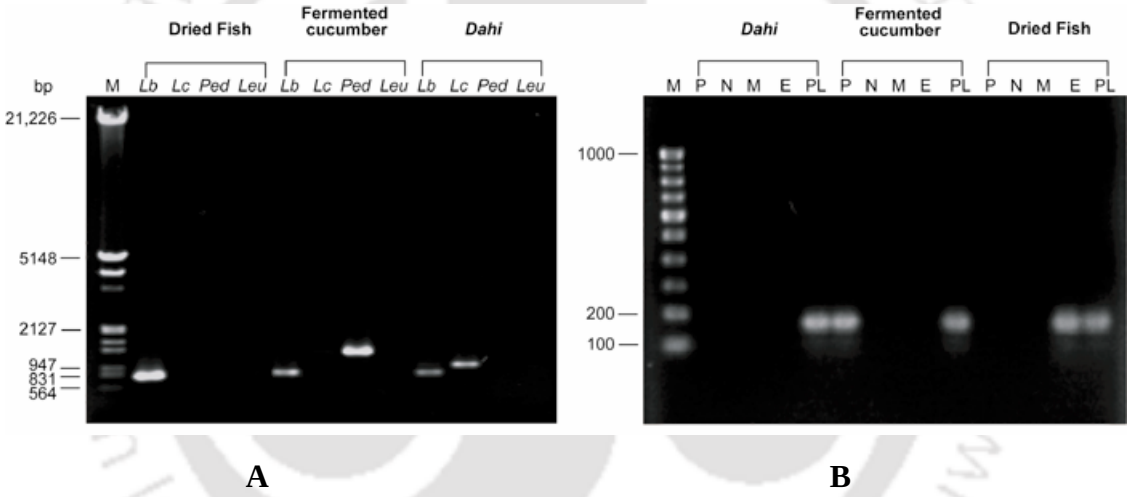

Figure 4a.3 PCR amplicons obtained from enriched indigenous samples with **(A)** 16S rRNA based genus-specific primers, Lb: *Lactobacillus*; Lc: *Lactococcus*; Ped: *Pediococcus*; Leu: *Leuconostoc*; Lane M: λ DNA *Eco*RI / *Hin*dIII double digest size marker; **(B)** Bacteriocin gene-specific primers, P: pediocin; N: nisin; M: mesentericin; E: enterocin-A; PL: plantaricin A; Lane M: 100 bp DNA ladder.

PCR could account for the presence of only two plantaricin A producers (CDRA27 and CDRA57) in *dahi* (Table 4a.1), whereas nisin producers could not be detected in *dahi* samples. In dried fish samples, plantaricin A (DF9) and enterocin A (DF14) producers could be detected by PCR. From Table 4a.1 it is also evident that in fermented cucumber, an overwhelming majority were pediocin producers (11 nos.) while a small number of

plantaricin A producers (5 nos.) were also discernible. The representative amplicons of the bacteriocin gene obtained from select LAB isolates is shown in Figure 4a.3. PCR-based screening of bacteriocin gene is a rapid and convenient tool to characterize antagonistic LAB isolates (Knoll *et al.,* 2008). PCR experiments revealed the preponderance of Class IIa bacteriocin producers amongst the anti-listerial LAB isolates, which was further substantiated by bacteriocin gene sequence of isolates DF14 (Accession no. FJ424062), CRA21 (Accession no. FJ424063) and CRA51 (Accession no. EU616745). Homology search by BLAST analysis identified the bacteriocin genes from isolate DF14, CRA21 and CRA51 as enterocin A, plantaricin A and pediocin, respectively. A representative result of the BLAST analysis of the bacteriocin gene for isolate DF14 is shown in Table 4a.2.

4a.3.3. Antimicrobial spectrum

The anti-listerial activity of the neutralized culture filtrate from select LAB isolates could be attributed to bacteriocin-like inhibitory substance (BLIS). From Table 4a.3 it is evident that the anti-listerial activity of BLIS present in the culture filtrate of isolates DF14, CRA21, CRA51 and CRA52 was comparatively higher as they produced a zone of inhibition measuring 16-18 mm. Isolate CRA52 revealed the highest average zone of inhibition of 18 mm. Some isolates such as CDRA60, DF9, DF14, CRA21, CRA28, CRA51, CRA52, and CRA61 also showed a significantly higher ($P<0.05$) anti-listerial activity than others. The results of ANOVA test is mentioned in Appendix (section 2). The variable inhibition observed against *L. monocytogenes* is possibly the reflection of differences in the susceptibility of the target cells or variation in the level of inhibitory molecule produced by the LAB isolates.

Table 4a.2

BLAST analysis for bacteriocin gene of LAB isolate DF14

Accession No.	Description	Maximum Score	Total Score	Query Coverage	E-value Value	Maximum Identity
FJ424062.1	Isolate DF14 enterocin (entA) gene, partial cds	196	196	100%	1e-47	100%
AB292463.1	*Enterococcus faecium* entA, entI, entF, entK, entR, orfA1, orfA2, orfA3, entT, entD, orfA4, orfA5 genes, complete cds	187	187	98%	6e-45	99%
AM746970.1	*Enterococcus faecium* partial enterocin A operon (entAIFK genes), strain MTCC 5153	187	187	98%	6e-45	99%
X94181.1	*E. faecium* entA and orf2 genes	187	187	98%	6e-45	99%
AF240561.1	*Enterococcus faecium* class IIa bacteriocin EntA (entA) and putative immunity protein EntI (entI) genes, complete cds	187	187	98%	6e-45	99%
AF099088.1	*Enterococcus faecium* enterocin A (entA), EntI (entI), EntF (entF), EntK (entK), EntR (entR), bacteriocin -like protein, EntT (entT), EntD (entD), and protease IV homolog genes, complete cds; and unknown genes	187	187	98%	6e-45	99%
AB038464.1	*Enterococcus faecium* genes for enterocin A, immunity protein against enterocin A, transposase complete cds	187	187	98%	6e-45	99%

ANOVA analysis also revealed that certain isolates displayed significantly higher ($P<0.05$) activity against a given pathogenic strain. Isolates DF14, CRA21, CRA51, CRA52 and CDRA27 were antagonistic to Gram-positive pathogens *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus cereus*. Inhibition of Gram-positive foodborne pathogens such as *Staphylococcus aureus* and *Bacillus cereus* by BLIS support earlier reports (Albano *et al.,* 2007; Jones *et al.,* 2008). It was also observed that isolates CRA51 and CRA52 could impede the growth of *Enterobacter aerogenes* whereas isolate CRA51 alone displayed antagonistic activity against *E. coli*. It was encouraging to observe that BLIS from certain LAB isolates (CRA51 and CRA52) were antagonistic to Gram-negative bacterial strains, considering only a few reports that suggest activity against Gram-negative bacteria (Omar *et al.,* 2006; Ponce *et al.,* 2008). None of the LAB isolates displayed antagonistic activity against *Pseudomonas aeruginosa* and *Yersinia enterocolitica*.

4a.3.4. Preliminary tests for phenotypic traits of potent anti-listerial LAB isolates

The results of preliminary tests to determine phenotypic traits of select potent anti-listerial LAB isolates are depicted in Table 4a.4. As evident from the results, all anti-listerial LAB isolates were Gram-positive and catalase-negative. On the basis of CO_2 production from glucose, out of seven potent anti-listerial isolates, *Lactobacillus* sp. DF9 and *Lactobacillus* sp. CRA61 were found to be heterofermenters (Table 4a.4). The anti-listerial LAB isolates demonstrated varied response under different NaCl concentration and temperature. All the tested LAB isolates could grow at pH 4.4 while they failed to grow at pH 9.6.

Table 4a.3

Antimicrobial activity of bacteriocin-like inhibitory substance (BLIS) from anti-listerial LAB isolates

Antagonistic LAB isolate[a]	Diameter of inhibition zone against pathogens (mm)[b]							
	L. monocytogenes **Scott A**	***S. aureus*** **MTCC 96**	***E. faecalis*** **MTCC 439**	***B. cereus*** **MTCC 1305**	***E. coli*** **MTCC 433**	***E. aerogenes*** **MTCC 2822**	***P. aeruginosa*** **MTCC 2488**	***Y. enterocolitica*** **MTCC 859**
Lactobacillus sp. CDRA10	10±0.4	0	0	0	0	0	0	0
Lactobacillus sp. CDRA19	12±0.4	10±0.4	0	0	0	0	0	0
Lactobacillus sp. CDRA27	10±0.3	10±0.3	10±0.3	10±0.2	0	0	0	0
Lactobacillus sp. CDRA39	10±0.3	0	0	0	0	0	0	0
Lactobacillus sp. CDRA57	12±0.4	0	0	0	0	0	0	0
Lactobacillus sp. CDRA60	**13±0.3***	0	0	0	0	0	0	0
Lactococcus sp. CDRA13	11±0.3	0	0	0	0	0	0	0
Lactobacillus sp. DF3	12±0.3	0	0	0	0	0	0	0
Lactobacillus sp. DF9	**13±0.4***	10±0.3	0	0	0	0	0	0
Lactobacillus sp. DF45	12±0.4	0	0	0	0	0	0	0
DF14	**16±0.5***	**12±0.4***	**12±0.3***	11±0.2	0	0	0	0
Lactobacillus sp. CRA3	10±0.3	0	0	0	0	0	0	0
Lactobacillus sp. CRA6	10±0.3	0	0	0	0	0	0	0
Lactobacillus sp. CRA16	11±0.4	0	0	0	0	0	0	0
Lactobacillus sp. CRA21	**16±0.5***	**11±0.2***	11±0.3	**12±0.4***	0	0	0	0
Lactobacillus sp. CRA23	11±0.3	0	0	0	0	0	0	0
Lactobacillus sp. CRA38	11±0.4	0	0	0	0	0	0	0
Lactobacillus sp. CRA39	10±0.2	0	0	0	0	0	0	0
Lactobacillus sp. CRA49	12±0.2	**11±0.3***	0	0	0	0	0	0
Lactobacillus sp. CRA52	**18±0.6***	**12±0.2***	**14±0.3***	**12±0.3***	0	11±0.2	0	0
Lactobacillus sp. CRA61	**14±0.4***	0	0	0	0	0	0	0
Pediococcus sp. CRA11	12±0.4	0	0	0	0	0	0	0
Pediococcus sp. CRA18	12±0.3	0	0	0	0	0	0	0
Pediococcus sp. CRA28	**13±0.4***	0	0	0	0	0	0	0
Pediococcus sp. CRA50	12±0.3	10±0.3	0	0	0	0	0	0
Pediococcus sp. CRA51	**16±0.5***	**12±0.4***	**13±0.3***	**12±0.2***	11±0.3	10±0.2	0	0
Pediococcus sp. CRA53	11±0.3	0	0	0	0	0	0	0

[a] LAB isolates showing a zone of inhibition ≥ 10 mm in colony overlay assay during initial screening against *L. monocytogenes* Scott A were selected.

[b] Diameter of the zone of inhibition in mm determined by agar well diffusion assay against target strains are shown as averaged value for three data sets ± standard deviation.

*Asterisk and boldface denotes significantly higher activity against a given target strain ($P<0.05$) compared to other isolates.

Table 4a.4

Tests for phenotypic traits of anti-listerial LAB isolates.

Bac$^+$ LAB isolates	Microscopy		Physiological and biochemical characteristics							
	Shape	Gram-staining	Catalase activity	CO_2 from glucose	NaCl tolerance		Growth at temp.		Growth at pH	
					6.5%	18%	10°C	45°C	4.4	9.6
Lactobacillus sp. DF9	rod	+	-	+	-	-	+	-	+	-
DF14	cocci	+	-	-	+	-	+	+	+	-
Lactobacillus sp. CRA21	rod	+	-	-	+	-	-	+	+	-
Pediococcus sp. CRA28	cocci	+	-	-	-	-	+	-	+	-
Pediococcus sp. CRA51	cocci	+	-	-	+	-	+	-	+	-
Lactobacillus sp. CRA52	rod	+	-	-	+	-	-	+	+	-
Lactobacillus sp. CRA61	rod	+	-	+	-	-	+	+	+	-

+: positive response or growth; - : negative response or no growth

Table 4a.5

Partial characterization of bacteriocin-like inhibitory substance (BLIS) produced by anti-listerial LAB isolates.

LAB isolate	Bacteriocin activity[a]								
	Enzymatic treatment		Dialysis		pH			Heat treatment	
	Trypsin	Catalase	12. 0 KDa	1.0 KDa	3.5	5.5	7.0	121°C/20 min	100°C/30 min
Lactobacillus sp. DF9	-	+	-	+	+	+	+	+	+
DF14	-	+	-	+	+	+	+	+	+
Lactobacillus sp. CRA21	-	+	-	+	+	+	+	+	+
Pediococcus sp. CRA28	-	+	-	+	+	+	+	+	+
Pediococcus sp. CRA51	-	+	-	+	+	+	+	+	+
Lactobacillus sp. CRA52	-	+	-	+	+	+	+	+	+
Lactobacillus sp. CRA61	-	+	-	+	+	+	+	+	+

[a] Bacteriocin activity was tested against *L. monocytogenes* Scott A by agar well diffusion assay.

4a.3.5. Characterization of antimicrobial compound

It is evident from Table 4a.5 that the anti-listerial activity of BLIS from select LAB isolates was completely abolished after treatment with trypsin, indicating the proteinaceous nature of the antimicrobial compound. The role of hydrogen peroxide as one of the components of BLIS was negated as catalase treatment failed to abolish antimicrobial activity. It is also clear from Table 4a.5 that the activity was retained in a 1.0 kDa dialysis bag, but lost when a 12.0 kDa dialysis bag was used. The pH and thermal stability of BLIS is also evident from Table 4a.5. A representative result of partial characterization of BLIS produced by isolate CRA51 is depicted in Figure 4a.4. Collectively the results shown in Table 4a.5 and Figure 4a.4 provide strong evidence for bacteriocin production by the anti-listerial isolates and supports earlier research work which report similar cardinal features of bacteriocin (Halami *et al.,* 2005; Todorov and Dicks, 2006).

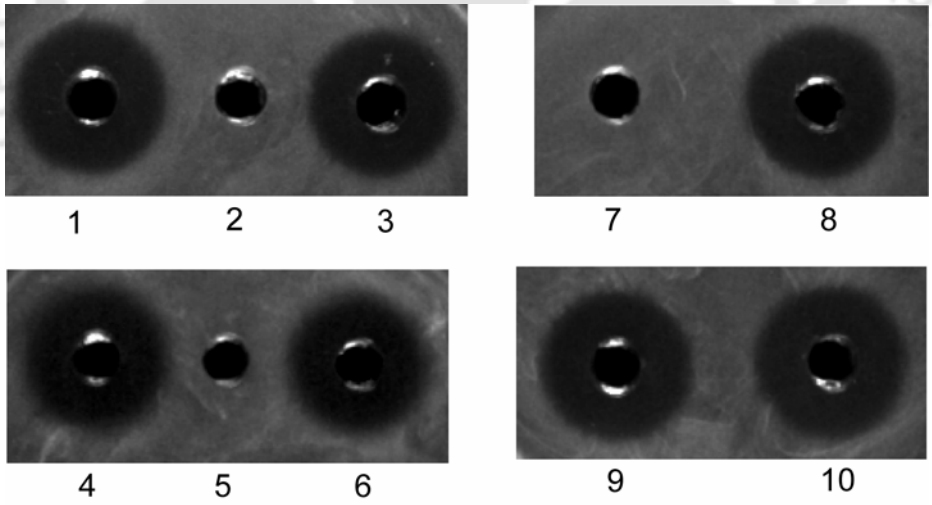

Figure 4a.4 Partial characterization of BLIS produced by *Pediococcus* sp. CRA51. Well Nos. **1:** Untreated BLIS, **2:** Dialyzed BLIS (12.0 kDa), **3:** Dialyzed bacteriocin sample (1.0 kDa), **4:** Catalase treated BLIS, **5:** Trypsin treated BLIS, **6:** Heat-inactivated trypsin added to BLIS, **7:** MRS broth adjusted to pH 3.5, **8:** BLIS pH 3.5, **9:** BLIS pH 5.5, **10:** BLIS pH 7.0. *Listeria monocytogenes* Scott A was used as target.

4a.3.6. Anti-listerial activity

4a.3.6.1. Growth inhibition studies

It can be seen from Figure 4a.5 that plantaricin A from strain CRA52, added at a concentration of 3200 AU/ml after 4 h of growth of *L. monocytogenes* (~1.6 $\log_{10}$ cfu/ml) significantly suppressed the growth of target cells. When bacteriocin was added after 8 h of growth which corresponds to late exponential growth phase (~ 5.9 $\log_{10}$ cfu/ml), the growth of the target cell was again observed to be affected and a plateau in the growth profile was attained. However, addition of bacteriocin at stationary phase of growth (~ 9.0 $\log_{10}$ cfu/ml) failed to demonstrate any impact on the growth of the target cell. Growth inhibition experiments revealed that the susceptibility of *Listeria* to plantaricin A produced by strain CRA52 is related to the age of the cells as indicated earlier by Todorov and Dicks (2006).

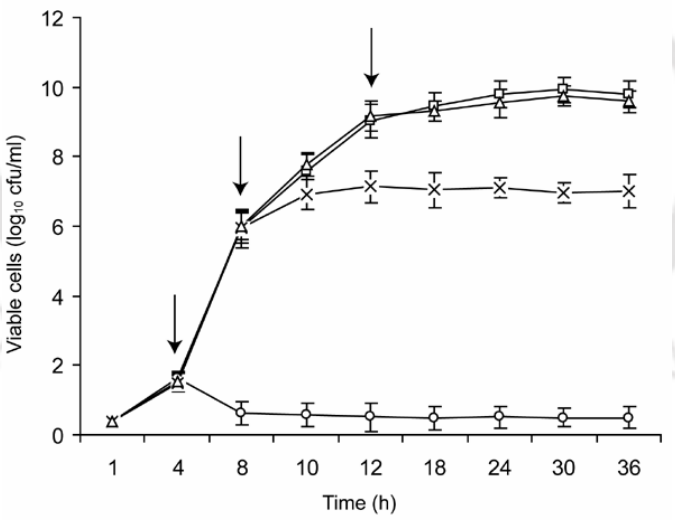

Figure 4a.5 Effect of plantaricin A produced by *Lactobacillus* sp. CRA52 (3200 AU/ml) on the growth of *Listeria monocytogenes* Scott A. Arrow indicates time of addition of bacteriocin to growing target cells. Growth profile of untreated target cells (—□—), target cells treated with bacteriocin at 4 h (—○—); 8 h (—×—) and 12 h (—△—) of growth phase.

4a.3.6.2. Fluorescence based studies

In the course of the investigation, a major aim was to conduct a comparative study on the efficacy of select anti-listerial bacteriocin from native LAB isolates and monitor the target cell damage as a function of bacteriocin concentration. For this purpose, cFDA-SE labelled target cells of *L. monocytogenes* Scott A were used and the extent of cellular damage was determined by measuring efflux of the fluorescent dye from cells treated with varying amounts of bacteriocin. cFDA-SE is a cell permeative dye with a strong and stable green fluorescence. Bacterial cells can readily take up cFDA-SE, and following uptake of the dye, the succinimidyl group conjugates with aliphatic amines of intracellular proteins. Fluorescence is detected due to accumulation of the fluorescent form of the dye following cleavage of the ester by intracellular esterase activity (Hoefel *et al.,* 2003). Treatment of CFDA-SE labelled target cells with bacteriocin would lead to pore formation in the target cells and subsequent leakage of the dye from damaged cells. The extent of dye leakage could then be a measure of dose-dependent cellular damage by bacteriocin. A schematic representing the mode of interaction of the fluorescent probe and its application in the dye leakage assay to quantify the effect of bacteriocin molecule on target bacterial strain is shown in Figure 4a.6.

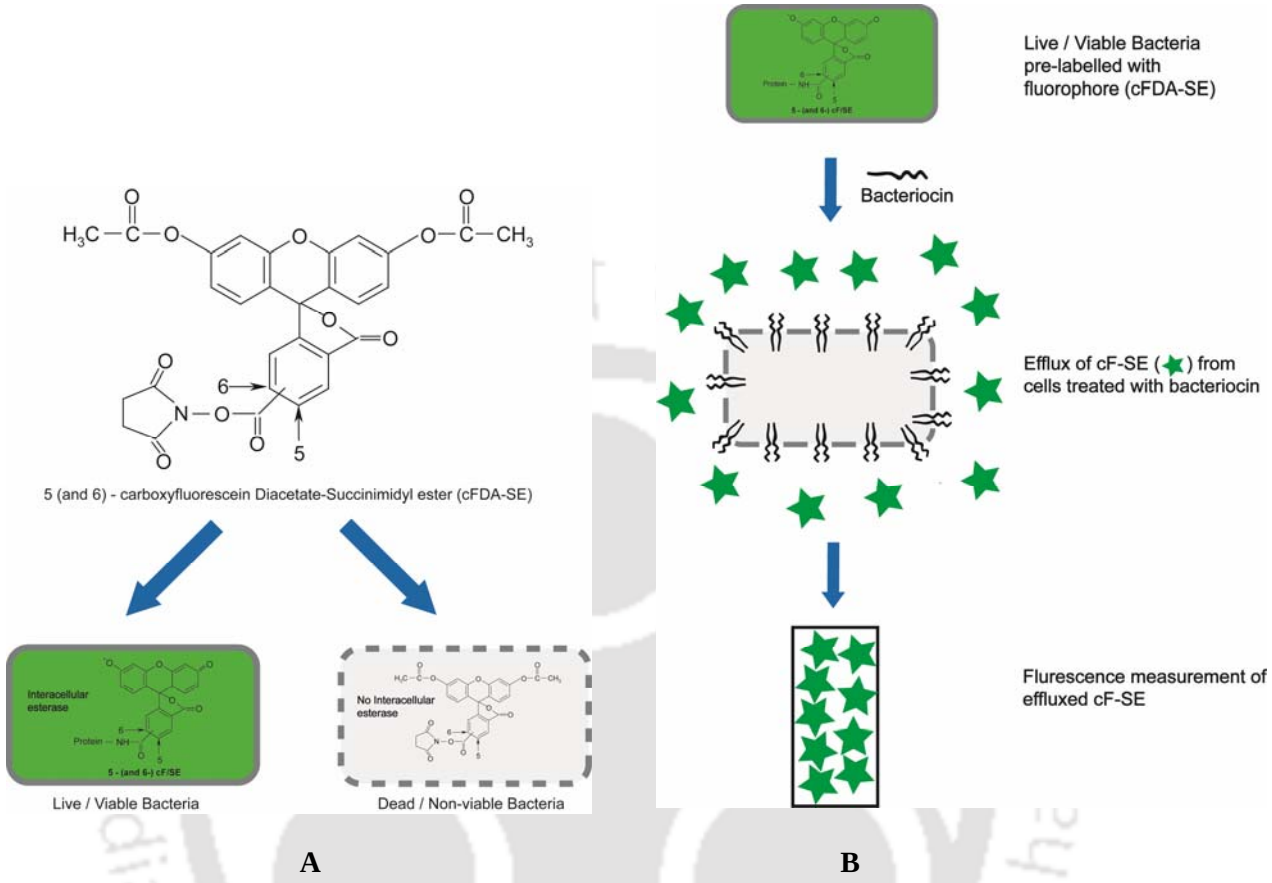

Figure 4a.6. (A) Schematic representation of the structure of cFDA-SE and its interaction with live and dead bacterial cells; **(B)** Dye-leakage assay to quantify the effect of bacteriocin treatment on target cell.

The result of the dye-leakage experiment is depicted in Figure 4a.7A. It is quite evident that leakage of the dye from bacteriocin treated cells of *L. monocytogenes* increased with the concentration of the added bacteriocin. Amongst the tested samples, plantaricin A produced by *L. plantarum* CRA52 exhibited the highest anti-listerial activity. For instance at a bacteriocin concentration of 800 AU/ml, the fluorescence intensity measured for the dye efflux was around 2.3 x 10^6 counts per second (cps) whereas the corresponding intensity at the highest concentration of bacteriocin (6400 AU/ml) amounted to a value of around

3.0 x 10^6 cps. It is also evident from Figure 4a.7A that the dose-dependent cellular damage in target cells of *L. monocytogenes* Scott A as observed in case of plantaricin A produced by *Lactobacillus* sp. CRA52 was also seen ubiquitously for bacteriocin tested from other isolates such as CRA18, CDRA57, CDRA60 and DF3. The difference obtained for fluorescence intensity for the bacteriocins of the tested isolates can be possibly accounted by the difference in the potency of the bacteriocin in damaging the target cells.

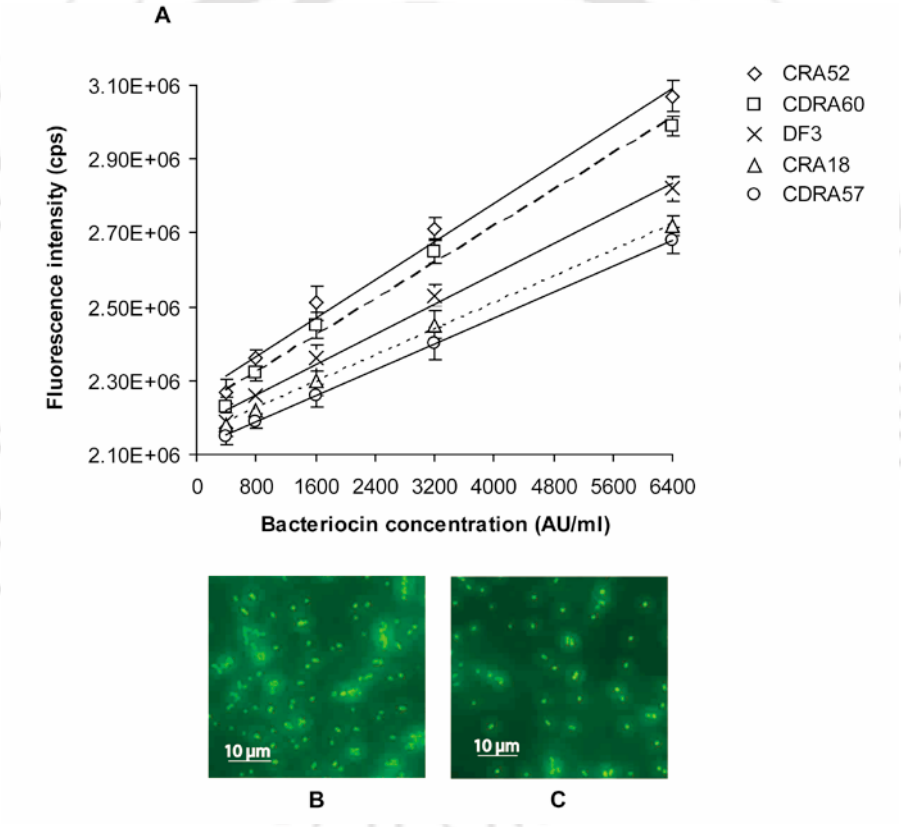

Figure 4a.7 (A) Estimation of anti-listerial activity of bacteriocin by fluorescent dye leakage assay; **(B)** Fluorescence image of untreated *Listeria monocytogenes* Scott A cells; **(C)** Fluorescence image of *Listeria monocytogenes* Scott A cells treated with 3200 AU/ml of plantaricin A obtained from *Lactobacillus* sp. CRA52.

In the present set of experiments, the use of cFDA-SE rendered a distinct advantage as the cross-linking of cFDA-SE with intracellular bacterial proteins minimizes the leakage of the dye from intact cells. Hence, the cause-and-effect phenomenon in terms of pore formation in the target cells following treatment with bacteriocin could be clearly deciphered by measuring efflux of the dye from damaged cells. Class IIa bacteriocins are known to induce permeabilization of the target cell, possibly by forming ion selective pores which result in dissipation of the proton motive force and depletion of intracellular ATP (Drider *et al.,* 2006). The results obtained in the present investigation clearly demonstrated the membrane permeabilizing attribute and dose-dependent anti-listerial activity of plantaricin A produced by isolate CRA52, corroborating with earlier studies on mode of action of bacteriocin (Atrih *et al.,* 2001; Budde and Rasch, 2001; Kristiansen *et al.,* 2005).

The cellular damage caused by plantaricin A was further substantiated by performing fluorescence microscopic investigation of cFDA-SE labelled cells of *L. monocytogenes* Scott A treated with 3200 AU/ml of bacteriocin. The results of the experiment are depicted in Figure 4a.7B and 4a.7C. It is quite evident from Figure 4a.7B that in case of control sample which consisted of untreated cells of *L. monocytogenes* Scott A, a large number of fluorescent cells could be observed indicating appreciable viability of the cells. The corresponding sample in Figure 4a.7C depicts a marked reduction in the number of fluorescent cells indicating a loss of viability as an effect of bacteriocin treatment. These results are in concordance with the results obtained from previous experiments (Figure 4a.7A) where cellular damage due to bacteriocin treatment was demonstrated by measuring dye leakage from membrane compromised cells of *L. monocytogenes* Scott A.

4a.3.6.3. Application of anti-listerial bacteriocin in paneer samples

The promise of plantaricin A as an anti-listerial agent was ascertained in paneer which is a widely consumed non-fermented dairy product devoid of any protective culture and is thus vulnerable to post-processing contamination. Further, the commercially available product is stored under refrigerated conditions, and hence contamination with psychrotrophic bacterial pathogens such as *Listeria monocytogenes* could pose serious health concerns. The potential of plantaricin A produced by *L. plantarum* CRA52 to inhibit the growth of *L. monocytogenes* Scott A inoculated in commercially available paneer samples was tested at two different inoculum levels (approximately 6.3 and 4.3 $\log_{10}$ cfu/g) of the target pathogen and varying bacteriocin concentrations (2560 and 1280 AU/g). It can be observed from Figure 4a.8 that the levels of *L. monocytogenes* Scott A increased steadily from an initial 6.3 $\log_{10}$ cfu/g to around 8.0 $\log_{10}$ cfu/g in the control samples after 5 days of incubation at 8^{o}C. This demonstrated that paneer was quite conducive to the growth of *Listeria*. Additionally, the psychrotrophic nature of *Listeria* and the absence of any competitive or protective microbial culture in paneer probably promoted growth of the target pathogen. Application of 1280 AU/g plantaricin A extract lead to a continuous decrease in the levels of *Listeria* till 60 h of incubation and a viable count of 5.53 $\log_{10}$ cfu/g was obtained. The final cell number obtained in the product at the end of 5 days storage period at 8^{o}C was 5.74 $\log_{10}$ cfu/g. It is also evident from Figure 4a.8 that at higher bacteriocin concentration of 2560 AU/g the inhibitory effect on *L. monocytogenes* Scott A was marked and a progressive decline in the viable cell population could be observed till 72 h of incubation, resulting in a viable cell count of 4.89 $\log_{10}$ cfu/g. The final cell number of the pathogen after 5 days of storage at 8^{o}C was 4.93 $\log_{10}$ cfu/g.

This observation clearly indicated that growth inhibition of *L. monocytogenes* in paneer samples was a function of the concentration of the bacteriocin used for treatment of the samples.

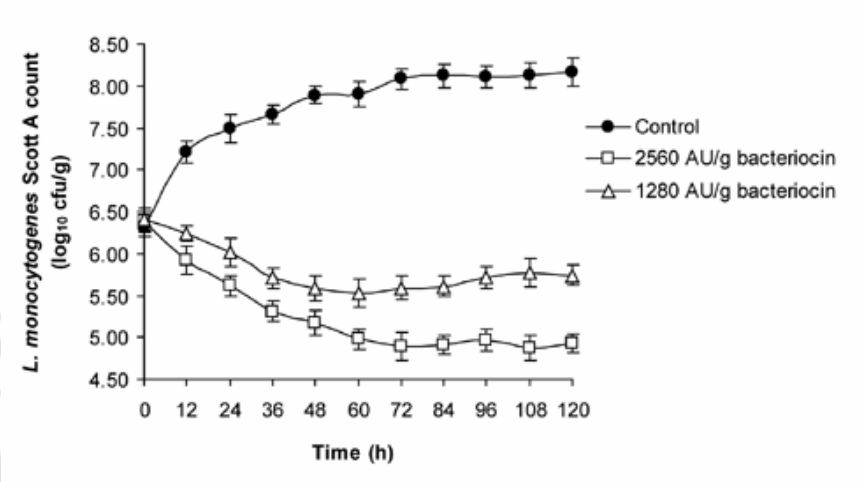

Figure 4a.8 Effect of plantaricin A produced by *Lactobacillus* sp. CRA52 on the growth of *Listeria monocytogenes* Scott A inoculated in commercial paneer samples and stored at 8°C for 5 days. Initial level of *L. monocytogenes* Scott A inoculated in paneer samples was 6.3 $\log_{10}$ cfu/g.

In case of the experiments with lower inoculum levels of *L. monocytogenes* it was observed that for control samples, an increase of viable *Listeria* cells by more than one log cycle (5.92 $\log_{10}$ cfu/g) was evident within 48 h, and after 5 days of storage at 8°C, the viable counts culminated at 6.14 $\log_{10}$ cfu/g (Figure 4a.9). However, in presence of 1280 AU/g plantaricin A in the paneer samples, the growth of the pathogen was impeded and the final viable count of the pathogen was estimated to be 2.87 $\log_{10}$ cfu/g. At a higher bacteriocin concentration of 2560 AU/g the viable count of *Listeria* in paneer revealed a progressive decline to reach a level of 2.07 $\log_{10}$ cfu/g.

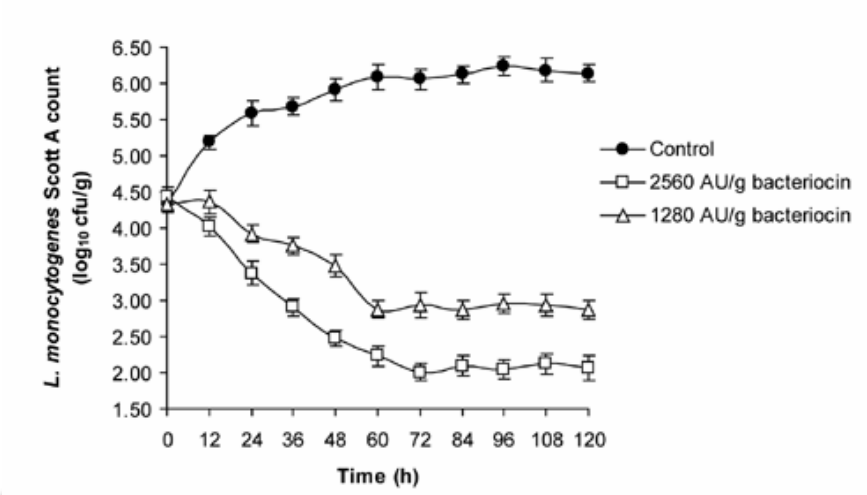

Figure 4a.9 Effect of plantaricin A produced by *Lactobacillus* sp. CRA52 on the growth of *Listeria monocytogenes* Scott A inoculated in commercial paneer samples and stored at 8°C for 5 days. Initial level of *L. monocytogenes* Scott A inoculated in paneer samples was 4.3 $\log_{10}$ cfu/g.

It is noteworthy from the experiments that the initial inoculum level of the target pathogen does play a significant role in determining the efficacy of the bacteriocin in suppressing the growth of the pathogen. This is evident from the fact that the percentage of surviving cells of *L. monocytogenes* Scott A in paneer treated with 2560 AU/g plantaricin A amounted to 77% (4.93 $\log_{10}$ cfu/g) and 46% (2.07 $\log_{10}$ cfu/g) at the end of storage period of 5 days when the initial inoculum levels of the pathogen used were 6.3 $\log_{10}$ cfu/g and 4.3 $\log_{10}$ cfu/g respectively (Figure 4a.8 and 4a.9). Besides the bacteriocin concentration and initial inoculum levels of target pathogen, there can be additional factors contributed by the paneer matrix that can influence the inhibitory action of the bacteriocin on the pathogen. Additional factors such as binding of bacteriocin to the paneer matrix resulting in depleted bioavailability of the molecule for target cell interaction and emergence of bacteriocin-resistant variants of *L. monocytogenes* as a consequence of continuous exposure to bacteriocin as reported by Naghmouchi *et al.* (2007) can influence the anti-listerial activity of the bacteriocin.

A large number of research investigations have revealed the application potential of bacteriocin to control the growth of *Listeria* in food samples. Schillinger *et al.* (2001) have reported the use of nisin alone and in conjunction with a protective culture to inhibit the growth of *Listeria* in tofu samples. In another study involving a dairy product, the combined effect of high-pressure (HP) treatment and bacteriocin-producing lactic acid bacteria (BP-LAB) on the survival of *Listeria monocytogenes* Scott A in cheeses made from raw milk has been demonstrated (Arques *et al.*, 2005). The application of *Enterococcus faecium* WHE 81, a multi-bacteriocin producer to smear soft cheese and control the growth of *Listeria* in the product has been demonstrated (Izquierdo *et al.*, 2009). The results obtained in the present investigation clearly demonstrated the promise of plantaricin A, an anti-listerial bacteriocin produced by strain *Lactobacillus* sp. CRA52 in mitigating the growth of *Listeria monocytogenes* in a perishable sample like paneer stored under refrigerated conditions.

4a.4. Conclusion

1. In the present investigation, anti-listerial bacteriocin producing LAB were isolated from enriched indigenous samples. The application of PCR facilitated genus identification as well as detection of Class IIa bacteriocin producers amongst the isolates.

2. Interestingly, many of the isolates exhibited a broad spectrum antimicrobial activity against foodborne bacterial pathogens and in some cases antagonism towards certain Gram-negative pathogens was noteworthy.

3. Partial characterization of the antimicrobial compound emphasized the presence of bacteriocin.

4. The mode of action of the bacteriocin produced by some of these isolates was demonstrated by a sensitive fluorescent dye-leakage assay.

5. A significant outcome of the present investigation was to demonstrate the promise of plantaricin A, an anti-listerial bacteriocin produced by strain *Lactobacillus plantarum* CRA52 in mitigating the growth of *Listeria monocytogenes* in a perishable sample like paneer stored under refrigerated conditions.

It is anticipated that some of the potent anti-listerial LAB strains isolated in the present investigation could find niche applications in food fermentation processes both as starter as well as bioprotective cultures. The subsequent objective of the present research investigation was to determine the genetic diversity of the anti-listerial LAB isolates. In the subsequent Chapter 4b, an arbitrarily-primed PCR (AP-PCR) based method is described for estimating the genetic diversity of the anti-listerial LAB isolates obtained from indigenous samples of *dahi*, dried fish and fermented cucumber.

CHAPTER 4b

Estimation of the genetic diversity of anti-listerial bacteriocin producing lactic acid bacteria by arbitrarily primed-PCR based fingerprinting method

ABSTRACT

The main goal of the investigations carried out in this chapter was to assess the genetic diversity of anti-listerial bacteriocin producing LAB isolates obtained in previous Chapter. To study the genetic diversity of anti-listerial LAB isolates, an 18-mer primer (ARAK5) specific for 16S rDNA conserved region of lactic acid bacteria (LAB) was designed and its discriminatory potentials were evaluated against standard LAB strains in molecular fingerprinting and strain tracking. Fingerprinting analysis based on AP-PCR in conjunction with ARAK5 primer generated discriminating banding patterns for LAB and non-LAB strains. Discriminatory potential of ARAK5 primer was validated by comparing fingerprinting profiles of 26 *Lactobacillus* reference strains obtained in AP-PCR with the fingerprints observed in rep-PCR using REP1R-I and REP2-I primer. The cluster pattern of the *Lactobacillus* strains obtained from both the methods was comparable and essentially in accordance with the taxonomic affiliation of the LAB strains. AP-PCR with selected primer enabled simultaneous tracking of the starter cultures *Lactococcus lactis* subsp. *lactis* MTCC 3041 and *Lactobacillus acidophilus* MTCC 447 in a culture-independent fashion in *dahi,* indicating the value of the designed primer for microbiological food quality assessment. The designed primer for AP-PCR based method clearly revealed the genetic diversity of the anti-listerial LAB isolates.

4b.1. Introduction

Lactic acid bacteria (LAB) are widely regarded as crusaders of food fermentation processes and bear immense commercial value because of their starter culture and probiotic attributes (Cagno *et al.,* 2008; Coolbear *et al.,* 2008; Ouwehand *et al.,* 2002; Zhou and Gill, 2005; Collado *et al.,* 2007; Gueimonde *et al.,* 2006; Sherman *et al.,* 2005). LAB are also known for their antagonistic virtues such as the production of bacteriocins, which are essentially antimicrobial peptides that are known to inhibit the growth of many foodborne pathogens (Cleveland *et al.,* 2001; Drider *et al.,* 2006; Castellano *et al.,* 2008; Jones *et al.,* 2008; Settanni and Corsetti, 2008). Hence there is a great demand for such bacteriocin producing LAB strains in food fermentation processes to ensure food safety and extended shelf-life of the product. While the choice of an LAB strain for food fermentation applications is primarily governed by the functional characteristics and technological properties of the strain that render unique sensorial and probiotic properties to the food product as well as ensure food safety, reliable identification and typing of LAB is crucial for strain authentication, strain tracking, determination of genetic diversity of the strains and microbiological quality control.

Conventional methods of detection and identification of LAB present in food samples mainly involve biochemical and phenotypic methods. However, these methods are arduous and error prone. Molecular techniques have provided powerful tools for the taxonomic identification and typing of LAB strains pertinent to food fermentation processes. Several molecular typing techniques have been developed for the identification and differentiation of LAB strains. Among these, DNA fingerprinting techniques such as pulsed-field gel electrophoresis (PFGE), ribotyping, and restriction fragment length polymorphism (RFLP) have been extensively applied for the intra-specific identification and genotyping of LAB

isolated from a wide gamut of fermented food (Coudeyras *et al.,* 2008; Randazzo *et al.,* 2004). In spite of the powerful taxonomic resolution offered by some of these techniques such as PFGE, routine application of these techniques are hampered due to the cost, level of sophistication involved and practical problems in large scale implementation. Random amplification based techniques such as RAPD, which utilizes short arbitrary primers has been widely acclaimed as a rapid, and inexpensive method for genotyping different strains of LAB (Pulido *et al.,* 2005; Rossetti and Giraffa, 2005; Ruiz *et al.,* 2008). A large number of researchers have also used other PCR-based techniques, such as repetitive element sequence-based PCR (rep-PCR) for molecular typing of LAB strains (De Angelis *et al.*, 2007; Gevers *et al.,* 2001). The 16S RNA gene, which is regarded as an evolutionary clock, has been a popular choice as a target for LAB identification as well as fingerprinting of LAB communities in food samples. The utility of 16S rRNA gene specific primers for the detection of LAB strains has been demonstrated (Settanni *et al.,* 2005). The rRNA heterogeneity among LAB species has also been judiciously exploited in PCR-DGGE, resulting in an insightful analysis of LAB population in a variety of food matrices (Randazzo *et al.,* 2009; Cocolin *et al.,* 2007; Cocolin *et al.,* 2006). Although a plethora of molecular techniques are available for identification and molecular typing of LAB strains, each method offers varying levels of discrimination and ease of operation. Clearly the essential criteria of an appropriate molecular typing method would be governed by the resolving power of the method, the speed, applicability to large number of samples and the reproducibility of the fingerprints.

In the previous chapter, anti-listerial bacteriocin producing LAB were isolated from indigenous samples of *dahi*, dried fish and fermented cucumber. The subsequent objective

was to determine the genetic diversity of the anti-listerial LAB isolates. In the present investigation, an arbitrarily primed PCR (AP-PCR) based molecular typing method is described. The method essentially uses a primer targeting a highly conserved region of the 16S rRNA gene of LAB strains. Application of the primer in AP-PCR successfully discriminated LAB and non-LAB strains and also generated robust fingerprints for several standard strains of *Lactobacillus* species. The application potential of the primer and the AP-PCR method was validated by detecting multiple LAB strains in a fermented milk product on the basis of characteristic fingerprinting patterns obtained for the strains. In the last section of this chapter, AP-PCR based fingerprinting analysis for estimation of the genetic diversity of bacteriocin producing natural isolates of LAB is described.

4b.2. Materials and Methods

4b.2.1. Strains and growth conditions

Bacterial strains used in the present study are enlisted in Table 4b.1. All LAB strains were grown in MRS broth (HiMedia, Mumbai, India) under static incubation at 37°C. The other bacterial strains such as *Bacillus cereus* MTCC 1305, *Escherichia coli* MTCC 433 and *Bacillus subtilis* MTCC 441 were cultured in nutrient broth (HiMedia, Mumbai, India) at 37°C and 180 rpm in a shaker incubator (New Brunswick, USA). *Staphylococcus aureus* MTCC 96 and *Listeria monocytogenes* Scott A were propagated in brain-heart infusion broth (HiMedia, Mumbai, India) at 37°C and 180 rpm in a shaker incubator. Stock cultures of LAB and non-LAB strains were maintained as mentioned in section 2.2.1 of Chapter 2.

Table 4b.1

Reference bacterial strains used in the present investigation.

Organism	Strain
Lactobacillus acidophilus	MTCC 447, NRRL B4495
Lactobacillus brevis	NRRL B4527, NCIM 2090
Lactobacillus casei	NCIM 2151, MTCC 1423
Lactobacillus casei subsp. *casei*	NRRL B1922
Lactobacillus delbrueckii subsp *delbrueckii*	NCIM 2025
Lactobacillus delbrueckii subsp. *bulgaricus*	NRRL B548
Lactobacillus delbrueckii subsp. *lactis*	MTCC 911
Lactobacillus fermentum	MTCC 903
Lactobacillus gasseri	NRRL B4240, NRRL B14168
Lactobacillus helveticus	NCIM 2126
Lactobacillus johnsonii	NRRL B2178
Lactobacillus lactis	NCIM 2368
Lactobacillus paracasei subsp. *paracasei*	NRRL B4564
Lactobacillus plantarum	MTCC 1325, MTCC 1746, MTCC 1407, MTCC 2083, NCIM 2592
Lactobacillus reuteri	NRRL B14171
Lactobacillus rhamnosus	MTCC 1408, NRRL B442
Lactobacillus sakei	NRRL B1917
Lactococcus lactis subsp. *chacetylactis*	MTCC 3042
Lactococcus lactis subsp. *lactis*	MTCC 440, MTCC 3041, MTCC 3038
Leuconostoc mesenteroides	NRRL B640
Leuconostoc mesenteroides subsp. *mesenteroides*	MTCC 107
Pediococcus acidilactici	CFR K7[a], NRRL B14009, NRRL 14958, NRRL B1153
Pediococcus pentosaceus	NCIM 2295
Enterococcus faecalis	MTCC 439
Escherichia coli	MTCC 433
Bacillus cereus	MTCC 1305
Bacillus subtilis	MTCC 441
Listeria monocytogenes	Scott A[a]
Staphylococcus aureus	MTCC 96

NRRL: Northern Regional Research Laboratory, Peoria, IL, USA; MTCC: Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India; NCIM: National Collection of Industrial Microorganism, National Chemical Laboratory (NCL), Pune, India.

[a] Culture provided by Dr. Prakash Halami, Central Food Technological Research Institute (CFTRI), Mysore, India.

4b.2.2. Isolation of bacterial genomic DNA

Standard LAB and non-LAB strains used in the present study were grown for 6 h in respective medium as per the conditions mentioned in section 2.2.1 of Chapter 2. Cells were harvested from a 1.0 ml aliquot of the culture by centrifugation at 3000 x g for 3 min at 4°C. Genomic DNA was extracted from the bacterial cells by urea-SDS-NaOH method as outlined in Figure 3.1 of Chapter 3.

4b.2.3. Selection of LAB specific 16S rRNA primer for arbitrarily primed PCR (AP-PCR) and in silico melting point analysis

The 16S rRNA full-length gene sequences of 114 LAB strains comprising of genus *Lactobacillus, Lactococcus, Pediococcus* and *Leuconostoc* as well as other bacterial strains belonging to genus *Bifidobacterium*, *Bacillus, Enterococcus, Escherichia, Listeria, Salmonella, Clostridium* and *Streptococcus* were retrieved from GenBank (http: //www. ncbi.nlm.nih.gov). The sequences were aligned using the web-based multiple alignment program (http://www.genomatix.de/cgi-bin/dialign/dialign.pl). A consensus region in the 16S rRNA gene having the sequence 5' AGCTAATCTCTTAAAACC 3' and unique to LAB strains was identified manually. The LAB specific consensus region was demarcated by using EditSeq and MegAlign subprograms of DNASTAR software package (Burland, 2000). An 18-mer primer named as ARAK5 having a nucleotide sequence 5' GGTTTTAAGAGATTAGCT 3' was selected to target the conserved region for AP-PCR based fingerprinting analysis. This primer was earlier used as reverse primer for detection of the genus *Pediococcus* (Chapter 2, Table 2.2).

Melting point (T_m) of the hybridized pair of ARAK5 primer and select conserved 16S rDNA region of bacterial strains were assessed *in-silico*. The two-state hybridization tool of the DINAMelt web server (http://frontend.bioinfo.rpi.edu/applications/hybrid/twostate.php) developed by Markham and Zuker (2005) was used for melting point analysis. The parameters for the calculations were: temperature 40 °C, Na^+ 1 M, Mg^{2+} 1.5 mM and strand concentration 0.01 mM. The difference of T_m (ΔT_m) of mismatch (MM) duplexes with respect to the perfect match (PM) duplex was derived by subtracting T_m of the MM duplexes from T_m of the PM duplex.

4b.2.4. Arbitrarily primed PCR (AP-PCR)

Amplification was performed in a programmable thermal cycler (GeneAmp Gold, Applied Biosystems, USA). PCR reactions were carried out in 25µl reaction volume containing 200 µM of each deoxynucleoside triphosphate (Sibenzyme, Novosibirsk, Russia), 1 unit of *Taq* DNA polymerase (Sibenzyme, Novosibirsk, Russia), 14µM of ARAK5 primer, 1 X *Taq* DNA polymerase buffer (Sibenzyme, Novosibirsk, Russia) and 2 ng template DNA. The amplification conditions were as follows: initial denaturation for 2 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 40°C for 1 min, extension at 68°C for 1 min 30 sec, followed by final 10 min incubation at 68°C. The PCR products were analyzed by agarose gel electrophoresis (0.8%).

4b.2.5. Evaluation of ARAK5 primer for LAB versus non-LAB discrimination

Template DNA was isolated from 12 standard LAB strains belonging to genus *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* as well as 6 non-LAB strains

L. monocytogenes Scott A, *E. faecalis* MTCC 439, *B. cereus* MTCC 1305, *B. subtilis* MTCC 441, *S. aureus* MTCC 96 and *E. coli* MTCC 433, using the method mentioned in section 4b.2.2. AP-PCR was performed with the isolated template DNA using ARAK5 primer under conditions specified in section 4b.2.4. The similarity of fingerprinting profiles obtained from the strains were assessed by determining Dice coefficients and cluster analysis was performed on the basis of unweighted pair group method using arithmetic averages (UPGAMA) using Quantity One software (BioRad, USA) as mentioned in section 3.2.5 of Chapter 3.

4b.2.6. Comparative analysis of rep-PCR and AP-PCR of standard Lactobacillus strains

Genomic DNA isolated from select standard strains of *Lactobacillus* was subjected to rep-PCR based amplification with REP1R-I (5' IIIICGICGICATCIGGC 3') and REP2-I (5' ICGICTTATCIGGCCTAC 3') primer. Reaction conditions for rep-PCR were similar to that mentioned in an earlier investigation (De Angelis *et al.*, 2007). The same *Lactobacillus* standard strains were also subjected to AP-PCR with primer ARAK5 as mentioned before. Estimation of Dice coefficient values and cluster analysis were performed as mentioned in section 4b.2.5.

4b.2.7. Detection of LAB strains in dahi samples

Pasteurized milk samples were procured from local retail outlets and boiled for 15 minutes. The samples were cooled to room temperature and replica samples (5 nos.) were inoculated at 2% level with saline suspensions of LAB cultures *Lactococcus lactis* subsp. *lactis* MTCC 3041 and *Lactobacillus acidophilus* MTCC 447 (10^7 cfu/ml each). The samples were incubated at 37^{o}C for 12-14 h for the formation of *dahi* (similar to curd). For detection

of LAB strains in *dahi* samples, DNA was extracted directly from *dahi* using the method described previously in Chapter 3 section 3.2.6. AP-PCR was performed with the extracted DNA following the method mentioned before. The banding profile obtained from *dahi* samples were compared with that obtained individually from pure cultures of LAB strains (MTCC 3041 and MTCC 447) used for the preparation of *dahi*.

4b.2.8. AP-PCR based fingerprinting of anti-listerial bacteriocin producing (Bac^+) LAB isolates

Twenty five anti-listerial bacteriocin producers (Bac^+) were selected which revealed potent activity against pathogens *Listeria monocytogenes* Scott A, *Staphylococcus aureus* MTCC 96 and *Bacillus cereus* MTCC 1305 and are described in the previous Chapter 4a of the thesis. AP-PCR based fingerprinting analysis of the anti-listerial LAB isolates was performed with ARAK5 primer. The fingerprinting profile of the antagonistic LAB strains were compared with the profiles of select standard LAB strains and cluster analysis was accomplished as mentioned before in section 4b.2.5.

4b.3. Results and Discussion

4b.3.1. Selection of a conserved region of 16S rRNA gene for primer design and in silico analysis

Multiple sequence alignment of 16S rRNA gene sequence of standard LAB strains along with non-LAB strains formed the basis of selecting a region in the 16S rRNA gene which is conserved amongst LAB strains. On manual examination, an 18 mer conserved region of 16S rRNA gene specific to LAB strains could be identified. Figure 4b.1 depicts the alignment of

this conserved region for some of the LAB as well as non-LAB strains and it is quite evident that the consensus region was omnipresent in a large number of LAB strains. A single base mismatch could be observed for certain LAB strains such as *L. reuteri* ATCC 55730, *L. brevis* I218, *L. rhamnosus* Lcr35, *L. plantarum* Lp1, *L. salivarius* subsp. *salivarius*, *L. garvieae* and *S. thermophilus* ATCC 19258. It is also apparent from the Figure 4b.1 that the degree of conservation diminished for certain LAB strains belonging to genus *Leuconostoc* wherein a mismatch was observed in the 3'end of the gene.

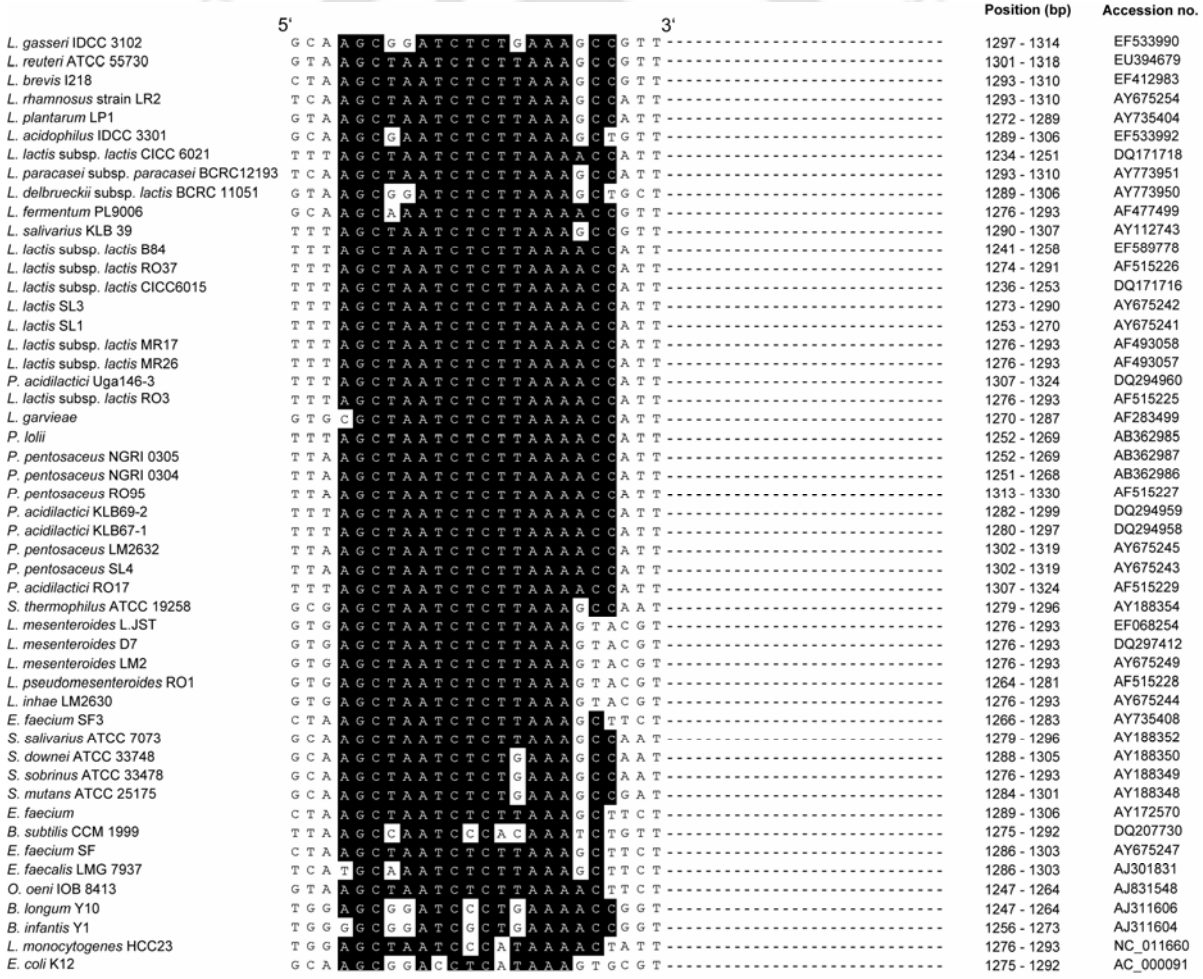

Figure 4b.1 Multiple sequence alignment of 16S rRNA gene from select LAB and non-LAB strains. The shaded portion indicates the 18-mer conserved target region (5'AGCTAATCTCTTAAAACC 3') selected for designing ARAK5 primer.

Interestingly, amongst the non-LAB bacterial strains, mismatches ranging from 3-7 bases could be observed for strains such as *L. monocytogenes* HCC23, *B. subtilis* CCM 1999 and *E. coli* K12.

Considering the implications of the mismatch on primer-target sequence annealing, an *in-silico* prediction of the melting temperature (T_m) of the duplex (hybridized regions of primer-target sequence) was accomplished. Essentially, a perfect match (PM) duplex, which is more thermostable has a higher T_m compared to other forms of duplex containing varying levels of mismatches (MMs). In the present study, the corresponding T_m values for the PM as well as other MMs were determined *in-silico* using the DINAMelt web server (http://frontend.bioinfo.rpi.edu/applications/hybrid/twostate.php) and the differences of T_m (ΔT_m) of PM with respect to each MM duplexes was compared. Table 4b.2 shows the estimated values of ΔT_m corresponding to different types of mismatches at varying positions for select LAB and non-LAB strains. The ΔT_m ranged from 3.6^{o}C to 34.6^{o}C for the considered mismatches. However, it is noteworthy that that the ΔT_m values were considerably low for LAB strains compared to their non-LAB counterparts. It may be construed that amongst the strains the differences in the ΔT_m value is a function of the number and position of mismatched bases as well as the type of mismatch. *In-silico* estimation of the thermal stability of primer-target sequence perfect match (PM) and mismatch (MM) duplexes has been accomplished earlier and a similar inference highlighting the effect of base mismatch was reported (Wu *et al.*, 2009). From Table 4b.2 it is also evident that the highest ΔT_m was obtained for mismatch type 6 (34.6^{o}C) corresponding to *B. subtilis* CCM 1999.

Table 4b.2

In silico prediction of ΔT_m for ARAK5 primer-16S rDNA target region duplex hybrid.

Strain	MM type[a]	Sequence of the duplex hybrid[b]	ΔT_m (°C)[c]
LAB			
L. reuteri ATCC 55730 *L. brevis* I218 *L. rhamnosus* LR2 *L. plantarum* LP1 *S. thermophilus* ATCC 19258 *S. salivarius* ATCC 7073	1	5' AGC TAA TCT CTT AAA **G**CC 3' 3' TCG ATT AGA GAA TTT **T**GG 5'	3.6
S. downei ATCC 33748 *S. sobrinus* ATCC 3347 *S. mutans* ATCC 25175	2	5' AGC TAA TCT CT**G** AAA **G**CC 3' 3' TCG ATT AGA GA**A** TTT **T**GG 5'	6.7
L. mesenteroides L.JST *L. mesenteroides* D7 *L. mesenteroides* LM2 *L. pseudomesenteroides* RO1	3	5' AGC TAA TCT CTT AAA **GTA** 3' 3' TCG ATT AGA GAA TTT **TGG** 5'	4.8
L. gasseri IDCC 3102	4	5'AGC **GG**A TCT CT**G** AAA **G**CC 3' 3'TCG **AT**T AGA GA**A** TTT **T**GG 5'	17.5
L. delbrueckii subsp. *lactis* BCRC 11051	5	5' AGC **GG**A TCT CTT AAA **G**C**T** 3' 3' TCG **AT**T AGA GAA TTT **T**G**G** 5'	14.9
Non-LAB			
B. subtilis CCM 1999	6	5'AGC **C**AA TC**C** C**A**C AAA **T**C**T** 3' 3'TCG **A**TT AG**A** G**A**A TTT **T**G**G** 5'	34.6
L. monocytogenes HCC23 7		5'AGC TAA TC**C** C**A**T AAA AC**T** 3' 3' TCG ATT AG**A** G**A**A TTT TG**G** 5'	19.7
E.coli K12	8	5'AGC **GG**A **C**CT C**A**T AAA **GTG** 3' 3' TCG **AT**T **A**GA G**A**A TTT **TGG** 5'	24.7
S. typhimurium	9	5'AGC **GG**A **C**CT C**A**T AAA **GTG** 3' 3' TCG **AT**T **A**GA G**A**A TTT **TGG** 5'	24.7
Clostridium sp.	10	5'AGC **A**AA TC**C** C**CA** AAA A**TG** 3' 3' TCG **A**TT AG**A** G**AA** TTT T**GG** 5'	25.9

[a]MM: Mismatch. [b]Sequence of base-paired region between ARAK5 primer (lower strand) and 16S rDNA target region (upper strand) from select bacterial strains. Mismatched bases in template and primer are indicated in boldface and underlined. The corresponding T_m values for each duplex were determined *in-silico* using the DINAMelt web server (http://frontend.bioinfo.rpi.edu/applications/hybrid/twostate.php). [c]ΔT_m (°C) was calculated by comparing the differences of T_m (ΔT_m) of perfect match (PM) with respect to each mismatch (MM) duplex.

Evidently the high ΔT_m value can be attributed to a large number of mismatch bases (5 nos.) and the distribution of the mismatched bases at various positions of the duplex hybrid. Overall, the *in-silico* prediction of ΔT_m of primer-target sequence hybrid indicates the possibility of greater thermal stability of the duplex for LAB strains in comparison to the non-LAB strains.

The 16S rRNA gene in LAB strains has been a popular choice as a target in previous investigations which essentially involved detection and community analysis (Dubernet *et al.,* 2002; Settanni *et al.,* 2005; Randazzo *et al.,* 2006; Delbes *et al.,* 2007). A number of research groups have also demonstrated the application of random primers and repetitive element primers for molecular fingerprinting of LAB strains (Hayford *et al.,* 1999; De Angelis *et al.,* 2007; Terzic-Vidojevic *et al.,* 2007; Gevers *et al.,* 2001). Based on the alignment results depicted in Figure 4b.1 it was conceived that the 18 mer region which was unique and noticeably conserved amongst LAB strains could possibly serve as a target for PCR-based fingerprinting analysis. The fact that certain non-LAB strains revealed a greater sequence deviation in the consensus region was also significant and indicated the possibility of generating LAB specific signature patterns and differentiate them from non-LAB strains. In an earlier study, an 18 mer primer targeting a universally conserved region of 16S rRNA gene with a wobble base at 17^{th} position was used to perform triplicate arbitrary primed PCR (TAP-PCR) and fingerprint various strains of LAB genera (Cussick and O'Sullivian, 2000).

4b.3.2. Fingerprinting profile for LAB and non-LAB strains

As evident from the multiple alignment results depicted in Figure 4b.1 the selected primer was essentially targeted to a 16S rRNA conserved region of LAB strains. The next aim was to

explore the possibility of using this primer in AP-PCR based fingerprinting experiments to generate discernible and robust banding patterns from select LAB strains. Besides, it was envisaged that since the primer high similarity to 16S rRNA conserved region present in LAB strains, it would probably yield discriminatory fingerprinting patterns for LAB and non-LAB strains. It is noteworthy from Figure 4b.2 that there was a clear differentiation between LAB and non-LAB strains as evident from the defined fingerprints and clustering patterns obtained from LAB and non-LAB strains. Interestingly, variation in the molecular size of bands obtained from LAB and non-LAB strains was also conspicuous. It was observed that the number of prominent bands obtained from non-LAB strains varied from 2-4, with *S. aureus* yielding the highest number of bands (4 nos.). Overall, major bands obtained from non-LAB strains were found to have an approximate molecular size of 540 bp, 830 bp and 1060 bp. In contrast, LAB strains used in this investigation revealed prominent bands of a higher molecular size in comparison to non-LAB strains.

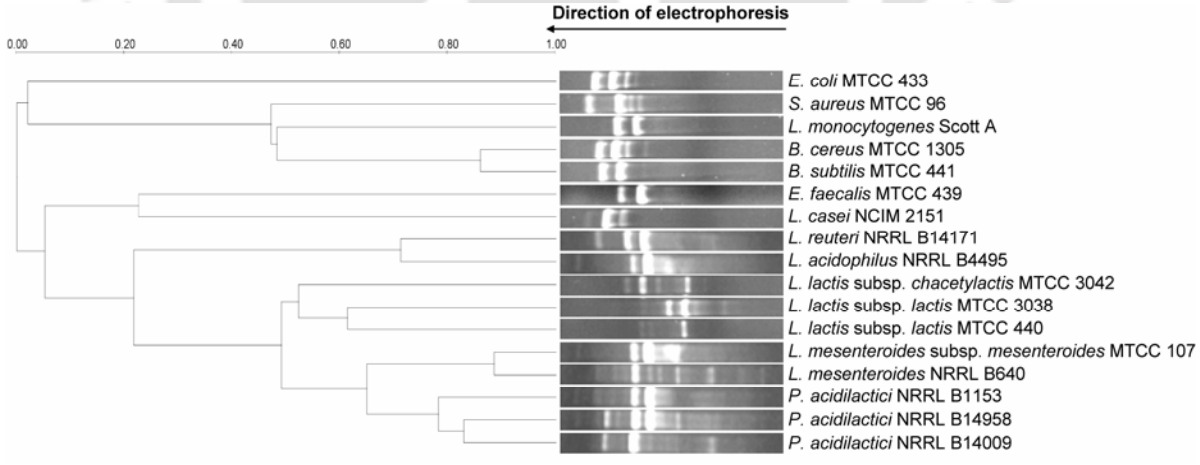

Figure 4b.2 UPGAMA based cluster analysis of the fingerprints generated for LAB and non-LAB strains with ARAK5 primer.

The sizes of the bands obtained from LAB strains were approximately 1214 bp, 1800 bp, 3000 bp and 3400 bp. Additionally, certain LAB strains like *P. acidilactici* NRRL B14009, *L. mesenteroides* NRRL B640 and *L. lactis* subsp. *lactis* MTCC 3038 also gave additional bands of even higher molecular size, albeit of faint intensity. The annealing temperature used in arbitrarily primed-PCR was 40^{o}C. This temperature was adopted based on experiments wherein varying annealing temperatures of 37, 40 and 42^{o}C were used and it was observed that an annealing temperature of 40^{o}C yielded stable bands and reproducible fingerprinting profiles. It is quite clear that primer annealing would occur for both non-LAB as well as LAB strains since annealing was performed at low stringency. However, larger number of bands obtained from LAB strains possibly indicates a greater number of priming sites available during AP-PCR. This could also imply that a greater number of invert repeat targets are available for the primer in case of LAB strains in comparison to their non-LAB counterpart. Also from the *in-silico* analysis (Table 4b.2) it is evident that the thermal stability of the primer-target region duplex was greater for mismatch duplexes present in LAB strains compared to the multiple base mismatches observed in case of non-LAB strains (Figure 4b.1 and Table 4b.2). Efficiency of chain extension in PCR is fundamentally governed by the thermal stability of the primer-target region duplex. Hence it likely that LAB strains would offer the possibility of generating longer PCR products, compared to the non-LAB strains. This is clearly manifested in the defined banding patterns observed in Figure 4b.2. Thus the application of ARAK5 primer in AP-PCR revealed the potential of the primer in differentiating LAB from non-LAB strains. It can also be seen from Figure 4b.2 that the LAB strains clustered together as evident from the high Dice coefficient values. For example, standard strains of *P. acidilactici* (NRRL B14009, NRRL B14958 and NRRL B1153)

displayed a high coefficient value of 0.83. Similarly, standard strains of *L. mesenteroides* (MTCC 107 and NRRL B640) revealed a high Dice coefficient value of 0.86 and thus clustered together. Hence the selected primer used in AP-PCR could not only differentiate broad taxonomic groups encompassing LAB and non-LAB strains, it also rendered reliable grouping of closely related LAB strains as evident from the cluster patterns obtained in the experiment. The discriminatory banding pattern obtained for LAB and non-LAB strains with ARAK5 primer has significant implications for food fermentation analysis. The fact that reliable fingerprinting and delineating cluster analysis of LAB strains could be achieved in the presence of other non-LAB strains suggests that the application of the selected primer in AP-PCR can perhaps confer a distinct advantage for specific detection of LAB strains in food samples, and in other niche applications wherein LAB strain monitoring in fermented food is desirable in the presence of a background population of non-LAB strains.

4b.3.3. Comparative analysis of fingerprinting profiles of Lactobacillus strains

Molecular fingerprinting analysis of LAB strains has been a subject of considerable interest for a large number of research groups and there is substantial literature report on the use of repetitive element based PCR methods (GTG_5, BOXA1R and REP1R-I/REP2-I) for fingerprinting of LAB strains (De Angelis *et al.,* 2007; Terzic-Vidojevic *et al.,* 2007). In the present study, the aim was to ascertain the application potential of the selected ARAK5 primer in generating discriminatory fingerprinting profiles for a set of standard *Lactobacillus* strains (26 nos.) and compare the profiles with that obtained using a conventional fingerprinting primer such as REP1R-I/REP2-I.

The results are depicted in Figure 4b.3 and 4b.4. Essentially, it was observed that the number of prominent bands obtained in arbitrarily primed-PCR using the ARAK5 primer for standard *Lactobacillus* strains varied (Figure 4b.3), whereas in case of rep-PCR using REP1R-I/REP2-I primer the number of discrete bands obtained from the strains was comparatively large (Figure 4b.4).

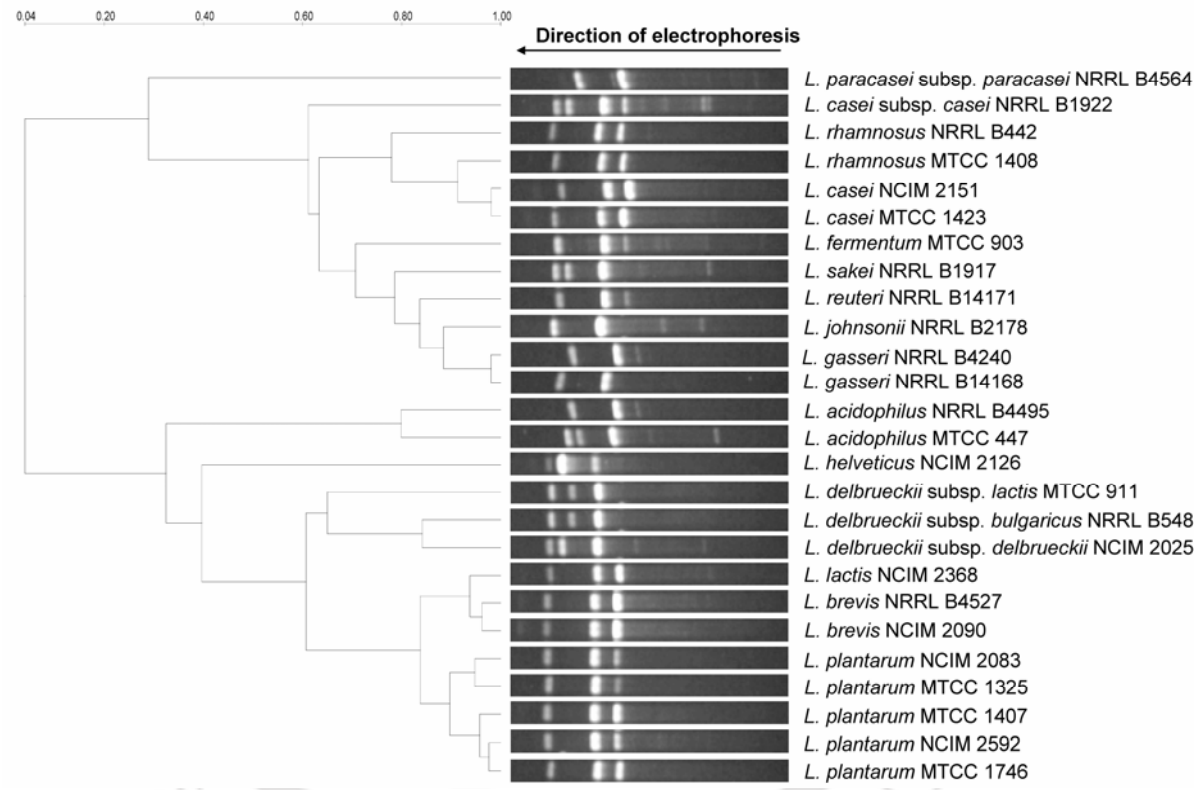

Figure 4b.3 Molecular fingerprints of standard *Lactobacillus* strains generated with ARAK5 primer in AP-PCR and UPGAMA based cluster analysis.

For instance from Figure 4b.3 it is evident that in case of *L. brevis* NRRL B4527 and *L. johnsonii* NRRL B2178, the number of prominent bands obtained in AP-PCR using ARAK5 primer was three and four, respectively. However, when the same strains were subjected to rep-PCR with REP1R-I/REP2-I primers the number of bands obtained was five for both the strains (Figure 4b.4). The propensity of obtaining large number of bands with

REP1R-I/REP2-I primer compared to ARAK5 primer is expected since the former is essentially a random primer targeting a repetitive element (Versalovic *et al.,* 1991) and hence it may be construed that it is likely to encounter a large number of priming sites. A comparative analysis of the fingerprinting profiles shown in Figure 4b.3 and 4b.4 also reveals that higher molecular size bands were more conspicuous for *Lactobacillus* strains in REP1R-I/REP2-I based PCR (Figure 4b.4). For example, in case of *L. plantarum* NCIM 2592, *L. gasseri* NRRL B4240, *L. delbrueckii* subsp. *bulgaricus* NRRL B548 and *L. fermentum* MTCC 903, high molecular size bands were observed in rep-PCR, whereas the same strains failed to yield any high molecular size band in AP-PCR (Figure 4b.3).

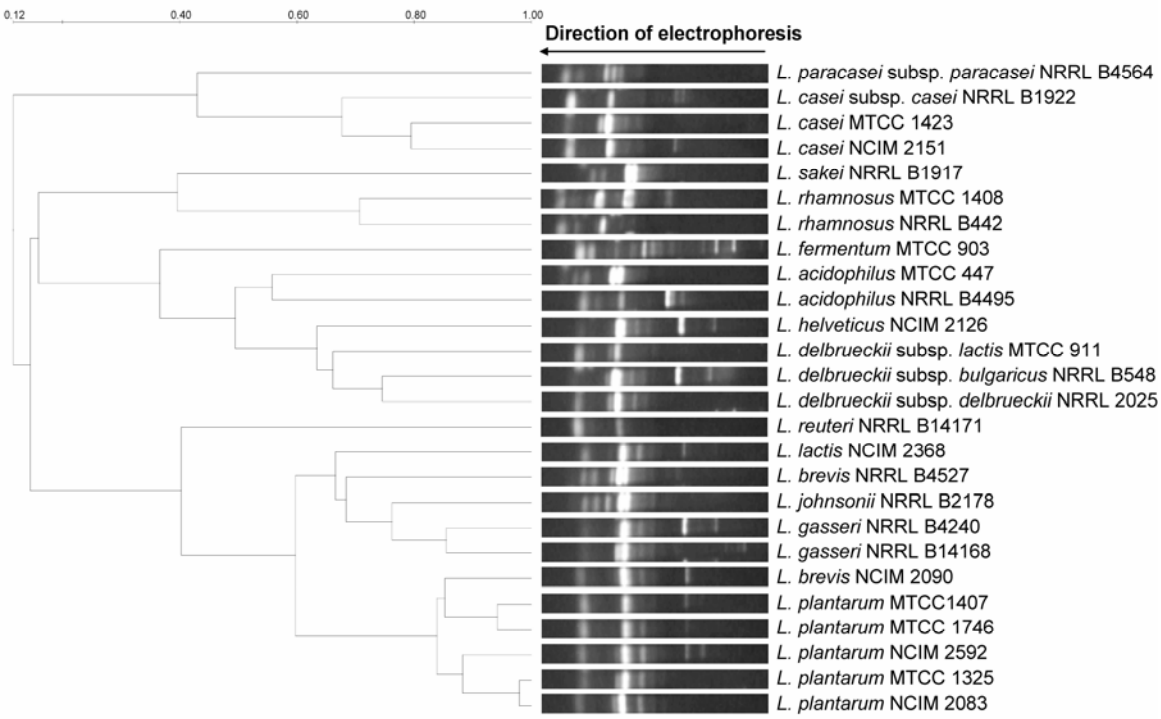

Figure 4b.4 Molecular fingerprints of standard *Lactobacillus* strains generated with REP1R-I/REP2-I primer in AP-PCR and UPGAMA based cluster analysis.

With regard to AP-PCR based fingerprinting analysis using ARAK5 primer, a pertinent point to be considered is that the number of bands obtained as well as the intensity

of the bands can be influenced by the number of ribosomal RNA (rrn) operons present in LAB strains. For example, it is established that the number of *rrn* operons vary in strains of *L. plantarum*, *L. lactis* and *L. johnsonii* (de Vries *et al.,* 2006) and hence these strains would display variations in the fingerprinting profiles in terms of number of bands and band intensity, when the ARAK5 primer targeting the 16S rRNA gene is used in arbitrarily primed-PCR.

It is also evident from Figure 4b.3 and 4b.4 that distinct clusters based on Dice coefficient values were obtained for reference *Lactobacillus* strains. In case of AP-PCR with ARAK5 primer, various species of *Lactobacillus* could be discriminated at around 0.90 Dice coefficient value (Figure 4b.3), whereas the same degree of discrimination could be obtained for *Lactobacillus* strains in rep-PCR using REP1R-I/REP2-I primers at an equivalent Dice coefficient value of 0.88. The overall clustering of reference strains of *Lactobacillus* was concordant with the expected pattern based on genetic relatedness amongst the various species of *Lactobacillus*. For instance, it is established that strains belonging to *L. casei*, *L. paracasei* and *L. rhamnosus* comprise the *L. casei* group (Song *et al.,* 2000; Ryu *et al.,* 2001). As seen from AP-PCR based fingerprinting profiles indicated in Figure 4b.3, *L. casei* subsp. *casei* NRRL B1922, *L. rhamnosus* NRRL B442 and MTCC 1408, *L. casei* NCIM 2151 and MTCC 1423 displayed similarity as evident from a Dice coefficient value of around 0.60 and thus clustered in close proximity. In rep-PCR based fingerprinting it can be observed that *L. casei* subsp. *casei* NRRL B1922, *L. casei* MTCC 1423 and NCIM 2151 clustered in close vicinity with a Dice coefficient value of around 0.65. It is noteworthy that reliability of the AP-PCR based method for estimation of genetic diversity and strain grouping was superior as strains of *L. rhamnosus* MTCC 1408 and NRRL B442 were grouped in the *L. casei* cluster in

accordance with expected genetic similarity (Figure 4b.3), whereas in case of rep-PCR based fingerprinting, these strains were delineated from the *L. casei* cluster (Figure 4b.4). Also, in case of strains of *L. gasseri* (NRRL B14168 and NRRL B4240) and *L. johnsonii* NRRL B2178, the similarity index was quite high based on Dice coefficient values (0.88) in AP-PCR (Figure 4b.3) whereas in case of rep-PCR analysis the same strains revealed a Dice coefficient value of 0.76 (Figure 4b.4). It can be mentioned that *L. johnsonii* and *L. gasseri* are genetically related and belong to the *L. acidophilus* group (Ryu *et al.,* 2001). Strains of *L. plantarum* (MTCC 1746, NCIM 2592, MTCC 1407, MTCC 1325 and NCIM 2083) were observed to cluster together in both AP-PCR and rep-PCR and exhibited high Dice coefficient values of 0.88 and 0.86, respectively. In case of *L. brevis*, it may be emphasized that in AP-PCR based fingerprinting analysis, the strains NCIM 2090 and NRRL B4527 clustered along with strains of *L. plantarum* which is conformity with the taxonomic proximity of these strains (Makarova and Koonin, 2007). However, in rep-PCR based analysis it was seen that only *L. brevis* NCIM 2090 grouped with strains of *L. plantarum*. The multiple banding patterns obtained for the other reference strain of *L. brevis* (NRRL B4527) were evidently different, which resulted in assignment of a distant cluster (Figure 4b.4). Overall, the cluster analysis results obtained from the standard LAB strains revealed that the AP-PCR method was at par with a conventional rep-PCR based fingerprinting technique. In certain instances, the results obtained from AP-PCR were even superior to rep-PCR, based on conformity with expected patterns from strains showing close phylogenetic affiliations. The reproducibility of the banding pattern obtained with AP-PCR using the selected ARAK5 primer was also evaluated for select *Lactobacillus* strains. Triplicate samples of template DNA isolated separately from each LAB strain was subjected to arbitrarily primed-PCR analysis. No

qualitative differences in banding patterns were observed and thus similar clusters of the strains were reproduced.

4b.3.4. Detection of LAB strains in dahi samples

It was evident from the results obtained in previous experiments that the ARAK5 primer could be used in AP-PCR to differentiate LAB from non-LAB strains as well as in fingerprinting applications. Thus the next objective was to use the primer and explore its potential in detecting LAB starter cultures in a fermented product. *Dahi*, a lactic cultured milk product was chosen as a model fermented product for the experiments. The result of the AP-PCR profile obtained from a representative *dahi* sample along with the profiles obtained from pure cultures of the LAB starter strains MTCC 3041 and MTCC 441 is depicted in Figure 4b.5. It is evident from the figure that discrete bands could be obtained from the *dahi* sample when subjected to AP-PCR (Figure 4b.5, lane 3).

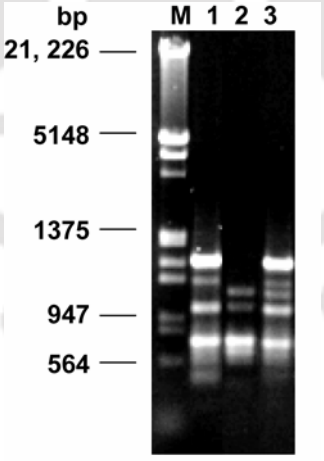

Figure 4b.5 Direct detection of the starter cultures *Lactococcus lactis* subsp. *lactis* MTCC 3041 and *Lactobacillus acidophilus* MTCC 447 in *dahi* using AP-PCR in conjunction with ARAK5 primer. Banding profiles obtained from pure culture of *Lactococcus lactis* subsp. *lactis* MTCC 3041 and *Lactobacillus acidophilus* MTCC 447 are shown in lane 1 and 2 respectively. Lane 3 indicates banding profile obtained from *dahi* sample. M: λ DNA double digest size marker.

Interestingly, AP-PCR with ARAK5 primer resulted in multiplex detection of the LAB strains in *dahi* as evident from the signature bands obtained from the LAB starter cultures used in the experiments. The presence and persistence of both the strains in the finished product was thus established. Similar results were obtained for all the five *dahi* samples, which indicated the reproducibility of the arbitrarily primed-PCR based detection method.

Lactic acid bacteria are central to food fermentation processes and starter culture applications of LAB strains hold significant implications for the food industry (Coolbear *et al.,* 2008). Strain tracking in a fermented product is a fundamental requirement to gain a useful insight of the behaviour of LAB starter strains during the course of fermentation and its prevalence in the finished product. Molecular methods are widely considered as sensitive and convenient means of studying the persistence and behaviour of LAB strains used for the formation of a fermented product. For instance, PCR-based methods have been used to monitor the persistence of LAB strains during cheese ripening (Bouton *et al.,* 2002). A large number of reports suggest the use of molecular techniques such as PCR-DGGE to monitor community development as well as strain persistence in food products (Randazzo *et al.,* 2002; Rantsiou and Cocolin, 2006; Cocolin *et al.,* 2006). In the present study, the presence of LAB starter strains in *dahi*, an LAB fermented milk product has been demonstrated. Significantly, diagnostic banding patterns corresponding to the LAB strains used for the formation of *dahi* samples were observed in AP-PCR, which enabled multiple strain detection in the finished product. Based on the results obtained in the present study, it is envisaged that the AP-PCR based method can be extended to other analogous applications to enhance confidence levels of microbiological quality control and monitoring persistence of starter LAB strains in fermented products.

4b.3.5. Estimation of genetic diversity of anti-listerial bacteriocin producing LAB isolates by AP-PCR

It is clear from the results obtained in previous sections that the AP-PCR based fingerprinting analysis based on the designed ARAK5 primer lead to explicit discriminatory patterns between LAB and non-LAB strains. Additionally the same primer also rendered species level differentiation of selected *Lactobacillus* reference strains. Thus it was conceived that the primer holds considerable promise in AP-PCR based fingerprinting analysis of native LAB strains. The application of rep-PCR for molecular fingerprinting of LAB strains has been demonstrated earlier in case of *L. sanfrancisensis* isolates (De Angelis *et al.,* 2007) and for molecular typing analysis of a probiotic strain of *L. rhamnosus* 35 (Coudeyras *et al.,* 2008). In a previous investigation highlighted in Chapter 3, fingerprinting analysis of bacteriocin producing LAB strains using BOXA1R primer in rep-PCR was performed.

In the present study, we were motivated to ascertain the application potential of the ARAK5 primer in arbitrarily primed-PCR based typing analysis of anti-listerial bacteriocin producing (Bac^+) LAB strains isolated previously from diverse indigenous source (Chapter 4a). The present endeavor was based on the premise that the promise of the primer in yielding discriminatory patterns in arbitrarily primed-PCR analysis could be extended to determine the genetic diversity of various native Bac^+ LAB strains and decipher strain specific signature patterns. The anti-listerial bacteriocin producing LAB strains (25 nos.) chosen for the study were previously isolated from *dahi*, dried-fish and salt-fermented cucumber and identified as *Lactobacillus* sp. and *Pediococcus* sp. by PCR (Chapter 4a).

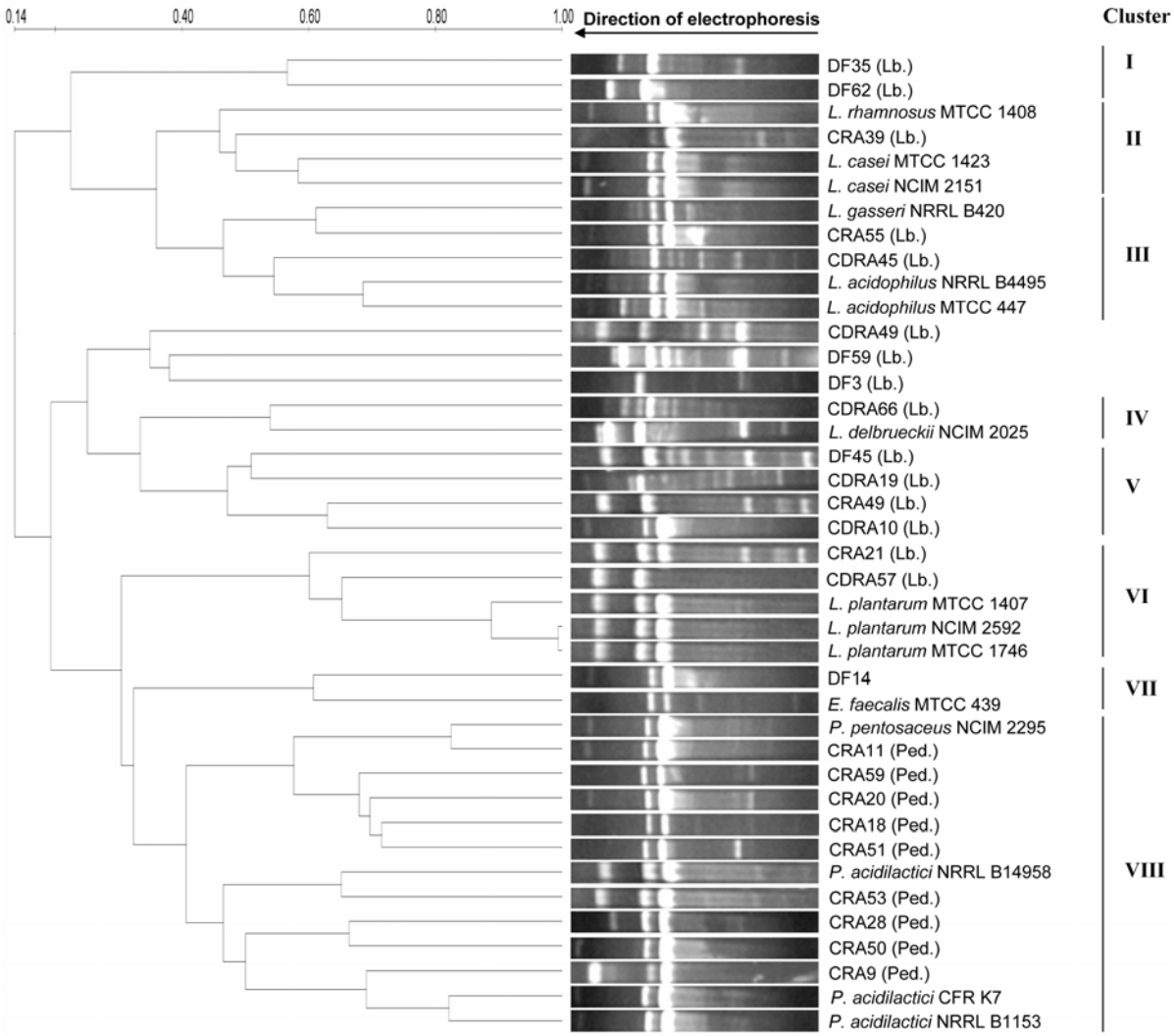

Figure 4b.6 UPGAMA based cluster analysis of native bacteriocin producing (Bac^+) LAB strains. Clusters were generated on the basis of Dice coefficient values of banding pattern obtained in arbitrarily primed-PCR. Bac^+ strains were isolated from *dahi* (CDRA), fermented cucumber (CRA) and dried fish (DF). Genus identity of Bac^+ native LAB isolates is indicated in parentheses (*Lactobacillus*: Lb; *Pediococcus*: Ped).

The fingerprinting profiles of native Bac^+ isolates were compared with the standard strains to calculate the Dice coefficient values and generate the clusters to identify their taxonomic affiliation (Figure 4b.6). It is quite apparent that the bacteriocin producing LAB strains yielded profiles which lead to generation of distinct clusters. It was interesting to observe that

while the reference strains chosen essentially yielded 2-4 prominent bands in AP-PCR, multiple bands could be seen for a number of native bacteriocin producing LAB strains (for example strains CDRA49, DF59, DF45, CRA21, CRA49), indicating a clear genetic heterogeneity existing amongst the anti-listerial bacteriocin producing (Bac^+) LAB isolates. At a Dice coefficient value of around 0.40, eight distinct clusters could be delineated. Cluster I, IV and VII consisted of two LAB strains of which cluster I included solely Bac^+ isolates (DF35 and DF62). The isolate DF14 grouped with the standard strain of *E. faecalis* MTCC 439 in cluster VII with a Dice coefficient value of 0.61. In cluster II, isolate CRA39 was the only Bac^+ isolate and this isolate was observed to group with reference LAB strains belonging to the *L. casei* group (Figure 4b.6). Cluster III included 2 Bac^+ LAB isolates (CRA55 and CDRA45) which grouped together with strains belonging to *L. gasseri* and *L. acidophilus*. Cluster V was unique and solely consisted of 4 bacteriocin producing LAB isolates of which the majority was obtained from *dahi* (CDRA19, CDRA49 and CDRA10). In cluster VI it was observed that 2 Bac^+ LAB strains (CRA21 and CDRA57) revealed reasonably high similarity and grouped with standard strains of *L. plantarum* (Dice coefficient value of around 0.60). Cluster VIII was the largest and consisted of 9 Bac^+ LAB strains and 4 standard LAB strains belonging to the genus *Pediococcus.*

In the present study, the overall clustering observed for the native anti-listerial Bac^+ LAB isolates was in agreement with their expected taxonomic affiliations. In routine fingerprinting analysis using random amplification protocols a low stringency annealing is adopted to enhance the possibility of generating multiple bands from bacterial strains. However, the primary concern is the criteria of discrimination amongst strains and the challenge is to obtain perceptible variations in the banding patterns from the strains and use

the patterns for generating reliable grouping. In the present study a primer specific to a conserved region of the 16S rRNA gene of LAB strains was chosen and used for fingerprinting analysis of anti-listerial bacteriocin producing LAB isolates. Significantly, the application potential of the primer in detecting polymorphism amongst Bac^+ LAB isolates obtained from diverse source was realized as prominent discriminating patterns were obtained in AP-PCR.

4b.4. Conclusion

1. In the present study a primer targeting a region of the 16S rRNA gene which is unique and conserved amongst a large number of LAB strains was selected. Application of the primer in AP-PCR based fingerprinting analysis resulted in significant discrimination of LAB and non-LAB strains.

2. The taxonomic value of the primer was further substantiated as defined and stable fingerprinting patterns could be obtained from a number of standard strains of *Lactobacillus* and the cluster analysis was comparable and even superior in some cases when compared to the results obtained with a conventional primer such as REP1R-I/REP2-I.

3. A significant application of the primer was realized when two standard LAB strains used as starter cultures for the preparation of the fermented milk product *dahi* could be simultaneously detected in the product in a culture-independent manner based on diagnostic banding patterns.

4. Finally, a salient achievement of the study was the application of the primer in AP-PCR based fingerprinting of select anti-listerial bacteriocin producing LAB strains and

determination of their genetic diversity. Overall, the study clearly revealed the versatility and the resolving strength of the primer and it is envisaged that there is an enormous scope of applying the AP-PCR based method described in this work for future food fermentation and microbiological quality control applications.

In the next chapter of the thesis, species level identification of three potent anti-listerial bacteriocin producing LAB isolates CRA21, CRA51 and DF14 is described. Further, bacteriocin purification and determination of the minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) of bacteriocin from select anti-listerial LAB isolates is also discussed. In the final part of the chapter a detailed comparative analysis of the antimicrobial activity of bacteriocin from three potent anti-listerial LAB isolates CRA21, CRA51 and DF14 is described.

CHAPTER 5

Comparative studies on the functional characterization of potent anti-listerial bacteriocin produced by lactic acid bacteria

ABSTRACT

The aim of the present study carried over in this part of thesis was to accomplish biochemical and molecular identification of three potent anti-listerial bacteriocin producing LAB strains and perform a comparative analysis of the properties of the anti-listerial bacteriocin. On the basis of results obtained from carbohydrate fermentation tests and analysis of 16S rRNA gene sequence, the potent anti-listerial LAB isolates were identified as *Lactobacillus plantarum* CRA21, *Pediococcus pentosaceus* CRA51 and *Enterococcus faecium* DF14. A cell adsorption method used for purification of pediocin produced by strain CRA51 indicated its molecular size as approximately 4.5 kDa, with retention of the biological activity of the peptide. The minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) of the purified bacteriocin elaborated by potent anti-listerial LAB isolates were in the range of 83.3-266.6 and 133.3-533.3 AU/ml, respectively. Flow cytometric analysis of carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) labelled target cells of *Listeria* clearly demonstrated the bactericidal effect of pediocin CRA51. cFDA-SE dye-leakage assay as well as propidium iodide (PI) uptake experiments revealed the membrane-permeabilizing effect of the bacteriocin produced by strains CRA21, CRA51 and DF14. These findings were also substantiated by fluorescence microscopy and transmission electron microscopic (TEM) analysis.

5.1. Introduction

Lactic acid bacteria play a pivotal role in food fermentation processes owing to their starter culture traits, ability to confer aroma and texture to food products (Di Cagno *et al.,* 2008; Coolbear *et al.,* 2008; Leroy and De Vuyst, 2004; van Hylckama Vlieg and Hugenholtz, 2007). Further as a consequence of production of antimicrobial compounds such as bacteriocin, LAB have been exploited for biopreservation (Castelano *et al.,* 2008; Drider *et al.,* 2006 ; Jagannath *et al.,* 2001; Jones *et al.,* 2008 ; Settani and Corsetti, 2008). Amongst several antimicrobial compounds produced by LAB, bacteriocins have come to the forefront as they are considered to be safe natural biopreservatives, are likely to be degraded by the proteases in the gastrointestinal tract and hold considerable promise as a primary hurdle for controlling food-borne pathogens (Cleveland *et al.,* 2001).

In the context of food safety, *Listeria monocytogenes* has emerged as a serious threat, as the pathogen can contaminate food at pre-and post-harvest stages of production. Besides, the psychrotrophic and acid tolerant nature of *Listeria* is of grave concern (Le Marc *et al.,* 2002). In recent years there has been a growing interest in application of bacteriocin producing LAB to counter the challenge of *Listeria* since bacteriocins such as nisin and pediocin-like Class IIa bacteriocins exhibited significant anti-listerial activity (Drider *et al.,* 2006; Montville and Chen, 1998).

In the previous investigation (Chapter 4a), several anti-listerial bacteriocin producing LAB isolates were obtained from indigenous samples of *dahi*, dried fish and fermented cucumber. Potent anti-listerial LAB isolates were also detected based on antimicrobial assay and food application experiments. In this investigation, species level identification of three potent anti-listerial bacteriocin LAB isolates is reported. Purification of the bacteriocin

molecule by a cell adsorption method and determination of minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) is also described. Application of flow cytometry and specific fluorescent probes to decipher the mode of actin of bacteriocin on target cells is also discussed.

5.2. Materials and Methods

5.2.1. Identification of potent anti-listerial LAB isolates

Three potent anti-listerial LAB isolates CRA21, CRA51 and DF14 were selected for species level identification. These isolates were chosen primarily based on the premise that the isolates revealed significant anti-listerial activity as scored on the basis of analysis of variance (ANOVA) statistical results (section 4a.3.3, Chapter 4a). The bacteriocins produced by these isolates were identified as plantaricin A, pediocin and enterocin A, respectively (section 4a.3.2, Chapter 4a). The tests performed for species level identification included standard biochemical tests and 16S rRNA gene sequence analysis and the details of the experiments are described in the following section.

5.2.1.1. Biochemical characterization

Standard sugar fermentation tests were conducted to identify the selected anti-listerial LAB isolates CRA21, CRA51 and DF14. These tests were conducted using the HiCarbohydrate^{TM} kit (HiMedia, Mumbai) and following the instructions provided by the manufacturer. In short, anti-listerial LAB isolates (CRA21, CRA51 and DF14) were grown overnight for 16 h at 37^{o}C under static condition. An aliquot of 1 ml from overnight grown culture was centrifuged at 5687 x g for 3 min at 4^{o}C and washed twice with 0.85% NaCl. The cell pellet was finally

resuspended in 1 ml of 0.85% NaCl and 50 µl aliquot was added to each well of the HiCarbohydrate^{TM} kit strip and kept for incubation for 24 h at 37^{o}C in static incubator. Following 24 h of incubation the strips were observed and the response of the cultures was scored based on the color change.

5.2.1.2. 16S rRNA gene sequence and phylogenetic analysis

DNA was isolated from the anti-listerial LAB isolates CRA21, CRA51 and DF14 using the urea-SDS-NaOH method as mentioned in section 3.2.2 in Chapter 3. For isolates CRA21 and CRA51, the 16S rRNA gene was amplified by PCR using *Lactobacillus* and *Pediococcus* genus specific primers as mentioned in Chapter 2 whereas for isolate DF14, PCR was performed with *Enterococcus* 16S rRNA gene specific primers (Deasy *et al.,* 2000). The PCR amplicons were sequenced at National Facility for Automated DNA Sequencing, Department of Biochemistry, University of Delhi South Campus (UDSC). Homology search for the sequences was performed by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The accession numbers for 16S rRNA gene sequence of isolates CRA21, CRA51 and DF14 were FJ424061, FJ424059 and FJ424060, respectively. The partial 16S rRNA gene sequence of isolate CRA21, CRA51 and DF14 were aligned with the available 16S rRNA gene sequence of strain belonging to genus *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* and *Enterococcus*. The phylogenetic tree was drawn on the basis of similarity in the aligned sequence by using Neighbor-joining algorithm available online (www.phylogeny.fr).

5.2.2. Scanning electron microscopy (SEM)

Scanning electron microscopic (SEM) analysis was performed to examine the morphology of potent anti-listerial LAB isolates CRA21, CRA51 and DF14. The LAB isolates were grown overnight in MRS broth at 37°C in static incubator. An aliquot of 1.0 ml culture broth (approximately 10^9 cfu) was centrifuged at 5687 x g for 5 min at 4°C. The cell pellet was washed twice with 10 mM phosphate buffer and resuspended in 2.5% glutaraldehyde solution for 2 h at 8°C for fixation. Fixed cells were washed twice with phosphate buffer to remove residual glutaraldehyde and cells were finally washed and resuspended in MilliQ sterile water. Resuspended cells were diluted 50 times in MilliQ sterile water and 5 µl aliquots of cell suspension was placed on SEM stubs and air dried under laminar hood. Subsequently, fixed and dehydrated cells were coated with gold plasmon and observed under scanning electron microscope (Leo, 1430 vp) at 15 kV and the image was acquired as Tagged image file format (TIFF) file.

5.2.3. Bacteriocin purification and comparative analysis of MIC and MKC

Purification of the bacteriocin produced by potent anti-listerial LAB isolates (6 nos.) was accomplished by the cell-adsorption method described by Halami *et al.* (2005) as follows: Briefly, MRS medium (1.0 litre) was inoculated with overnight grown anti-listerial bacteriocin producing LAB isolates and incubated at 37°C for 24 h in static condition. The culture broth was heat inactivated at 70°C for 30 min and the pH of the culture broth was adjusted to 6.0 and stirred for 2 h to allow adsorption of bacteriocin present in the culture broth onto the producer cells. The cell-adsorbed bacteriocin was recovered by centrifugation at 8000 x g for 15 min at 4°C. The cells were washed twice by resuspending in 25 ml of

10 mM phosphate buffer (pH 6.0), followed by centrifugation at 8000 x g for 15 min at 4°C and draining off the buffer washings. Subsequently the cells were resuspended in 25 ml of 100 mM NaCl solution and the pH of the solution was adjusted to 2.0 by gradual addition of 5% phosphoric acid. The mixture was stirred in the cold room at 4°C for 18 h and then centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was filtered through a 0.22 µm sterile disposable filter (Millipore, USA) and dialyzed exhaustively for 24 h against de-ionized water using a 1.0 KDa cut-off dialysis bag (Sigma, USA). The solution was freeze-dried in a lyophilizer (ALPHA 1-4 LD, CHRIST, Germany) and the purified sample was stored in -20°C till subsequent use. Purity of the bacteriocin preparation was analyzed by Tricine SDS-PAGE (Schagger & von Jagow, 1987) and determination of bacteriocin activity in the gel was accomplished by the method described by Bhunia *et al.* (1987).

Bacteriocin titer in the purified sample was ascertained against *L. monocytogenes* Scott A by a spot-on-lawn assay (Halami *et al.,* 2000) and activity was expressed in terms of arbitrary units per ml (AU/ml). The minimum inhibitory concentration (MIC) of the purified bacteriocin samples (reconstituted in 10mM phosphate buffer, pH 7.0) from select anti-listerial LAB isolates was determined by adding a double dilution series of the bacteriocin solution into culture tubes inoculated with *Listeria monocytogenes* Scott A. MIC was defined as the highest dilution of the bacteriocin sample that prevented growth of the target bacteria as observed by the lack of visual turbidity (Pucci *et al.,* 1988). An aliquot (1% v/v) from all the culture tubes which lacked visual turbidity was re-inoculated into fresh broth medium and incubated to observe the target cell growth. Minimum killing concentration (MKC) of the bacteriocin sample was expressed as the highest dilution (lowest bacteriocin concentration) that prevented growth of the target bacterial cells following re-inoculation, as observed by the

lack of visual turbidity. The MIC and MKC values were expressed as average values of three independent experiments and ANOVA analysis was performed as mentioned before (section 4a.2.5, Chapter 4a).

5.2.4. Functional assay for bacteriocin

5.2.4.1. Flow cytometry

The effect of pediocin CRA51 on the viability of *L. monocytogenes* Scott A was also studied by flow cytometry (FCM), which was performed on a FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with a 15-mW, 488-nm, air-cooled argon ion laser. Target cells of *L. monocytogenes* Scott A were pre-labelled with cFDA-SE following the protocol as mentioned in section 4a.2.9.2 of Chapter 4a. Labelled *Listeria* cells were treated with 6400 AU/ml purified pediocin CRA51 sample for 6 h at 37°C in circulating water bath incubator (Amersham, USA). Following treatment, treated cells were analyzed along with unlabelled-untreated cells as well as labelled-untreated (control) cells. For FCM analysis, approximately 10^6 cells per ml were delivered at a low flow rate setting (150-500 cells per second) and the FACS instrument was adjusted to acquire 20,000 fluorescent events. All the signals were collected in logarithmic mode. Green fluorescence of cFDA-SE stained cells was detected in the FL1 channel (band pass filter of 530 nm). Before analysis of treated samples, appropriate voltage and threshold parameter were adjusted for unlabeled-untreated cells. The corresponding signal of unlabeled-untreated cells was set in the lower left quadrant in order to negate cellular autofluorescence. Data were acquired and analyzed with the CellQuest Pro software (BD CellQuest™ Pro Version 6.0, Becton-Dickinson, USA) and the WinMDI software program WinMDI ver 2.9 (http://en.bio-

soft.net/other/WinMDI). Acquisition of fluorescence data was accomplished by setting a gate in the forward-angle light scatter (FSC) vs. sideward scatter (SSC) plot, which facilitated discrimination of bacterial cells from other artifacts. The data were analyzed with the aid of statistical tables, which indicated the percentages of stained cells determined by the detector.

5.2.4.2. Fluorescent-dye based assay to determine target cell viability and membrane damage

A comparative analysis of the potency of the bacteriocin produced by anti-listerial LAB isolates CRA21, CRA51 and DF14 was performed using the fluorescent probes: 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) and propidium iodide (PI). cFDA-SE essentially stains viable cells owing to intracellular esterase activity of viable bacteria, whereas PI is a DNA-intercalating dye which is normally impermeable to viable cells. However, membrane compromised bacterial cells can take up PI, which upon intercalation with bacterial DNA shows intense red fluorescence. A schematic representation of bacterial cell interaction with cFDA-SE and PI is shown in Figure 5.1. Target cells of *Listeria monocytogenes* Scott A and *Staphylococcus aureus* MTCC 96 were labeled with cFDA-SE (M_r 557.46; Sigma-Aldrich, USA) following the method outlined by Lee *et al.* (2004). In short, overnight grown cells of *L. monocytogenes* Scott A and *S. aureus* MTCC 96 were recovered by centrifugation at 3,000 x g for 10 min. The cell pellet was washed twice with sterile phosphate buffer and labeled with cFDA-SE (final concentration of 50 μM) at 37°C for 20 min. Termination of the labeling reaction was accomplished by pelleting and washing of bacterial cells twice with phosphate buffer to remove excess cFDA-SE molecules.

The labeled target cells were resuspended in 1.0 ml sterile buffer and 10^6 cfu/ml of labeled cells were used for bacteriocin assay.

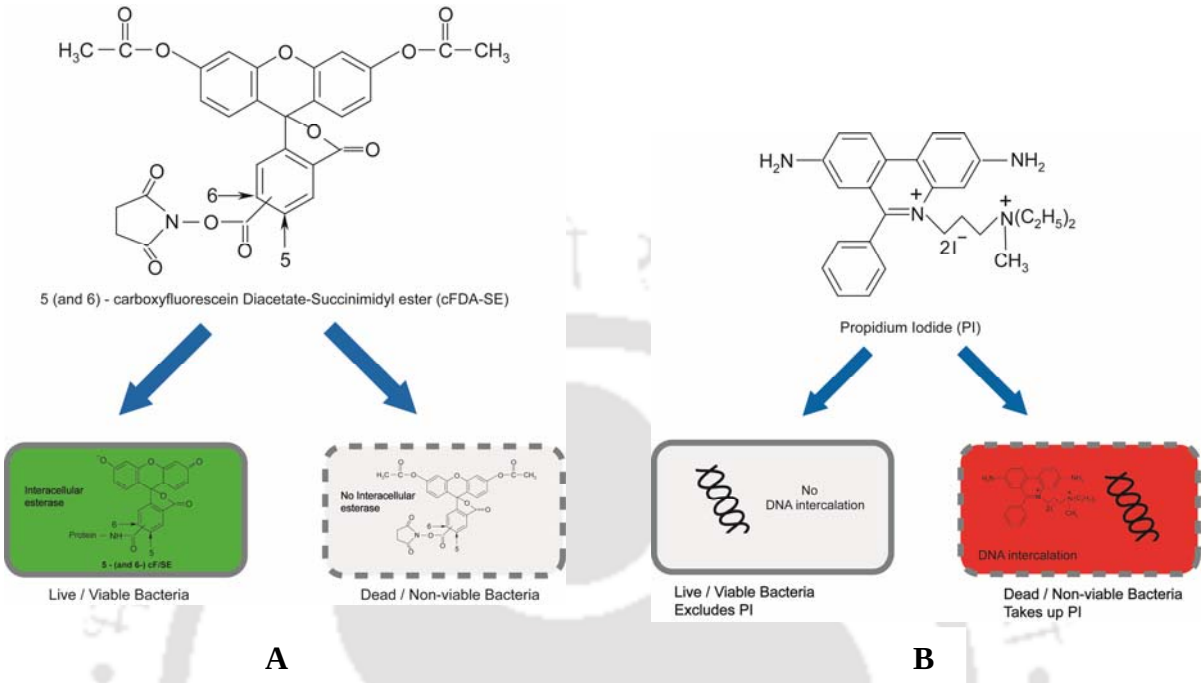

Figure 5.1 Schematic representation of fluorophore used for differential staining of live and dead bacterial cell **(A)** carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) and **(B)** propidium iodide (PI).

Purified bacteriocin sample obtained from isolates CRA21, CRA51 and DF14 were added at varying concentrations (400, 800, 1600, 3200 and 6400 AU/ ml) separately to cFDA-SE labeled cells of *L. monocytogenes* Scott A and *S. aureus* MTCC 96 in phosphate buffer and incubated at 37°C for 6 h in a water bath (Amersham, USA). A control sample of labelled cells was incubated in buffer under same conditions without bacteriocin. Following 6 h of incubation, all the samples were centrifuged at 8000 x g for 5 min. Leakage of carboxyfluorescein from cells was determined by measuring fluorescence of the cell free supernatant at λ_{ex} of 488 nm and λ_{em} at 518 nm in a fluorescence spectrophotometer (FluoroMax-3, HORIBA). The fluorescence measurements were recorded after subtracting

the fluorescence of effluxed dye from control cells. Fluorescence measurement were taken for three independent samples and expressed as mean values.

The integrity of the membrane of bacteriocin-treated target cells was determined by using DNA binding dye, propidium iodide (PI) of relative molecular weight M_r 668 (Sigma-Aldrich, USA). A 1.5 mM stock solution of PI was prepared in sterile MilliQ water and stored at 4°C. Cells of *L. monocytogenes* Scott A and *S. aureus* MTCC 96 (10^6 cfu/ml) were treated with varying concentrations (400, 800, 1600, 3200 and 6400 AU/ ml) of the purified bacteriocin preparation from isolate CRA21, CRA51 and DF14 as mentioned previously. Target cells incubated in phosphate buffer under same conditions without bacteriocin was included as control. Following bacteriocin treatment, cells were washed with phosphate buffer to remove bacteriocin and PI was added to the cells at a final concentration of 30 µM. After 10 min of incubation in circulating water bath incubator (Amersham, USA) set at 37°C, samples were centrifuged and washed in distilled water to remove excess dye. Fluorescence was measured with a fluorescence spectrophotometer (FluoroMax-3, HORIBA) at λ_{ex} 535 nm and λ_{em} 617 nm. Fluorescence data for each sample were normalized with the optical density at 617 nm. The values obtained for untreated cells were subtracted from all experimental values. A schematic representation of the working principle of fluorescent dye based assay to decipher mode of action of bacteriocin and quantification of target cell damage is indicated in Figure 5.2.

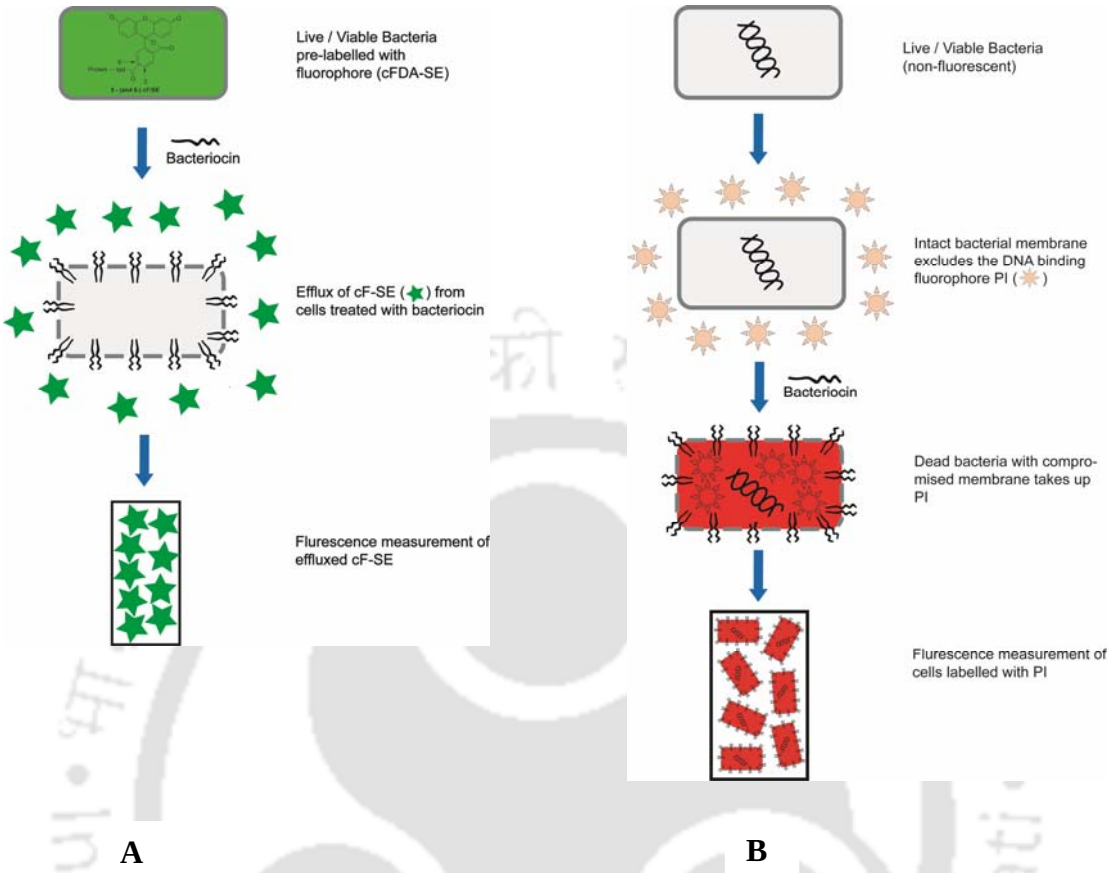

Figure 5.2 Schematic representation of the principle of fluorescent dye based assay to ascertain mode of action of bacteriocin and quantification of target cell damage with fluorophore. **(A)** 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) and **(B)** Propidium iodide (PI).

5.2.4.3. Fluorescence microscopy

Fluorescence microscopic studies were performed to study the effect of bacteriocin on target cells of *Listeria monocytogenes* Scott A. *Listeria monocytogenes* Scott A cells were labeled with cFDA-SE as mentioned in section 5.2.4.2. A purified bacteriocin sample obtained from isolate CRA21, CRA51 and DF14 was added to labeled target cells (10^6cfu/ml) at a concentration of 3200 AU/ml and incubated for 6 h at 37°C. Control samples comprised of labeled target cells incubated in phosphate buffer under the same conditions without bacteriocin. Treated as well as control target cells were fixed in 2.5% glutaraldehyde and

washed twice with phosphate buffer. A 10 µl aliquot of the fixed sample was spotted on a clean glass slide, air dried and observed under fluorescence microscope (Axioskop2MAT, Carl Zeiss, Oberkochen, Germany) with a filter that allowed blue light excitation at 445-495 nm and a long pass filter above 518 nm as an emission wavelength. Images of the treated and control cells were recorded to study the effect of bacteriocin. In case of PI staining, unlabelled target cells of *L. monocytogenes* ScottA were treated with bacteriocin sample from isolate CRA21, CRA51 and DF14 as mentioned previously. Following treatment with bacteriocins, cells were washed to remove bacteriocin, labelled with PI as mentioned in section 5.2.4.1 and observed under fluorescence microscope with green light excitation at 495-570 nm and long pass filter above 617 nm. In case of PI staining, control samples were given similar treatments in the absence of bacteriocin.

5.2.4.4. Transmission electron microscopy

The effect of pediocin produced by *Pediococcus* sp. CRA51 on target cells of *Listeria monocytogenes* Scott A was also evaluated by transmission electron microscope (TEM). Target cells were grown for 6 h in BHI broth medium prior to treatment with purified pediocin CRA51. The cells were washed twice with 10 mM phosphate buffer (pH 7.0) and once with MilliQ water to remove residual medium components. *Listeria* cells (~10^6 cfu) were kept for interaction with 6400 AU of purified pediocin obtained from isolate CRA51 in 1.0 ml of 10 mM phosphate buffer (pH 7.0) for 6 h at 37°C in water bath incubator. Treated cells were washed once with buffer and fixed in 2.5% of glutaraldehyde for 2 h. Following fixation, cells were centrifuged, washed with MilliQ water and resuspended in 10 µl of the same.

A 2.0 µl aliquot of treated cells was spotted on carbon coated TEM grid (Pacific Asia, USA) and air-dried in laminar hood. Samples of untreated cells were given similar treatment without pediocin CRA51. The treated and untreated cells of *Listeria monocytogenes* Scott A were examined through a transmission electron microscope (Jeol JEM 2100, Japan) operating at 200 kV.

5.3. Results and Discussion

5.3.1. Species-level identification of anti-listerial LAB isolates and cell morphology

The results of the sugar fermentation tests for potent anti-listerial isolates CRA21, CRA51 and DF14 are shown in Table 5.1. Based on standard tests outlined by Kandler and Weiss (1986), Garvie (1986) and Devriese and Pot (1995); anti-listerial isolates CRA21, CRA51 and DF14 were identified as *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Enterococcus faecium*, respectively. These results were further substantiated by partial 16S rRNA gene sequencing for isolates CRA21, CRA51 and DF14. BLAST analysis indicated that the 16S rRNA gene fragment obtained from isolates DF14, CRA21 and CRA51 (accession nos. FJ424060, FJ424061 and FJ424059) exhibited 99% homology with respect to *Enterococcus faecium* KT4S13 (accession no. AB481104), 99% homology with *Lactobacillus plantarum* I041703 (accession no. AB510750) and 100% homology with *Pediococcus pentosaceus* ATCC 25745 (accession no. CP000422), respectively.

Table 5.1

Sugar fermentation and biochemical tests for anti-listerial LAB isolates

Characteristics	Anti-listerial LAB isolates[a,b]		
	DF14	CRA21	CRA51
Acid production from:			
Ribose	+	-	-
Lactose	-	-	+
Xylose	-	-	-
Maltose	+	+	+
Fructose	+	+	+
Dextrose	+	+	+
Galactose	+	-	+
Mannose	+	+	+
Raffinose	-	-	-
Rhamnose	-	-	-
Trehalose	+	+	+
Melibiose	+	-	-
Cellobiose	+	+	+
Melezitose	-	-	+
Sucrose	+	+	+
L-Arabinose	-	-	-
D-Arabinose	+	-	-
Sorbose	-	-	-
Inulin	+	+	+
Salicin	+	+	+
Glycerol	-	-	-
Dulcitol	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Mannitol	+	-	-
Adonitol	-	-	-
Xylitol	-	-	-
Sodium gluconate	-	-	-
Glucosamine	+	-	+
α-Methyl-D-glucoside	-	-	-
α-Methyl-D-mannoside	-	-	+
ONPG decarboxylase	-	-	-
Esculin hydrolysis	+	-	-
Citrate utilization	-	-	-
Malonate utilization	-	-	-

[a] Biochemical properties of LAB isolates were determined with HiCarbohydrate™ Kit KB009, HiMedia, Mumbai, India. [b] DF: dried-fish; CRA: salt-fermented cucumber

The 16S rRNA sequence based phylogenetic analysis for isolates DF14, CRA21 and CRA51 shown in Figure 5.3 reiterate the species level identification of these LAB isolates.

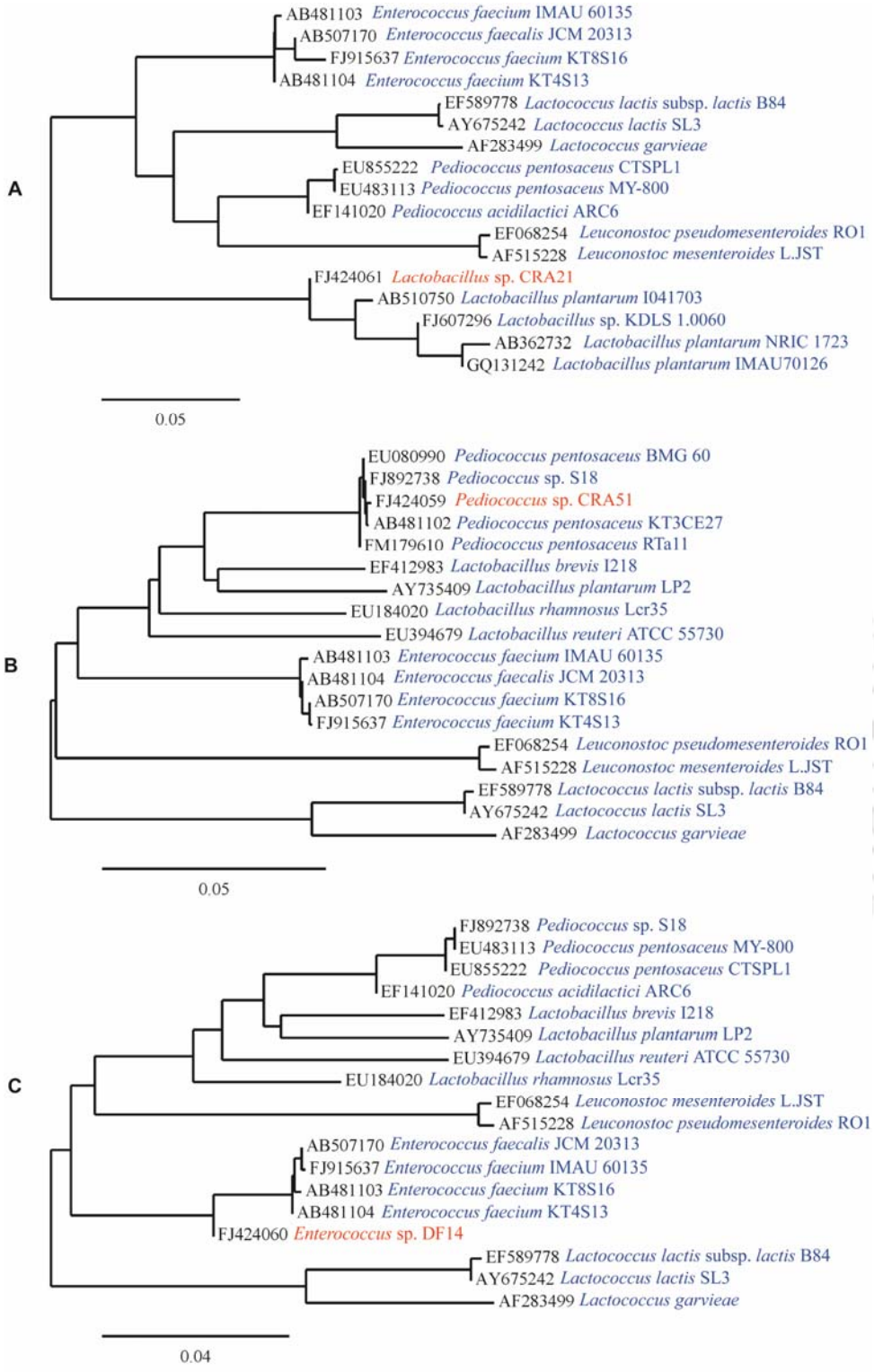

Figure 5.3 16S rRNA gene sequence based phylogenetic tree constructed for anti-listerial LAB isolates *Lactobacillus* sp. CRA21 **(A)**, *Pediococcus* sp. CRA51 **(B)**, and *Enterococcus* sp. DF14 **(C)**. Phylogenetic tree was constructed by using neighbour-joining algorithm (www.phlogeny.fr).

The cell morphology of isolates CRA21, CRA51 and DF14 were ascertained by SEM analysis. The images of SEM analysis are depicted in Figure 5.4. From the figure it is evident that *Lactobacillus plantarum* CRA21 was rod-shaped (Figure 5.4A). It can also be observed that the typical cocci-shape in case of *Pediococcus pentosaceus* CRA51 and *Enterococcus faecium* DF14 was conspicuous (Figure 5.4B and C).

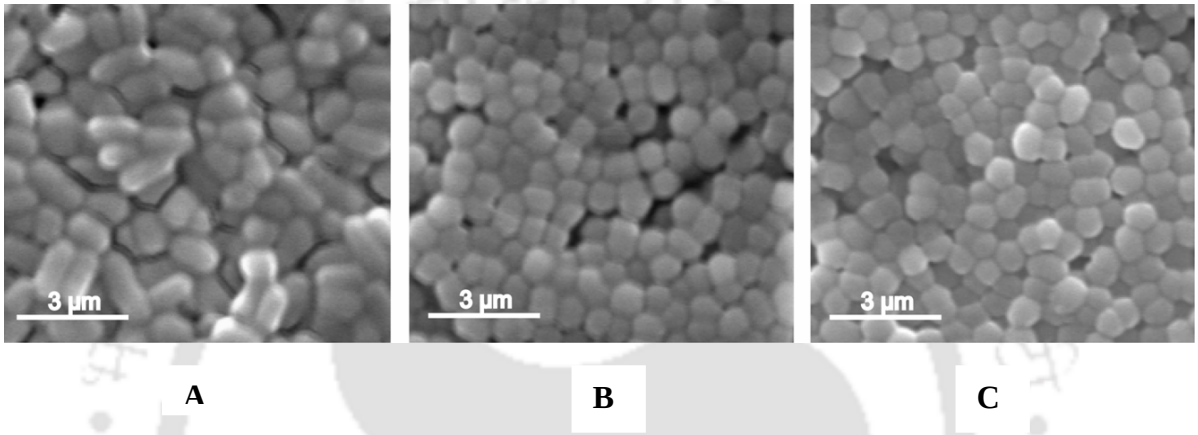

Figure 5.4 Scanning electron micrograph of anti-listerial LAB isolates. **(A)** *Lactobacillus plantarum* CRA21; **(B)** *Pediococcus pentosaceus* CRA51; **(C)** *Enterococcus faecium* DF14. Magnification: 2.5 KX; Voltage: 15 kV.

5.3.2. Bacteriocin purification

Purification of bacteriocin produced by select anti-listerial LAB isolates (DF9, DF14, CRA21, CRA28, CRA51 and CRA61) by cell-adsorption method resulted in appreciable yield and purification fold. Bacteriocin, being a cationic peptide could be readily adsorbed on producer cells at a pH of 6.0 wherein the bacterial cell surface bears a net negative charge. Subsequently bacteriocin molecule could be selectively desorbed at an acidic pH. An analogous method has been adopted earlier by other research groups (Yang *et al.,* 1992; Halami *et al.,* 2005). A schematic representation of the principle steps involved in the purification of bacteriocin molecule is depicted in Figure 5.5.

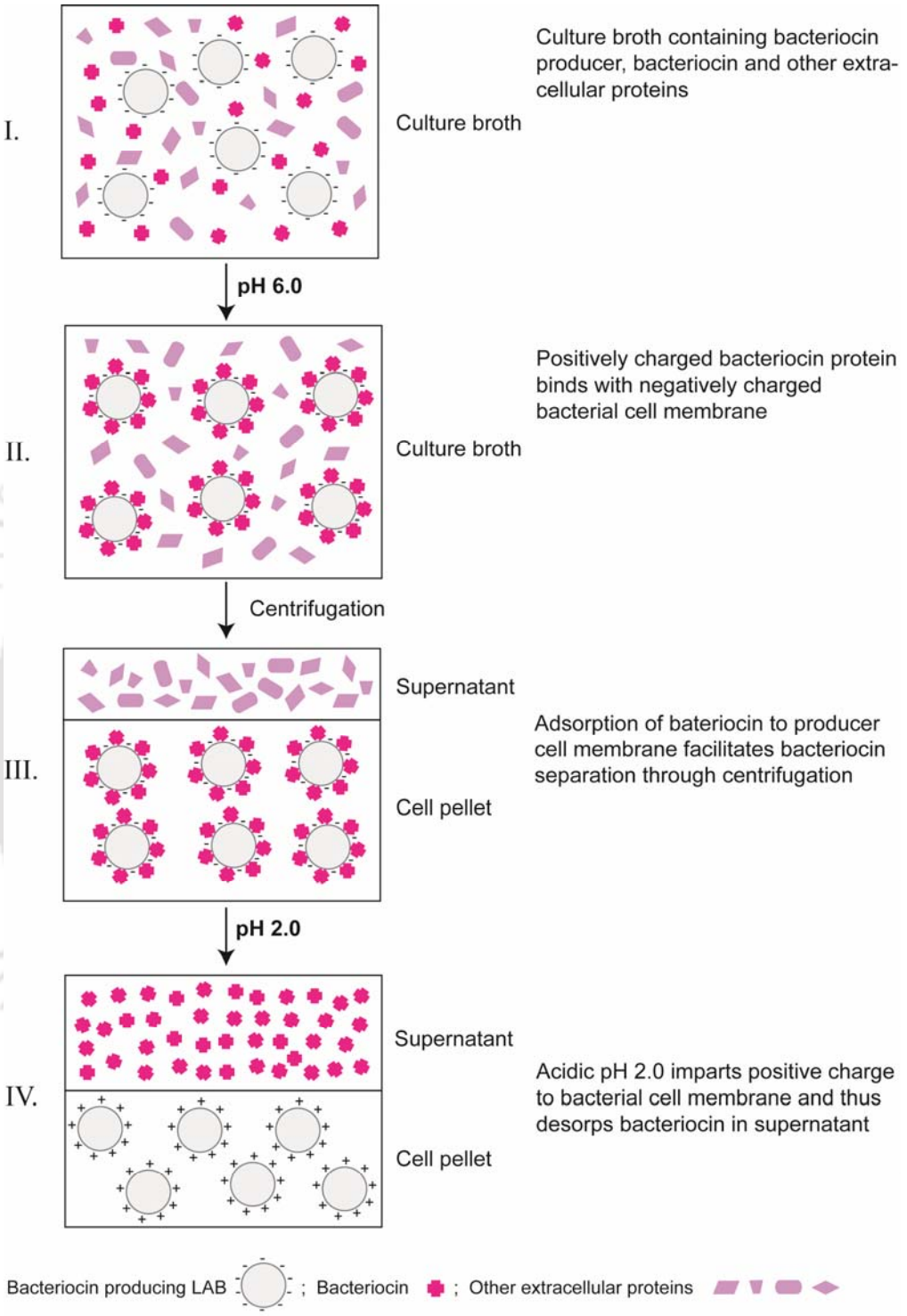

Figure 5.5 Schematic representation of cell adsorption based purification of bacteriocin produced by anti-listerial LAB isolate.

A representative result for the purification of pediocin CRA51 produced by *Pediococcus pentosaceus* CRA51 is shown in Table 5.2. Bacteriocin activity in the purified sample for the anti-listerial LAB isolates ranged from 102400-204800 AU/ml.

Table 5.2

Summary of pediocin CRA51 purification from *Pediococcus pentosaceus* CRA51

Source	Volume (ml)	Protein (mg ml^{-1})	Activity (AU ml^{-1})	Total activity (AU)	Specific activity (AU mg^{-1})	Yield (%)	Purification fold
Culture broth	1000	2.3	3200	3.2×10^6	1391	100	1
pH 2.0 extract	25	0.40	204800	5.12×10^6	512000	162	368

Tricine SDS-PAGE analysis of purified pediocin produced by *Pediococcus pentosaceus* CRA51 revealed a single band corresponding to an approximate molecular size of 4.6 kDa (Figure 5.6A), with corresponding biological activity against *L. monocytogenes* Scott A (Figure 5.6B).

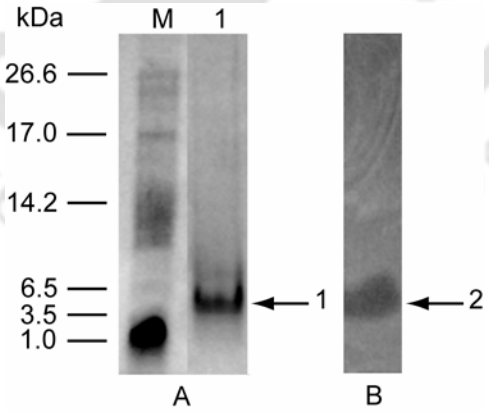

Figure 5.6 (A) Tricine-SDS-PAGE for purified pediocin produced by *Pediococcus pentosaceus* CRA51; **(B)** Gel overlay assay of purified pediocin showing anti-listerial activity. Lane M is a low molecular weight marker (Sigma-Aldrich, USA). Arrow '1' indicates purified pediocin and arrow '2' depicts anti-listerial activity of purified pediocin.

5.3.3. MIC and MKC of purified bacteriocin

The average MIC of purified bacteriocin from 6 anti-listerial LAB isolates varied from 83.3-266.6 AU/ml whereas MKC ranged from 133.3-533.3 AU/ml (Table 5.3). The varying levels of MIC and MKC observed amongst the LAB strains reflect the differences in the susceptibility of the target strain of *Listeria* and corroborates with earlier results of colony overlay and agar well diffusion assay (Chapter 4a). On the basis of ANOVA analysis, bacteriocin produced by *Enterococcus faecium* DF14, *Lactobacillus plantarum* CRA21, *Pediococcus pentosaceus* CRA51 and *Lactobacillus plantarum* CRA52 exhibited significant anti-listerial activity ($P<0.05$). Results of ANOVA test are elaborated in Appendix (section 2.3). The bacteriocin gene in these isolates were earlier identified as enterocin (DF14), pediocin (CRA51) and plantaricin A (CRA21, CRA52), which are categorized as Class IIa bacteriocins. Compelling anti-listerial activity of Class IIa bacteriocin is a well documented phenomenon (Drider *et al.*, 2006).

Table 5.3

MIC and MKC for purified anti-listerial bacteriocins

LAB strain	**Bacteriocin**	***Listeria monocytogenes* Scott A**[a]	
		MIC (AU/ml)	**MKC (AU/ml)**
Lactobacillus plantarum DF9	Plantaricin A	266.6	533.3
Enterococcus faecium DF14	Enterocin A	**83.3***	**166.6***
Lactobacillus plantarum CRA21	Plantaricin A	**83.3***	**166.6***
Pediococcus acidilactici CRA28	Pediocin	266.6	533.3
Pediococcus pentosaceus CRA51	Pediocin	**83.3***	**166.6***
Lactobacillus plantarum CRA61	Plantaricin A	266.6	533.3

[a] MIC and MKC for *L. monocytogenes* is represented as an averaged value for triplicate samples.
*Asterisk and boldface denotes significantly higher activity against target strain ($P<0.05$) compared to other isolates.

5.3.4. Functional assessment of anti-listerial bacteriocin

5.3.4.1. Flow cytometric analysis

Flow cytometric analysis were carried out to study the effect of bacteriocin treatment on target cells of *Listeria monocytogenes* Scott A. Flow cytometry has been used earlier by other research groups to study effect of bacteriocin treatment on target cells (Budde *et al.,* 2001) and viability of lactic acid bacteria (Bunthof *et al.,* 2001; Volker *et al.,* 2008). Figure 5.7 shows the distribution of fluorescence intensities for cells of *Listeria monocytogenes* Scott A after exposure to purified pediocin CRA51 (6400 AU/ml) for 6 h (Figure 5.7, Panel B) compared to control sample without bacteriocin treatment (Figure 5.7, Panel A).

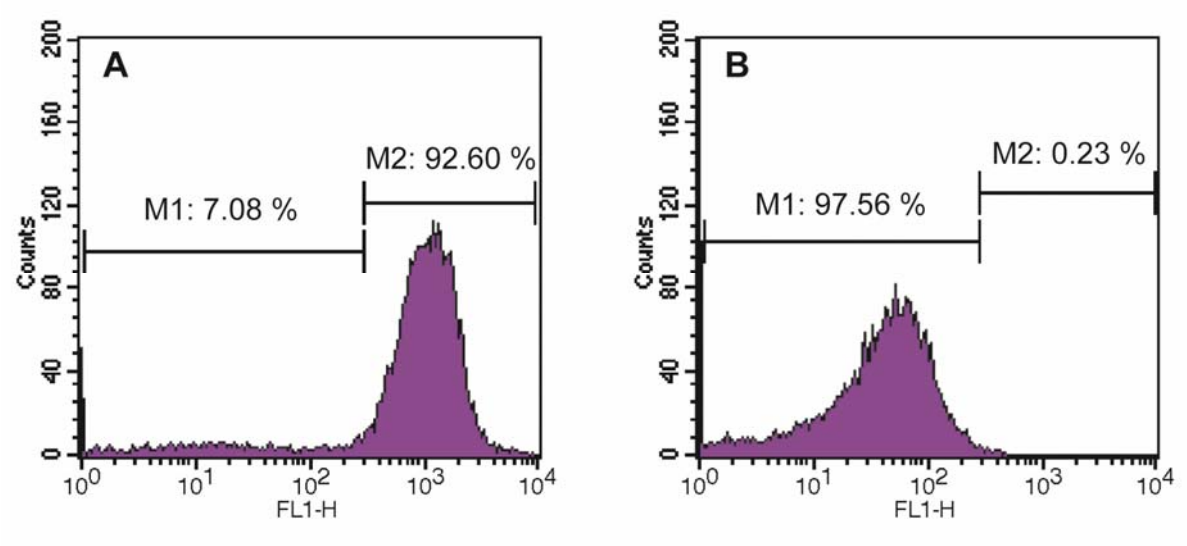

Figure 5.7 Flow cytometric analysis of the effect of pediocin CRA51 on cFDA-SE labelled *Listeria monocytogenes* Scott A cells. **(A)** Untreated cells and **(B)** cells treated with 6400 AU/ml of pediocin CRA51. Data represented as fluorescence histogram of cFDA-SE labelled *Listeria monocytogenes* Scott A cells. M1: Marker 1; M2: Marker 2

It is quite evident from figure 5.7 that in case of control samples (Panel A) the fluorescence histogram and statistical analysis revealed the presence of an overwhelming population of viable *Listeria* cells exhibiting strong cFDA fluorescence (M2: 92.6%). Following treatment

with pediocin CRA51, there was a substantial change in the profile of the fluorescence histogram (Panel B). There was a distinct shift in the position of the peak indicating decrease in the intensity of cFDA-SE fluorescence obtained from bacteriocin treated *Listeria* and a broader fluorescence distribution was observed. The marked reduction in the fluorescence intensity (FI) of the treated cell compared to untreated cell possibly indicates membrane damage of target cells of *Listeria* by bacteriocin molecule and subsequent leakage of the fluorescent dye from damaged cells. This would eventually result in the decrease in the population of fluorescently labelled viable cells. Similar results were also reported by Budde *et al.* (2001). The results obtained from flow cytometric analysis clearly pointed out the utility of the experiment in ascertaining the membrane damage of *Listeria* upon exposure to pediocin produced by a native LAB isolate *Pediococcus pentosaceus* CRA51.

5.3.4.2. Assessment of target cell viability and membrane damage by cFDA-SE/PI staining

To ascertain the effect of bacteriocins from isolate CRA21, CRA51 and DF14 on the viability of *Listeria* and *Staphylococcus* and determine membrane damage of bacteriocin treated target cells, experiments were conducted with the fluorescent dyes cFDA-SE and PI. The target cells were treated with various concentrations of bacteriocin and dye leakage in case of cFDA-SE and PI uptake were measured. The results of the experiments are shown in Figure 5.8, 5.9 and 5.10. The essential observation from the experiments was that in case of cFDA-SE staining, there was a progressive increase in the leakage of the dye, which was proportional to the dose of the bacteriocin used for the experiments. The linearity of the response was clearly manifested as R^2 values in all the cases were close to 0.99. cFDA-SE is cell permeable and following uptake of the dye, the succinimidyl group conjugates with aliphatic amines of

bacterial intracellular proteins. Fluorescence is detected due to accumulation of the fluorescent form of the dye following cleavage of the ester by intracellular esterase activity (Hoefel *et al.,* 2003). Treatment of cFDA-SE labelled target cells with bacteriocin would lead to pore formation in the target cells and subsequent leakage of the dye from damaged cells. The extent of dye leakage could then be a measure of dose-dependent cellular damage by bacteriocin as reported earlier by Budde *et al.* (2003). The results shown in Figures 5.8, 5.9 and 5.10 are in accordance with previous results discussed in section 4a.3.6.2 of Chapter 4a.

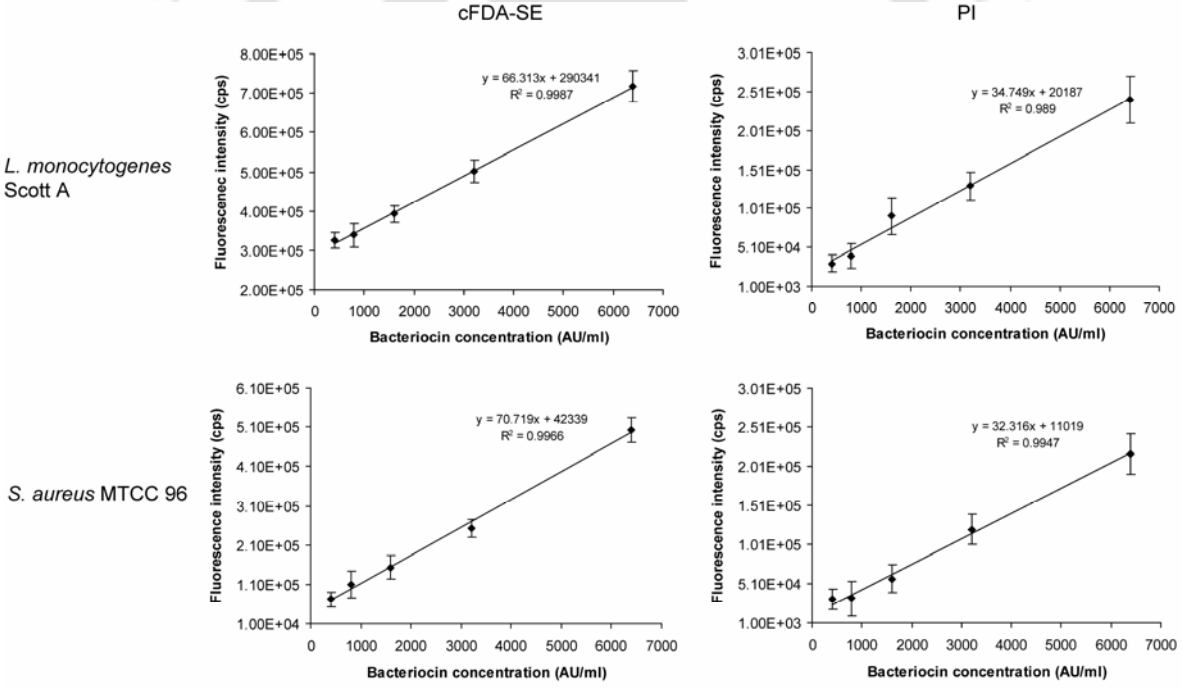

Figure 5.8 Fluorescence based quantification of the effect of plantaricin A produced by *Lactobacillus plantarum* CRA21 on pathogenic bacterial strains *Listeria monocytogenes* Scott A and *Staphylococcus aureus* MTCC 96. The measurements included cFDA-SE dye leakage and PI uptake in bacteriocin treated target cells.

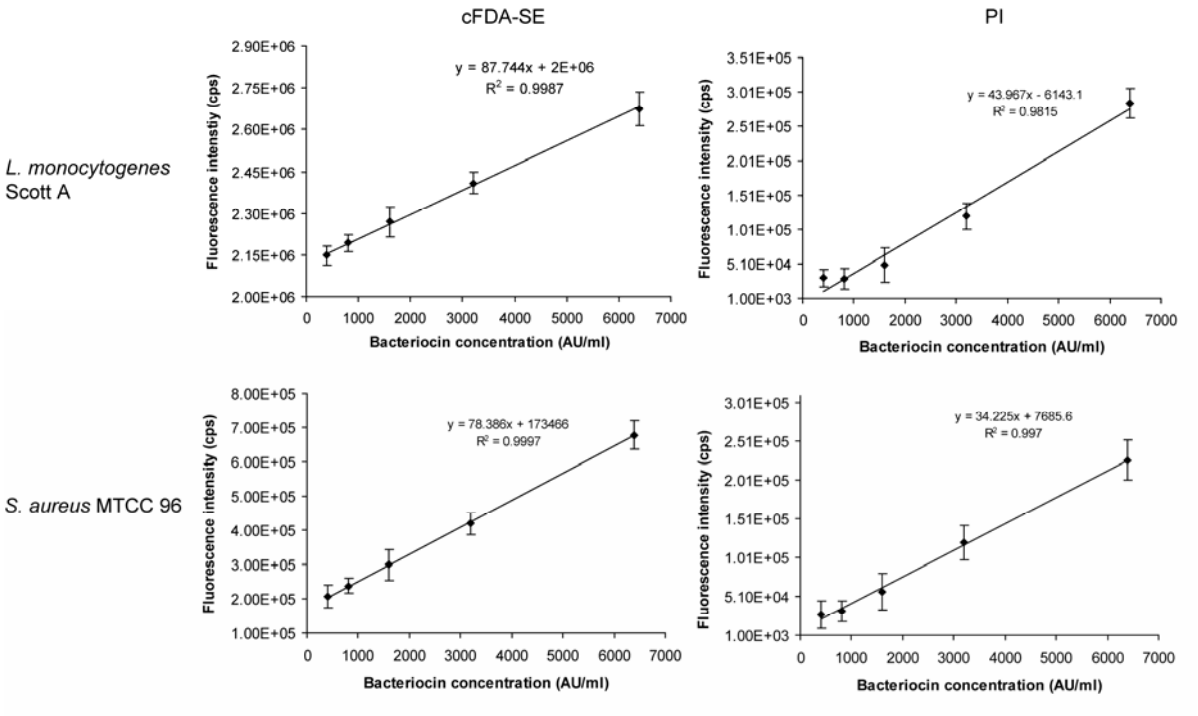

Figure 5.9 Fluorescence based quantification of the effect of pediocin produced by *Pediococcus pentosaceus* CRA51 on pathogenic bacterial strains *Listeria monocytogenes* Scott A and *Staphylococcus aureus* MTCC 96. The measurements included cFDA-SE dye leakage and PI uptake in bacteriocin treated target cells.

Additional evidence of membrane damage of target cells by bacteriocin treatment was obtained by conducting experiments wherein uptake of propidium iodide (PI) by treated cells was measured as a function of bacteriocin dose. PI is used as an indicator of membrane integrity and has been used in an earlier study to determine membrane damage (Virto *et al.,* 2005). PI is able to enter cells only if the membrane is permeabilized or compromised. Upon entry into cells, PI binds to single- and double-stranded nucleic acids, yielding an intense red fluorescence. Data for the uptake of PI by bacteriocin treated cells of *Listeria monocytogenes* and *Staphylococcus aureus* are shown in Figures 5.8, 5.9 and 5.10. The uptake of PI by bacteriocin treated cells exhibited similar dose-dependent pattern as the leakage of cFDA-SE

reiterating that there was permeabilization of bacterial cell membrane when cells were treated with bacteriocin.

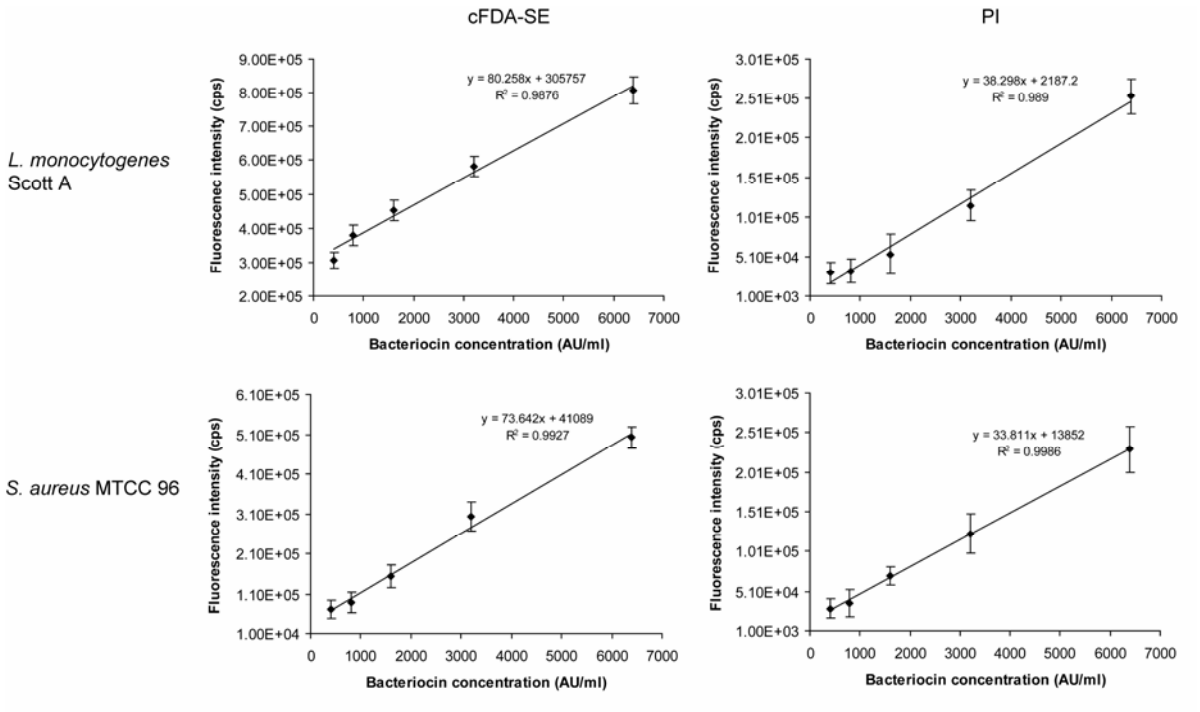

Figure 5.10. Fluorescence based quantification of the effect of enterocin A produced by *Enterococcus faecium* DF14 on pathogenic bacterial strains *Listeria monocytogenes* Scott A and *Staphylococcus aureus* MTCC 96. The measurements included cFDA-SE dye leakage and PI uptake from bacteriocin treated target cells.

To compare the overall results pertaining to cFDA-SE dye leakage and PI uptake, the slopes of the responses shown in Figures 5.8, 5.9 and 5.10 were calculated using Microsoft Office Excel Program. The results of the slope calculations are indicated in Table 5.4. It is noteworthy that pediocin produced by strain CRA51 exhibited the highest slope values (cFDA-SE and PI response) indicating highest antimicrobial activity against the target pathogens *Listeria* and *Staphylococcus*. Following pediocin, the highest potency was exhibited by enterocin A and plantaricin A produced by strains DF14 and CRA21, respectively.

Table 5.4

Comparison of antagonistic activity of purified bacteriocins obtained from native LAB isolates

LAB strain	Bacteriocin	Slope value [a]			
		L. monocytogenes Scott A		*S. aureus* MTCC 96	
		cFDA-SE	PI	cFDA-SE	PI
L. plantarum CRA21	Plantaricin A	66.31	34.75	70.72	32.32
P. pentosaceus CRA51	Pediocin	87.74	43.97	78.39	34.22
E. faecium DF14	Enterocin A	80.26	38.29	73.64	33.81

[a] Slope values were obtained from the plots between bacteriocin concentration (AU/ml) and fluorescence intensity (cps) shown in Figures 5.8, 5.9 and 5.10.

5.3.4.3. Microscopic analysis

Fluorescence and transmission electron microscopic analysis were conducted to substantiate the mode of action of pediocin CRA51 on target cells of *Listeria monocytogenes* Scott A. The results of the experiments are indicated in Figure 5.11. It can be observed from Figure 5.11, Panel A that in case of untreated cells, a large number of cFDA-SE stained *Listeria* were observed indicating that the cells were viable. However, upon treatment with 3200 AU/ml pediocin CRA51 for 6 h, the number of fluorescently labelled *Listeria* decreased substantially indicating a profound loss in viability. To determine the mode of action of pediocin CRA51 on target cells of *Listeria*, the bacteriocin treated cells were stained with propidium iodide which is normally impermeable to viable cells and is known to enter dead cells or cells with compromised membrane. As evident from Figure 5.11, Panel B, in case of untreated target cells only a few cells revealed uptake of PI. These cells are probably the few dead cells originally present in the culture.

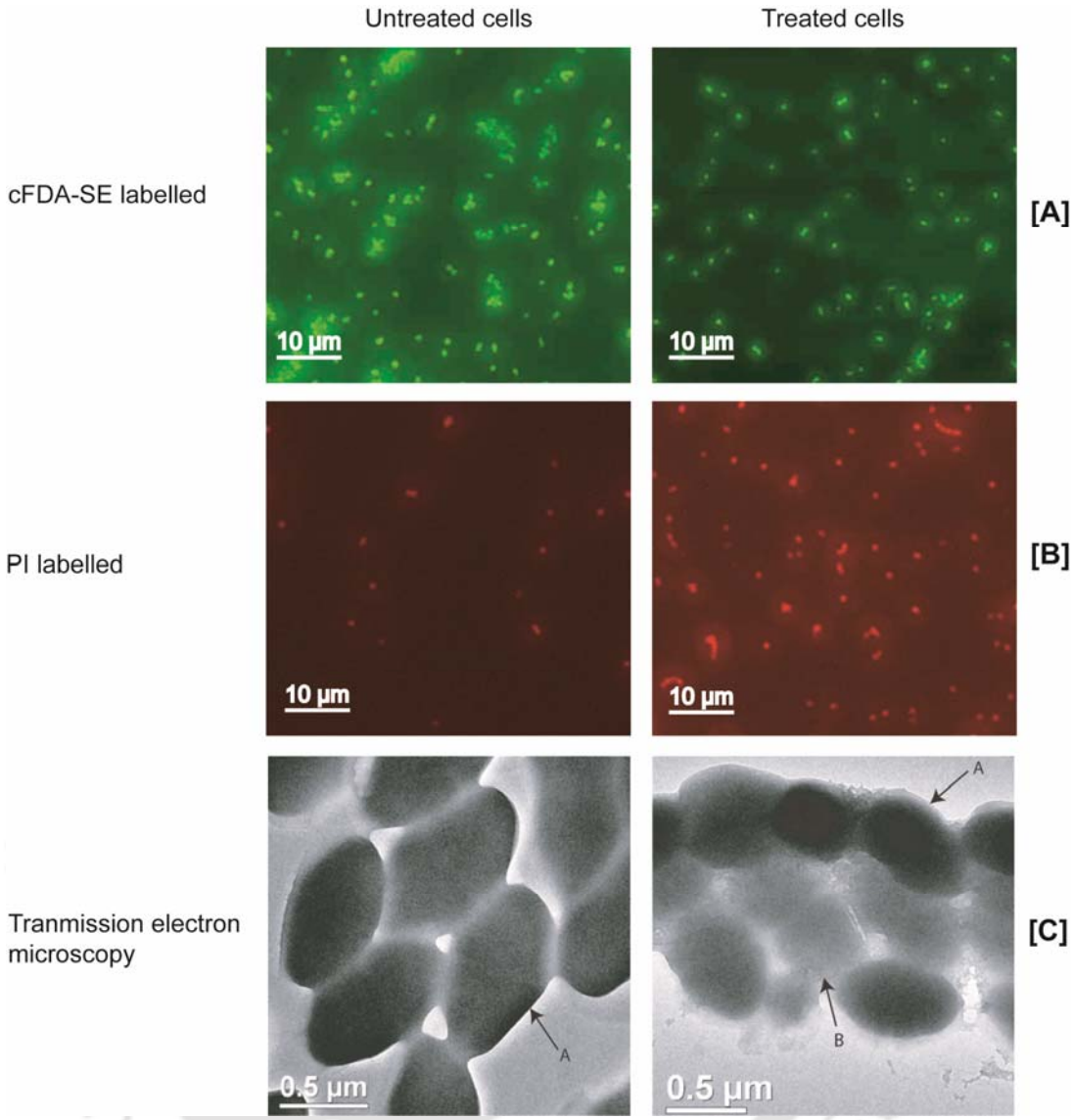

Figure 5.11 (A) and (B): Fluorescence microscopic images of *Listeria monocytogenes* Scott A; **(C)** Transmission electron microscopic images of *Listeria monocytogenes* Scott A. Arrow 'A' indicates intact cells and arrow 'B' indicates membrane damaged cell.

It is noteworthy to observe that upon treatment with pediocin CRA51, a large number of *Listeria* cells exhibited red fluorescence indicating uptake of the dye as a consequence of membrane damage. Collectively the fluorescence microscopic results corroborated with the results obtained in flow cytometry and dose-dependent fluorescence measurement experiments (section 5.3.4.1 and 5.3.4.2).

Transmission electron micrographs of target cells of *Listeria* were captured to determine membrane damage upon treatment with pediocin CRA51. It is clear from Figure 5.11, Panel C (indicated by arrow A) that in case of untreated samples, cells of *Listeria* generally displayed intact cell walls and prominent electron-dense layers within the cells. However, on treatment with 6400 AU pediocin CRA51, significant structural perturbations such as cell wall loosening was evident as observed by decreased electron density (Arrow B)**.** The decreased electron density also points out to the possibility of membrane damage followed by leakage of intracellular constituents which eventually leads to formation of cell voids and diminishes the absorption of primary electrons. Transmission electron microscopic studies elaborating bacteriocin induced target cell membrane damage has been reported earlier (Benech *et al.,* 2002; Bhunia *et al.,* 1991).

5.4. Conclusion

1. In the present investigation, species level identification of three potent anti-listerial bacteriocin producing LAB were accomplished by standard biochemical tests and 16S rRNA gene sequence analysis.

2. Bacteriocin from select anti-listerial LAB isolates was purified by cell adsorption method and the minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) of the purified bacteriocin was determined.

3. The anti-listerial activity of pediocin produced by *Pediococcus pentosaceus* CRA51 has been demonstrated by flow cytometric analysis.

4. Based on experiments with fluorescence probes cFDA-SE and PI, membrane permeabilizing activity of three bacteriocin molecules (plantaricin A, pediocin and enterocin

A) produced by anti-listerial LAB strains *Lactobacillus plantarum* CRA21, *Pediococcus pentosaceus* CRA51 and *Enterococcus faecium* DF14 was established. These results were further substantiated by transmission electron microscope analysis.

On the basis of potent anti-listerial activity exhibited by some of the native LAB strains, it is envisaged that some of these isolates can be exploited as potential probiotic strains. In this regard, a key attribute for probiotic applications would be the ability to adhere to intestinal cells and ensure gut colonization. The next chapter of the thesis reports the investigation undertaken to assess the *in vitro* adhesion potential of some of the potent anti-listerial LAB strains.

CHAPTER 6

Quantitative assessment of the *in vitro* adhesion potential of potent anti-listerial bacteriocin producing lactic acid bacteria

ABSTRACT

The aim of this investigation was to ascertain the *in vitro* adhesion potential of anti-listerial bacteriocin producing LAB strains which were isolated and characterized in the previous investigations (Chapter 4a, 4b and 5). The selection of the anti-listerial strains for the adhesion experiments was based on acid and bile salt tolerance exhibited by some of the LAB strains. Conventional plating based adhesion assay revealed that anti-listerial LAB isolates obtained from dried fish (DF3, DF9, DF14 and DF45) exhibited significant *in vitro* adhesion potential to cultured HT-29 cells and the adhesion was comparable to some of the standard probiotic strains such as *Lactobacillus acidophilus* NRRL B4495, *Lactobacillus johnsonii* NRRL B2178, and *Lactobacillus rhamnosus* GG. These results were further substantiated by conducting the adhesion experiments by using cFDA-SE labeled anti-listerial LAB strains. The *in vitro* adhesion potential was further validated by microscopic observation and counting of adhered LAB on HT-29 cells, which reiterated earlier observations. Interestingly, fluorescence microscopic observation of adhered LAB cells revealed a difference in the adherence pattern of the anti-listerial LAB isolates *Lactobacillus plantarum* DF9 and *Enterococcus faecium* DF14 when compared to the reference strain of *Lactobacillus rhamnosus* GG.

6.1. Introduction

Lactic acid bacteria (LAB) have been in focus for their probiotic virtues as they are known to influence the activity and composition of the intestinal microbiota (Ouwehand *et al.,* 2002). Probiotic LAB are essentially selected on the basis of their ability to adhere to the intestinal mucosa, acid and bile resistance, and antagonism against pathogenic bacteria. The ability to adhere to intestine is a key selection criterion, because it is implicated in persistence, enhanced healing of the damaged mucosa (Elliott *et al.,* 1998), antagonism against pathogens (Reid and Burton, 2002) and immunomodulation (Zhou and Gill, 2005). Thus an essential prerequisite of an efficient probiotic strain is adhesion to mucus and epithelial cells.

A number of *in vitro* models have been routinely employed to ascertain the adhesion of probiotic LAB to epithelial cells. Of these, colon adenocarcinoma cells HT-29 and Caco-2 are the most ideal choice (Gopal et al. 2001; Sambuy *et al.,* 2005). Adhesion of bacteria to epithelial cells has been studied by visualizing the adhered cells with gram-staining (Tuomola and Salminen, 1998) or Giemsa staining (Forestier *et al.,* 2001). Adhesion has been studied by using radioactive labeling of bacterial cells (Coconnier *et al.,* 1998). Recently, fluorescent dyes with specific binding to nucleic acids have been employed (Fuller *et al.,* 2000).

In the previous investigation (Chapter 4a, 4b and 5), a large number of anti-listerial bacteriocin producing LAB strains were isolated and characterized. The next aim which encompasses the last objective of the present research work was to ascertain the *in vitro* adhesion potential of the anti-listerial bacteriocin producing LAB strains on HT-29 cells. In this chapter the adhesion of the anti-listerial LAB determined by conventional plating methods, fluorescence-based techniques and microscopic analysis are reported.

6.2. Materials and methods

6.2.1. Bacterial growth conditions and maintenance

Reference LAB strains and native anti-listerial bacteriocin producing LAB strains isolated and characterized in a previous investigation (Chapter 4a, 4b and 5) were used for the present study. The list of the LAB strains used is shown in Table 6.1.

Table 6.1

Lactic acid bacterial strains used in the present study

Bacteria	Strain
Reference LAB strains	
Lactobacillus acidophilus	NRRL B4495
Lactobacillus johnsonii	NRRL B2178
Lactobacillus rhamnosus	GG
Lactobacillus helveticus	NCIM 2126
Lactobacillus gasseri	NRRL B4240
Anti-listerial bacteriocin producing LAB strains [a]	
Lactobacillus sp.	DF3
Lactobacillus plantarum	DF9
Enterococcus faecium	DF14
Lactobacillus sp.	DF45
Lactobacillus sp.	CDRA10
Lactobacillus sp.	CDRA57
Lactobacillus sp.	CDRA60
Lactobacillus sp.	CRA3
Lactobacillus sp.	CRA6
Lactobacillus plantarum	CRA21
Pediococcus acidilactici	CRA28
Lactobacillus sp.	CRA38
Lactobacillus sp.	CRA39
Pediococcus pentosaceus	CRA51

[a] Anti-listerial LAB strains were isolated from indigenous samples and characterized previously (Chapter 4 and 5)

6.2.2. Acid and bile salt tolerance of anti-listerial bacteriocin producing LAB

A 1.0 ml aliquot LAB culture (10^9 cfu/ml) was transferred into 9.0 ml PBS. The pH was adjusted to 2.0, 2.5 and 3.2 using 0.1N HCl and the cells were incubated at 37°C for 3 h. In control samples the cells were incubated in PBS (pH 7.2) under the same condition. After incubation, viable bacterial counts were determined by plating serial dilutions of the culture in PBS (pH 7.2) on MRS agar.

LAB that survived the acid tolerance study were centrifuged (5000 x g, 5 min), washed with PBS (pH 7.2) and then grown in 9.0 ml MRS broth with and without 0.3% (w/v) oxgall bile (HiMedia, Mumbai) 24 h. Bile tolerance was estimated by comparing the viable LAB bacteria count in MRS with and without bile salt.

6.2.3. HT-29 cell culture

For *in vitro* adhesion studies, human HT-29 colon adenocarcinoma cells were procured from National Animal Cell Repository, National Centre of Cell Sciences (NCCS), Pune, India. HT-29 cells were grown in 24-well microtitre plate (Greiner, CELLSTAR, Germany) in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% (v/v) foetal bovine serum (Sigma), 100 U ml^{-1} penicillin (Sigma) and 100 U ml^{-1} streptomycin (Sigma) in a humidified 5% CO_2 incubator (HERA Cell 150, Heraeus, USA), until the cells were approximately 90% confluent. Monolayers of HT-29 cells were used at late post-confluence culture after 15 days, with a change of medium every 2 days. At least 24 h prior to adhesion experiment, monolayer was washed twice with PBS, and fresh DMEM medium devoid of any antibiotic was added.

6.2.4. Quantitation of in vitro adhesion of anti-listerial LAB

Potent anti-listerial bacteriocin producing LAB strains described in earlier investigation (Chapter 4a, 4b and 5) were selected to evaluate their *in vitro* adhesion potential to cultured HT-29 cells. Standard LAB strains included in the experiments for comparative analysis of adhesion potential were *L. acidophilus* NRRL B4495, *L. gasseri* NRRL B4240, *L. helveticus* NCIM 2126, *L. johnsonii* NRRL B2178 and *L. rhamnosus* GG (Table 6.1). Plating based *in vitro* adhesion study was performed according to the method described by Matijasic *et al.* (2003). LAB cells from 18 h MRS cultures were obtained by centrifugation at 3500 g for 10 min and washed once with PBS buffer (pH 7.2) and once with DMEM without FBS and antibiotic. After washing the HT-29 monolayer twice with PBS, 1.0 ml of LAB suspension (approximately10^8 cfu/ml) was added to each well and incubated for 1 h in atmosphere with 10 % CO_2, at 37 °C. The non-adhered LAB was removed by 3-fold washing with PBS. In order to enumerate adhered LAB, each well was treated with 1 mL 0.05 % Triton X-100 for 15 min. Mixtures of lysed HT-29 cells and LAB were plated on MRS agar. The enumeration was done after 48 h incubation at 37 °C in microaerophilic atmosphere. Adhesion for the LAB strains was expressed as % adhesion in comparison to the adhesion observed for *Lactobacillus rhamnosus* GG. Data points from three independent experiments were averaged and standard deviations were calculated. The scheme of methodology adopted to quantify *in vitro* adhesion based on plating assay is outlined in Figure 6.1.

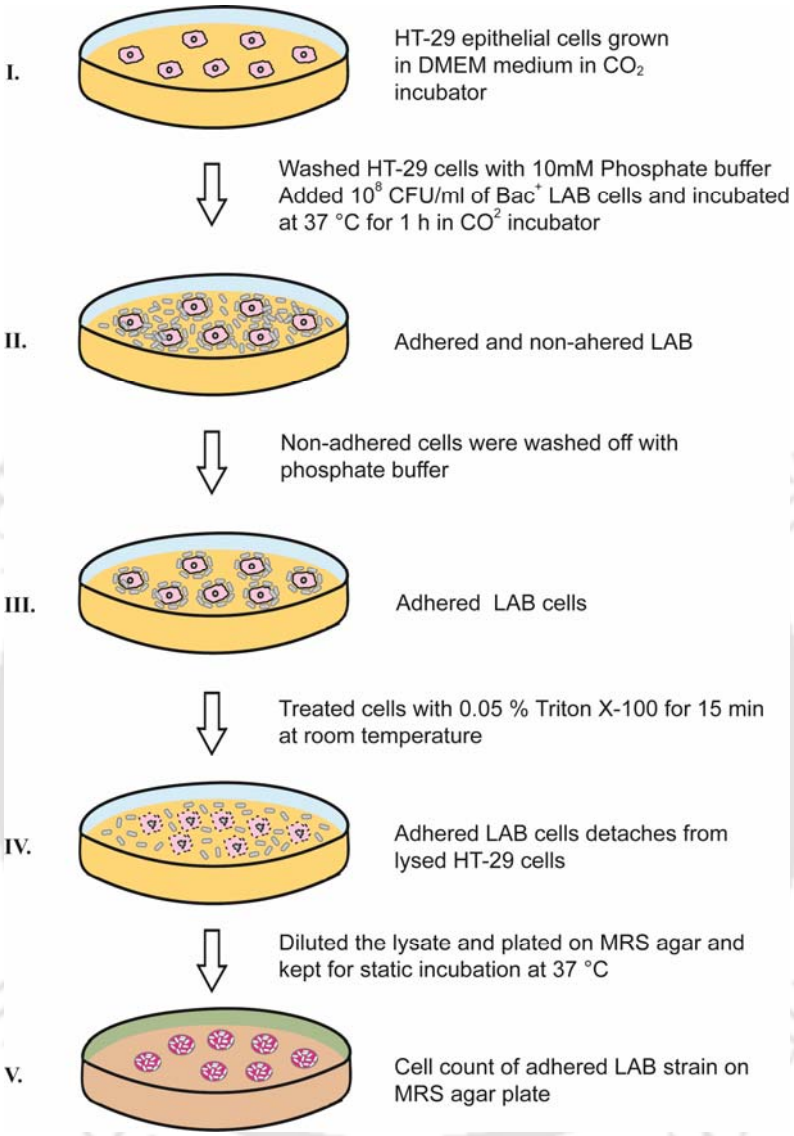

Figure 6.1 Schematic representation of plating based quantification of *in vitro* LAB adhesion to HT-29 cells.

6.2.5. Fluorescence-based assay for in vitro adhesion of LAB to HT-29 cells

In these experiments cFDA-SE labelled LAB strains (Table 6.1) were used. The protocol followed for cFDA-SE labeling of LAB strains was as described earlier (Chapter 4a, section 4a.2.9.2). HT-29 cell were grown as mentioned in section 6.2.3 and washed with PBS prior to interaction with cFDA-SE labelled LAB strains. A 100 µl aliquot of LAB cells (approximately 10^6 cfu) resuspended in DMEM medium devoid of antibiotic was added to cultured HT-29 cells in separate microtitre wells. After 1 h of incubation at 37^{o}C, the non-adhered labelled bacterial cells were withdrawn from the wells and their fluorescence intensity was quantified in a fluorescence spectrophotometer (FluoroMax-3, HORIBA) at λ_{ex}488 nm and λ_{em}518 nm (Chapter 4a, section 4a.2.9.2). The recorded values were referred as fluorescence intensity of non-adhered LAB cells (F_{NA}). The initial fluorescence of cFDA-SE labelled LAB cells added to HT-29 cells was also recorded and referred to as total fluorescence (F_T). The fluorescence intensity of adhered cells (F_A) was obtained by calculating the difference of total fluorescence of added cells and the fluorescence of non-adhered cells ($F_A = F_T - F_{NA}$). Data points from three independent experiments were averaged and standard deviations were calculated. Adhesion for the LAB strains was expressed as % adhesion in comparison to the adhesion observed for *L. rhamnosus* GG. A schematic representation of the fluorescence-based method for quantification of *in vitro* adhesion of anti-listerial LAB strains to HT-29 cells is indicated in Figure 6.2.

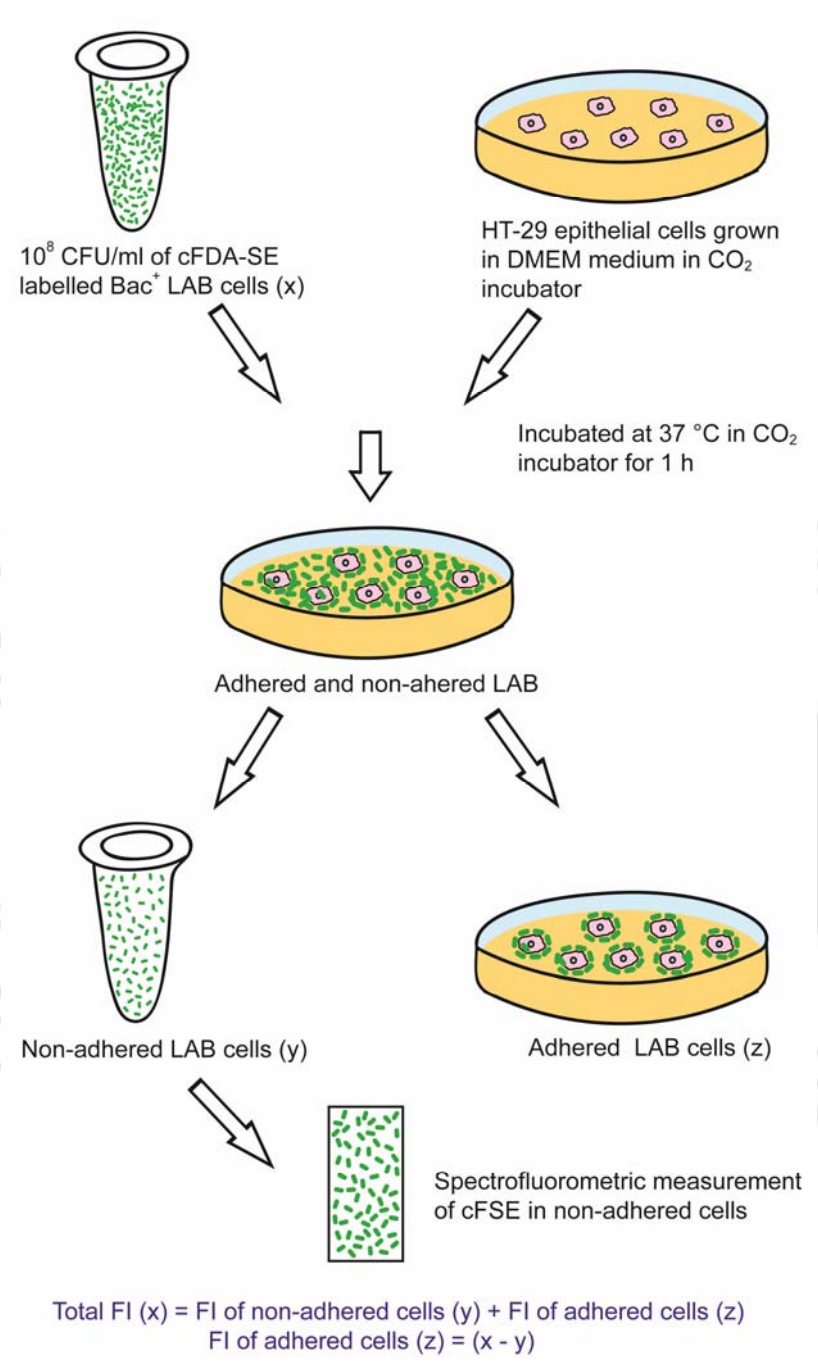

Figure 6.2 Schematic representation of fluorescence-based quantification of LAB adhesion to HT-29 cells under *in vitro* condition. FI indicates fluorescence intensity.

6.2.6. Fluorescence microscopic analysis

Fluorescence microscopic study was also conducted for the anti-listerial LAB strains to observe their *in vitro* cell adhesion potential. LAB strains were grown overnight in MRS broth and labelled with cFDA-SE fluorescent probe following the method mentioned previously (Chapter 4a, section 4a.2.9.2). cFDA-SE labelled LAB cells were resuspended in the serum- and antibiotic-free DMEM medium. HT-29 cells grown as monolayer in 24 well microtitre plates were washed twice with PBS. A 100 µl aliquot of labelled LAB cells (10^6 cfu) were added to the HT-29 monolayer. After 1 h of incubation, the cells were washed twice with PBS. Following removal of non-adhered bacterial cells, the number of adhered cells was assessed by visual microscopic counts of green fluorescent LAB cells under a fluorescent inverted microscope (Model ECLIPSE TS100, Nikon, Japan). The number of adhered bacteria was counted in 25 randomly selected microscopic fields and the data was represented as number of LAB cells adhered per 100 HT-29 cells. All adhesion based experiments were conducted in triplicate for each strain and standard deviations for the data points were calculated.

6.2.7. Statistical analysis

In case of *in vitro* adhesion experiments analysis of variance (ANOVA) test was accomplished (section 2d of Appendix) using the web-based statistical program Instat Plus version 3.36 (http://www.reading.ac.uk/ssc/software/instat/instat.html).

6.3. Results and Discussion

6.3.1. Acid and bile salt tolerance of anti-listerial LAB strains

When the anti-listerial LAB strains were subjected to survival tests at various pH, there was no significant change in the viability of the cells indicating that all the native anti-listerial LAB strains were acid resistant. Subsequently the anti-listerial LAB strains were subjected to growth in the presence of bile salt. On the basis of viable cell count it was observed that strains CDRA10, CDRA57, CRA3 and CRA6 revealed a considerable drop in cell viability following growth in 0.3% bile salt for 24 h (Table 6.2). For all the other native anti-listerial LAB strains the viable cell count varied from 9.23 ± 0.10 to 8.77 ± 0.24 log $_{10}$ cfu/ml. These strains were thus considered as bile salt tolerant and were selected for all the subsequent adhesion experiments.

Table 6.2 Effect of bile salt on the growth of anti-listerial LAB strains

LAB strain	Viable cell count (log_{10} cfu/ml) [a]
Lactobacillus sp. DF3	9.03 ± 0.10
Lactobacillus sp. DF9	8.97 ± 0.24
Enterococcus faecium DF14	9.23 ± 0.10
Lactobacillus sp. DF45	9.15 ± 0.08
Lactobacillus sp. CDRA10	5.72 ± 0.17
Lactobacillus sp. CDRA57	5.13 ± 0.20
Lactobacillus sp. CDRA60	8.83 ± 0.18
Lactobacillus sp. CRA3	5.47 ± 0.04
Lactobacillus sp. CRA6	5.89 ± 0.12
Lactobacillus plantarum CRA21	9.11 ± 0.14
Pediococcus acidilactici CRA28	8.77 ± 0.24
Lactobacillus sp. CRA38	8.81 ± 0.10
Lactobacillus sp. CRA39	9.03 ± 0.24
Pediococcus pentosaceus CRA51	8.82 ± 0.10

[a] Viable cell count was estimated by plating in MRS agar after 24 h of growth in MRS broth with 0.3% bile salt

6.3.2. In vitro adhesion of anti-listerial LAB cells to HT-29 monolayer

The anti-listerial LAB strains which exhibited good acid and bile salt tolerance were selected for investigating the adhesion potential to HT-29 cells. The *in vitro* adhesion potential of select anti-listerial bacteriocin producing native LAB to HT-29 cells was determined by the well established plating method. The result of the experiments is indicated in Figure 6.3. The use of Triton X-100 facilitated release of adhered LAB from the surface of HT-29 cells as reported earlier (Matijasic *et al.*, 2003). It is evident from the figure that the adhesion levels of native LAB strains exhibited a great deal of variability. Based on ANOVA analysis four anti-listerial LAB strains DF3, DF9, DF14 and DF45 exhibited significantly higher adhesion potential on to HT-29 cells ($P<0.05$) when compared to other isolates. It may be mentioned that all these LAB strains were isolated from dried fish samples. Amongst these isolates, strain DF14 revealed the highest adhesion potential wherein % adhesion was close to 88% of the positive control strain of *Lactobacillus rhamnosus* GG. Anti-listerial LAB isolates which were obtained from *dahi* (CDRA60) and fermented cucumber (CRA21, CRA28, CRA38, CRA39, CRA51) revealed poor adhesion potential compared to the isolates obtained from dried fish.

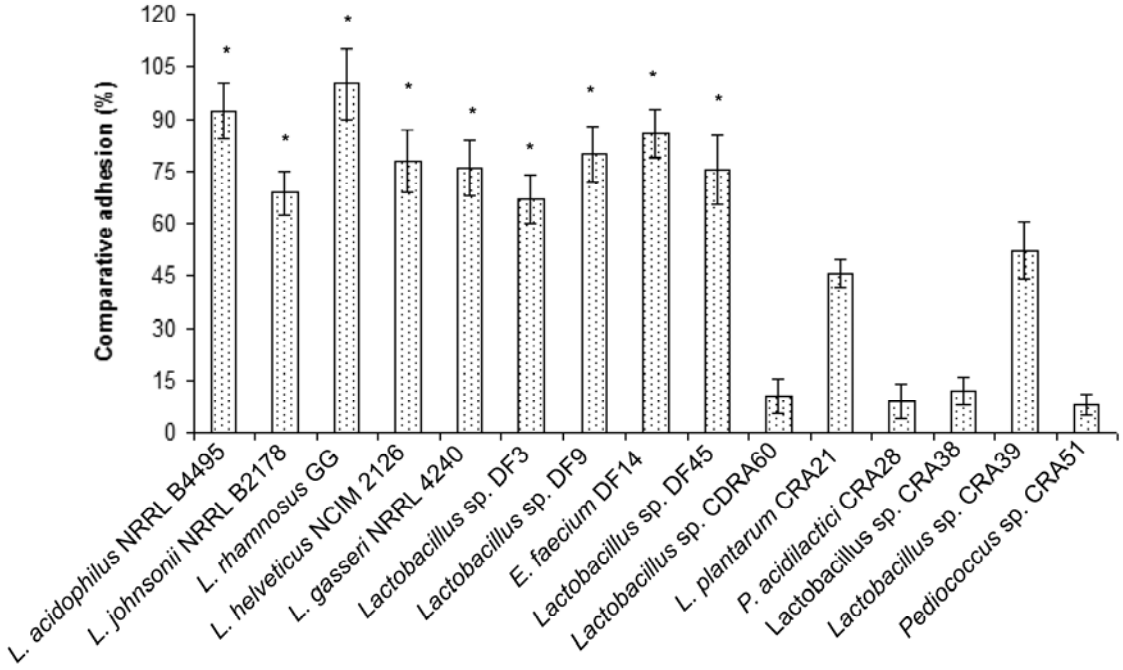

Figure 6.3 Quantification of *in vitro* adhesion potential of reference and anti-listerial bacteriocin producing LAB to HT-29 cells based on plating assay. Data is represented as comparative adhesion (%). The adhesion potential of *Lactobacillus rhamnosus* GG was assumed as 100%. *Represents LAB with significant adhesion potential based on statistical analysis (ANOVA). CRA: strains isolated from salt-fermented cucumber; DF: strains isolated from dried fish.

Adhesion to intestinal epithelial cells is regarded to be an essential prerequisite for probiotic microorganisms to be effective. Adhesion initiates the process of colonization of the human intestinal tract (Alander *et al.,* 1999) and it has been implicated in many of the health benefits attributed to probiotics. Thus, the ability to adhere to epithelial cells has been suggested as a crucial attribute of probiotic bacterial strains (Collado *et al.,* 2005; Ouwehand *et al.,* 1999).

From the results shown in Figure 6.3 it was evident that adhesion potential of the anti-listerial LAB was strain dependent and were in agreement with earlier observations (Collado *et al.,* 2005; Forestier *et al.,* 2001; Gopal *et al.,* 2001; Gueimonde *et al.,* 2006; Tuomola *et al.,* 1999).

6.3.3. Fluorescence based quantification of LAB adhesion

Adhesion potential of anti-listerial LAB strains were also studied by fluorescence-based method wherein the LAB cells were labelled with cFDA-SE prior to adhesion assay. The results of the experiments are indicated in Figure 6.4. It is evident from the figure that the amongst the anti-listerial LAB, strains DF3, DF9, DF14 and DF45 revealed significantly higher adhesion potential to HT-29 cells, based on ANOVA analysis ($P<0.05$). These results corroborated with earlier results obtained by plating method (Figure 6.3). It can also be observed from Figure 6.4 that the fluorescence-based method also provided a measure of the non-adherent cells. This enhanced the confidence levels of the measurements and provided an accurate quantitation of adherent cells. As evident from the figure, LAB strains which revealed poor adhesion potential to HT-29 cells exhibited a corresponding strong fluorescence of non-adhering cells. Likewise the anti-listerial LAB strains with strong adhesion potential displayed lower levels of fluorescence from non-adhering cells as expected.

Bacterial adhesion to host epithelial cells is an important prerequisite for LAB strains with probiotic implications. The results from the present study show that the fluorescence-based method of analysis of LAB adhesion to HT-29 cells is a specific, sensitive and quantitative method for measuring bacterial adhesion. The cFDA-SE method allows homogeneous labeling of each bacterial strain and thus facilitates reliable measurement of

individual bacterial fluorescence. Alternate methods of using fluorescently labelled antibody techniques to identify adherent bacteria are prone to several variables which limit their utility in quantitative assessment of bacterial adherence. These variables may include binding characteristics of the primary and secondary antibodies, levels of expression of bacterial antigens and their recognition by the primary antibody. Additional factors like bacterial cell clumping may preclude accurate measurement.

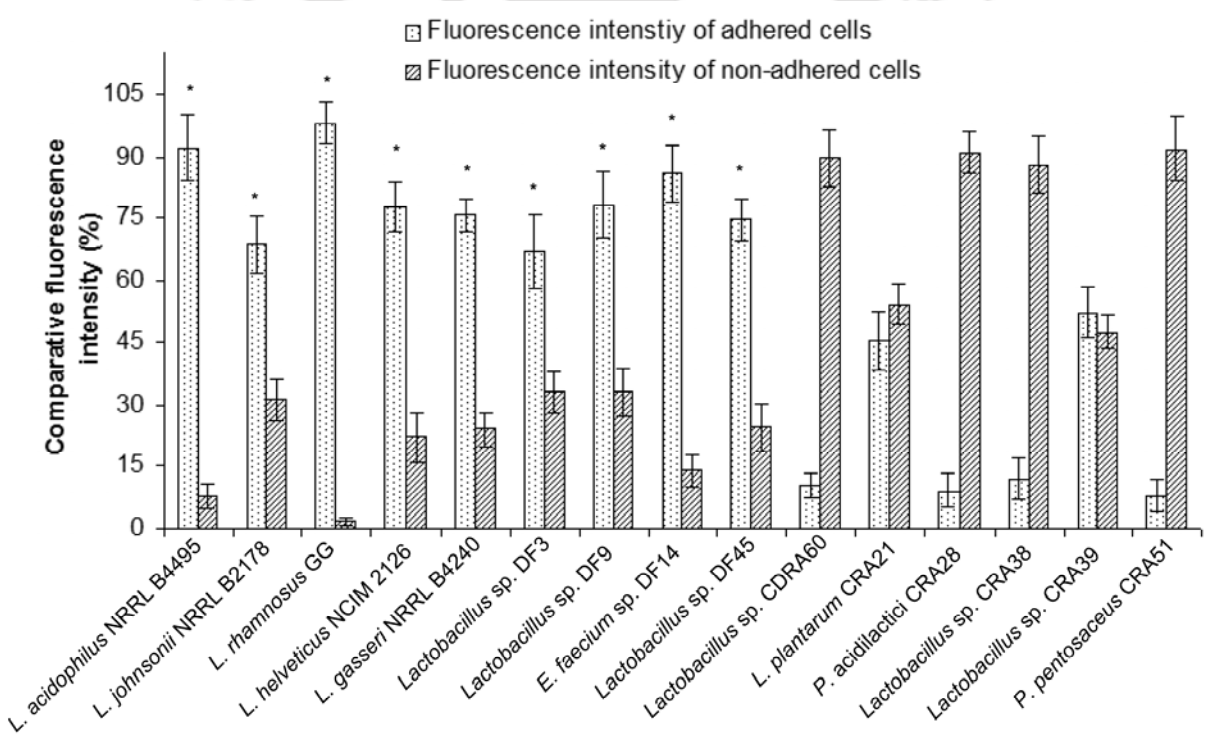

Figure 6.4 Quantification of *in vitro* adhesion of cFDA-SE labelled reference and anti-listerial bacteriocin producing LAB to HT-29 cells. Comparative fluorescence intensity (%) is used as an index of adhesion. The fluorescence intensity of adhered *Lactobacillus rhamnosus* GG was assumed as 100%. *Represents LAB with significant adhesion potential based on statistical analysis (ANOVA). CRA: strains isolated from fermented cucumber; DF: strains isolated from dried fish.

In contrast CFDA-SE labeling has none of these limitations and accurate quantification of the number of LAB cells adhering to HT-29 was consistently determined by obtaining the difference in fluorescence intensity of non-adhered LAB cells from total fluorescence intensity which was a measure of the bacterial cells used for the adhesion assay. It may also be mentioned that cFDA-SE is specific to viable cells as it is dependent on intracellular esterase activity (Hoefel *et al.,* 2003). Thus the fluorescence-based method of estimating LAB adhesion essentially provides a measure of viable cell adhesion onto HT-29 cells and thus enhances the analytical value of this method.

6.3.4. Fluorescence microscopic study of LAB adhesion

The adhesion potential of anti-listerial LAB strains was also estimated by fluorescence microscopic method. The number of adherent cells was counted per 100 epithelial cells and expressed as adhesion index as indicated in Table 6.3. It can be seen fro the table that the reference strain *L. rhamnosus* GG exhibited maximum adhesion potential on HT-29 cells as indicated by an adhesion index of approximately 160. Amongst the native anti-listerial bacteriocin producing LAB, *Enterococcus faecium* DF14 exhibited the highest adhesion potential followed by strain DF9 and DF3. These results are in agreement with the previous results wherein the adhesion of anti-listerial LAB strains was measured by plating and cFDA-SE fluorescence (Figures 6.3 and 6.4). Overall the adhesion index values observed for anti-listerial LAB strains in the present investigation are comparable with those reported previously for other probiotic strains (Gopal *et al.,* 2001; Tsai *et al.,* 2008).

Table 6.3

In vitro adhesion of anti-listerial bacteriocin producing LAB strains on HT-29 cells estimated by fluorescence microscopic method

LAB strain[a]	Adhesion index [b,c]
L. acidophilus NRRL B4495	141 ± 12
L. johnsonii NRRL B2178	102 ± 9
L. rhamnosus GG	160 ± 14
L. helveticus NCIM 2126	112 ± 10
L. gasseri NRRL B4240	122 ± 11
Lactobacillus sp. DF3	101 ± 8
Lactobacillus plantarum DF9	121 ± 10
Enterococcus faecium DF14	139 ± 12
Lactobacillus sp. DF45	99 ± 7
Lactobacillus sp. CDRA60	15 ± 2
Lactobacillus plantarum CRA21	60 ± 4
Pediococcus acidilactici CRA28	12 ± 1
Lactobacillus sp. CRA38	16 ± 2
Lactobacillus sp. CRA39	80 ± 4
Pediococcus pentosaceus CRA51	11 ± 1

[a] DF and CRA indicates LAB strains isolated from sun dried-fish and salt-fermented cucumber samples respectively. [b] Adhesion index represents bacterial cells adhered per 100 HT-29 cells counted in 25 random microscopic fields. [c] Data is represented as mean value ± standard deviation of adhered LAB cells.

Fluorescence microscopic observation of adhered LAB strains on HT-29 cells was also performed and the results of the same are evident in Figure 6.5. It is clear from the figure that a number of LAB cells were adhering to the monolayer of HT-29 cells after extensive washing. This observation eliminated the possibility of mere physical entrapment of LAB cells and indicated that the adhesion was not non-specific.

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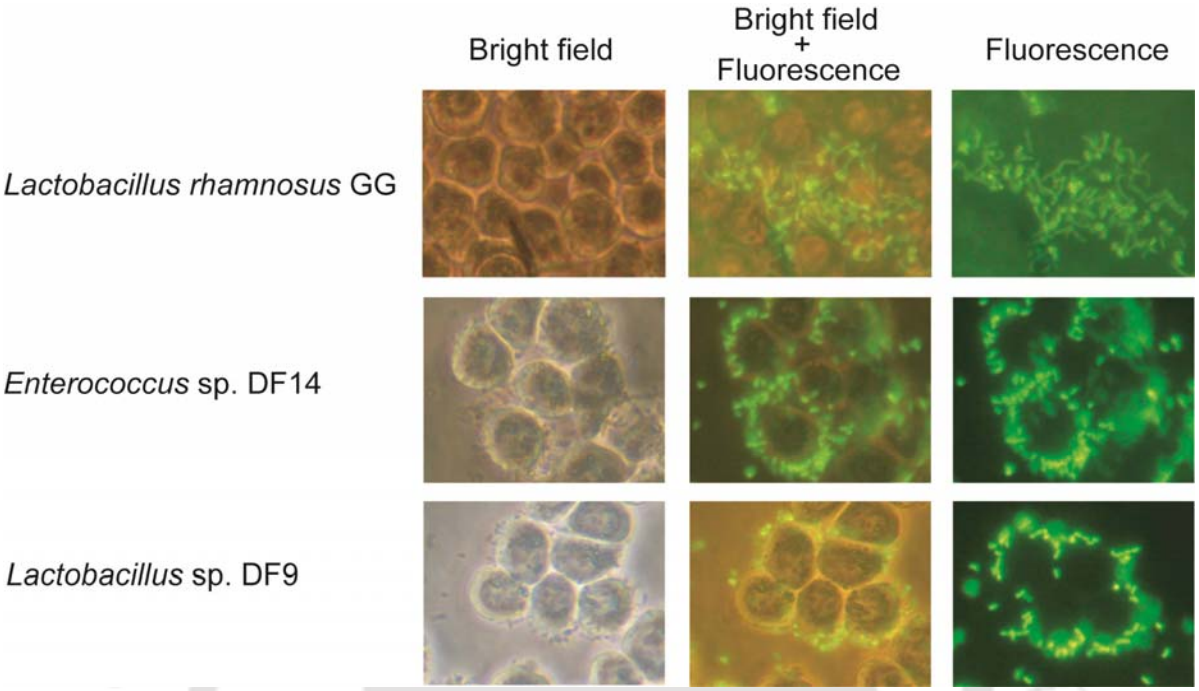

Figure 6.5 Epifluorescence micrograph of cFDA-SE labeled *L. rhamnosus* GG and anti-listerial bacteriocin producing LAB cells adhered to HT-29 cells. The image was captured with 400X magnification.

From Figure 6.5 it was also interesting to observe that the pattern of anti-listerial LAB adhesion on HT-29 cells revealed a distinct difference fro that observed for *L. rhamnosus* GG. In case of the standard strain the adhered cells revealed a diffused spread over the entire cell surface of the epithelial cell. In contrast, the native anti-listerial LAB strains DF14 and DF9 clearly revealed a localized adhesion pattern as they tend to cluster around the periphery margins of the HT-29 cells. This points out to a clear strain variation in the adhesion patterns and points out to the possible difference in the mechanism of LAB adhesion on HT-29 cells.

6.4. Conclusion

1. Acid tolerance and bile salt tolerance tests identified anti-listerial LAB strains DF3, DF9, DF14, DF45, CDRA60, CRA21, CRA28, CRA38, CRA39 and CRA51 as potential probiotic strains.

2. Cell culture based comparative adhesion assay revealed that some of the anti-listerial bacteriocin producing strains isolated from dried fish (DF3, DF9, DF14 and DF45) exhibited significant cell adhesion potential.

3. A fluorescence based adhesion assay was developed for ascertaining the adhesion potential of anti-listerial bacteriocin producing LAB strains. Essentially, the results obtained in the experiments corroborated with earlier results obtained by plating method.

4. Experimental evidence for cell adhesion was also obtained by microscopic enumeration of adhered LAB cells as well as fluorescence microscopic analysis

Summary and Future Scope

SUMMARY AND FUTURE SCOPE

Application of a PCR-based approach could monitor time-dependent succession of LAB in the fermenting cucumber samples and the succession of the LAB population could be rationally linked to the emergence of bacteriocin-producing LAB strains. The PCR-based method developed in the study could be extended to other fermenting samples and the information could form a basis of selecting the optimum time period to isolate bacteriocin producing strains from a naturally fermenting sample.

A facile DNA isolation method from LAB strains was developed which provided compatible templates for PCR-based detection and typing of LAB strains. Application of this method led to reliable molecular fingerprinting analysis of bacteriocin producing LAB strains isolated from fermented cucumber. The DNA extraction method outlined in the present investigation can be adopted on a routine basis in food microbiology laboratories for PCR-based detection of LAB in various indigenous fermented foods as well as in studies that aim to establish the genetic diversity of native LAB strains by PCR-based fingerprinting.

A large number of anti-listerial bacteriocin producing LAB strains were isolated from indigenous source. PCR facilitated genus identification as well as the detection of Class IIa bacteriocin producers amongst these bacteriocin producing LAB isolates. Some of the anti-listerial bacteriocin producing LAB exhibited broad antimicrobial spectrum. A fluorescence-dye leakage assay revealed the potency of anti-listerial activity of the bacteriocin producing LAB strains. The food application potential of an anti-listerial bacteriocin produced by a native LAB isolate in inhibiting the growth of *Listeria monocytogenes* in a widely consumed perishable Indian dairy product like paneer was clearly demonstrated. It is anticipated that some of the potent anti-listerial

LAB strains isolated in the present investigation could find niche applications in food fermentation processes both as starter as well as bioprotective cultures.

An AP-PCR method with a primer targeting a conserved region of the 16S rRNA gene of LAB strains was developed which resulted in significant discrimination of LAB and non-LAB strains. This method could ascertain the genetic diversity of native anti-listerial bacteriocin producing LAB strains on the basis of robust and discriminating fingerprinting patterns. Overall, the study clearly revealed the versatility and the resolving strength of the method and there is an enormous scope of applying the AP-PCR based method described in this work for future food fermentation and microbiological quality control applications.

Gene sequencing analysis revealed the identity of three potent anti-listerial bacteriocin producing LAB strains as well as their bacteriocin. A comparative analysis based on fluorescence, microscopy and flow cytometry revealed the potency of the anti-listerial bacteriocin produced by the LAB strains.

The probiotic potential of select anti-listerial bacteriocin producing LAB strains was ascertained on the basis of *in vitro* cell adhesion assay on HT-29 cells. Significantly, some of the natural LAB strains isolated in the course of the research work revealed comparable adhesion potential with respect to standard probiotic LAB strains. These strains hold considerable promise as probiotics and mucosal delivery systems.

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APPENDIX

1. Quantity One Software based similarity analysis:

Quantity One Software Versioin 4.5 (Bio-Rad, USA) was used for similarity comparison of banding profile of lane-based samples in a gel. To study the cluster analysis and draw phylogenetic tree banding sets of each samples in a lane were matched. Similarity comparisons were performed on the basis of Dice coefficient values with Unweighted Pair Group Method using Arithematic Averages (UPGAMA) algorithm. The method for computing similarity in Quantity One is the Dice Coefficient. The formula for the Dice Coefficient is:

$$sim = 200 \times \frac{\sum_{i=1}^{B} Min(s_{i,} t_i)}{\sum_{i=1}^{B} Min(s_i + t_i)}$$

$$dist = 100 - sim$$

where,

sim repersents similarity between samples in two lane.

dist represents dissimilarity between samples in two lane.

S and T are vectors representing two lanes in the same band set that are being compared.

B represents no. of elements (band sets) in each vector.

To compute similarity, a vector is constructed that represents the bands identified in the lane. The vector depends on the comparison options selected. If the search was done on classified (matched) bands only, then the vector S contains B elements (S = (s1, s2, s3 ... sB)), where B is the number of band types in the lane's band set. The values for s1, s2, s3 ... sB have the following values:

Weighting Off or Unweighted Search (Density of bands is not considered)

s_i = 1 if the i'th band type is found in the lane.

s_i = 0 if it is not found.

Weighting On or Weighted Search (Density of band is considered)

If the band set has a normalizing band type, then:

s_i = The normalized density of the band assigned to the i'th band type.

s_i = 0 if the lane does not have a band assigned to the i'th type.

2. Statistical analysis of data based on one way Analysis of Variance (ANOVA):

2a. Statistical analyses (by an ANOVA test with the criterion of a P value of <0.05) of bacteriocins producing zone of inhibition (ZOI) against various pathogens

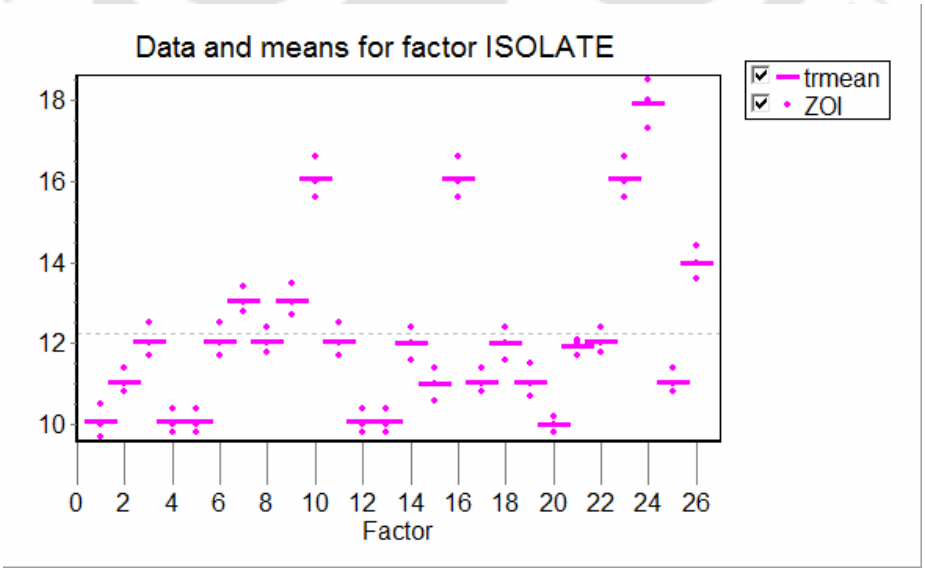
2a.1 Pathogen: *Listeria monocytogenes* Scott A

```
OneWay Analysis of Variance

YVAr 'ZOI'
: ONEway 'ISOLATE'

          ANOVA TABLE for ZOI against L. monocytogenes ScottA

Source   DF             SS             MS           F       Prob.
-----------------------------------------------------------------
ISOLATE   25         350.54          14.022         97.0     0.000
Residual  52           7.52         0.14462
-----------------------------------------------------------------
Total     77         358.06
-----------------------------------------------------------------

Overall Mean = 12.24   s (Residual) = 0.3803
Coefficient of Variation = 3.1 %
```

MAIN EFFECTS

ISOLATE

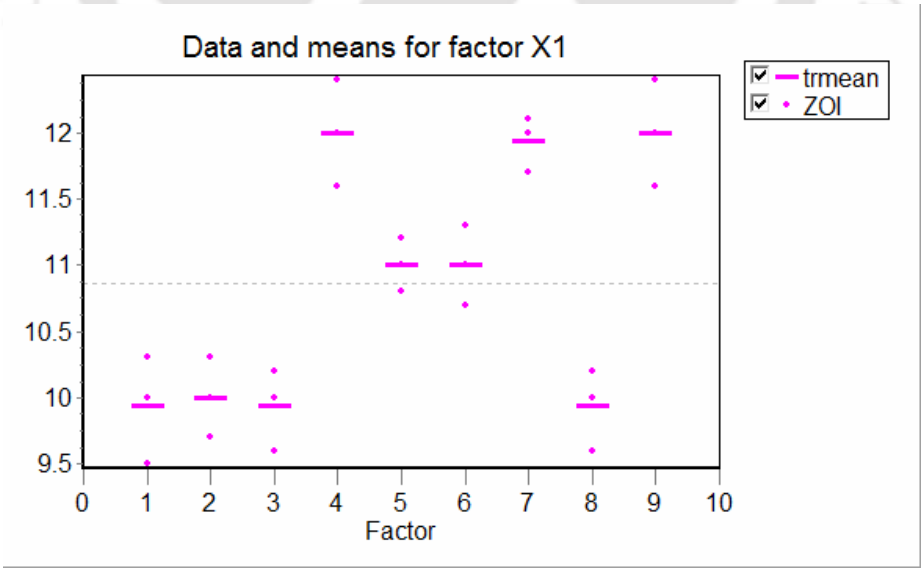
Level	Mean	Count	S.E.	
CDRA10	10.07	3	0.2196	[1]
CDRA13	11.07	3	0.2196	[2]
CDRA19	12.07	3	0.2196	[3]
CDRA27	10.07	3	0.2196	[4]
CDRA39	10.07	3	0.2196	[5]
CDRA57	12.07	3	0.2196	[6]
CDRA60	13.07	3	0.2196	[7]
DF3	12.07	3	0.2196	[8]
DF9	13.07	3	0.2196	[9]
DF14	16.07	3	0.2196	[10]
DF45	12.07	3	0.2196	[11]
CRA3	10.07	3	0.2196	[12]
CRA6	10.07	3	0.2196	[13]
CRA11	12	3	0.2196	[14]
CRA16	11	3	0.2196	[15]
CRA21	16.07	3	0.2196	[16]
CRA23	11.07	3	0.2196	[17]
CRA28	12	3	0.2196	[18]
CRA38	11.07	3	0.2196	[19]
CRA39	10	3	0.2196	[20]
CRA49	11.93	3	0.2196	[21]
CRA50	12.07	3	0.2196	[22]
CRA51	16.07	3	0.2196	[23]
CRA52	17.93	3	0.2196	[24]
CRA53	11.07	3	0.2196	[25]
CRA61	14	3	0.2196	[26]



2a.2 Pathogen : *Staphylococcus aureus* MTCC 96

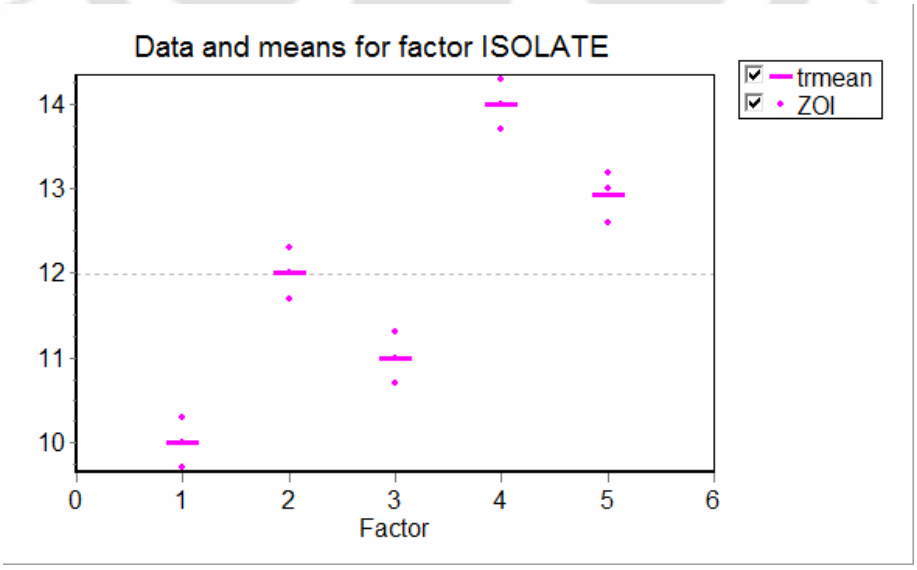
OneWay Analysis of Variance

```
YVAr 'ZOI'
: ONEway 'ISOLATE'

          ANOVA TABLE for ZOI

Source   DF             SS             MS           F       Prob.
-------------------------------------------------------------------
ISOLATE   8         21.319         2.6648        25.7     0.000
Residual  18        1.8667         0.1037
-------------------------------------------------------------------
Total     26        23.185
-------------------------------------------------------------------

Overall Mean = 10.86   s (Residual) = 0.322
Coefficient of Variation = 3.0 %

      MAIN EFFECTS
      ------------


        ISOLATE
    Level     Mean   Count   S.E.

  CDRA19     9.933     3   0.1859 [1]
  CDRA27        10     3   0.1859 [2]
     DF9     9.933     3   0.1859 [3]
    DF14        12     3   0.1859 [4]
   CRA21        11     3   0.1859 [5]
   CRA49        11     3   0.1859 [6]
   CRA52     11.93     3   0.1859 [7]
   CRA50     9.933     3   0.1859 [8]
   CRA51        12     3   0.1859 [9]
```



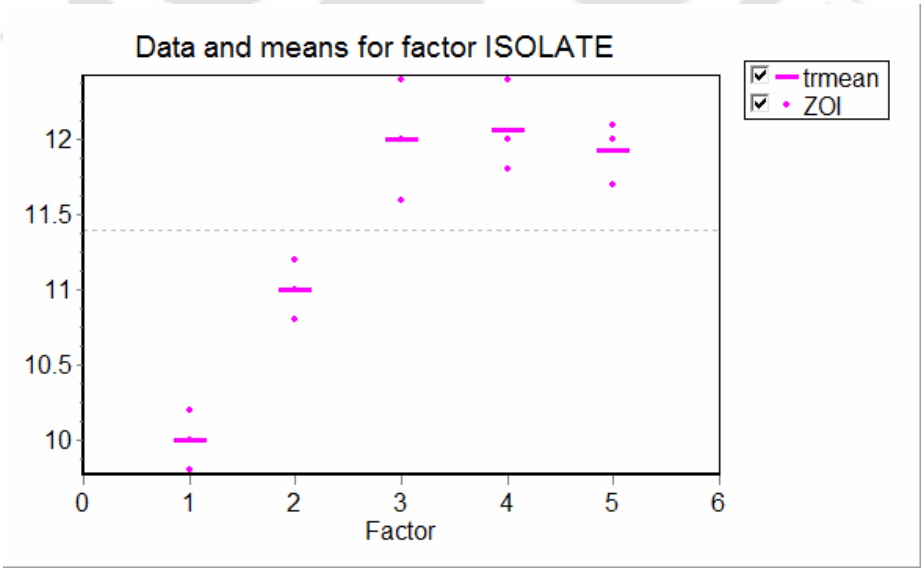
2a.3 Pathogen : *Enterococcus faecalis* MTCC 439

```
OneWay Analysis of Variance
YVAr 'ZOI'
: ONEway 'ISOLATE'

          ANOVA TABLE for ZOI

Source   DF             SS             MS           F       Prob.
-------------------------------------------------------------------------
ISOLATE    4         29.611         7.4027        81.6      0.000
Residual  10        0.90667        0.09067
-------------------------------------------------------------------------
Total     14         30.517
-------------------------------------------------------------------------

Overall Mean = 11.99    s (Residual) = 0.3011
Coefficient of Variation = 2.5 %

      MAIN EFFECTS
      ------------


        ISOLATE
    Level     Mean    Count    S.E.

  CDRA27         10      3    0.1738 [1]
    DF14         12      3    0.1738 [2]
   CRA21         11      3    0.1738 [3]
   CRA52         14      3    0.1738 [4]
   CRA51      12.93      3    0.1738 [5]
```



2a.4 Pathogen : *Bacillus cereus* MTCC 1305

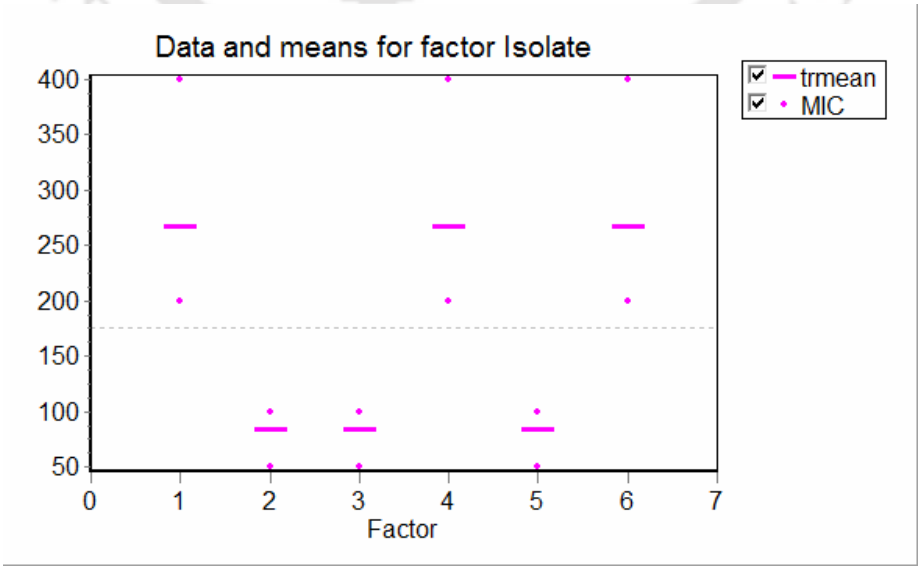
```
OneWay Analysis of Variance
YVAr 'ZOI'
: ONEway 'ISOLATE'

          ANOVA TABLE for ZOI

Source   DF             SS             MS           F       Prob.
-----------------------------------------------------------------------
ISOLATE    4         9.6267         2.4067        31.9     0.000
Residual  10        0.75333        0.07533
-----------------------------------------------------------------------
Total     14          10.38
-----------------------------------------------------------------------

Overall Mean = 11.4   s (Residual) = 0.2745
Coefficient of Variation = 2.4 %

The following observations have large residuals
Observation   Value   Residual       se     Ratio     %RSS
      7        11.6       -0.4    0.224     -1.78     21.2
      9        12.4        0.4    0.224      1.78     21.2


      MAIN EFFECTS
      ------------


        ISOLATE
    Level     Mean   Count   S.E.

  CDRA27       10       3   0.1585 [1]
    DF14       11       3   0.1585 [2]
   CRA21       12       3   0.1585 [3]
   CRA52    12.07       3   0.1585 [4]
   CRA51    11.93       3   0.1585 [5]
```



2b. Statistical analyses (by an ANOVA test with the criterion of a P value of <0.05) of bacteriocins for its minimum inhibitory concentration (MIC) against Listeria monocytogenes Scott A.

OneWay Analysis of Variance

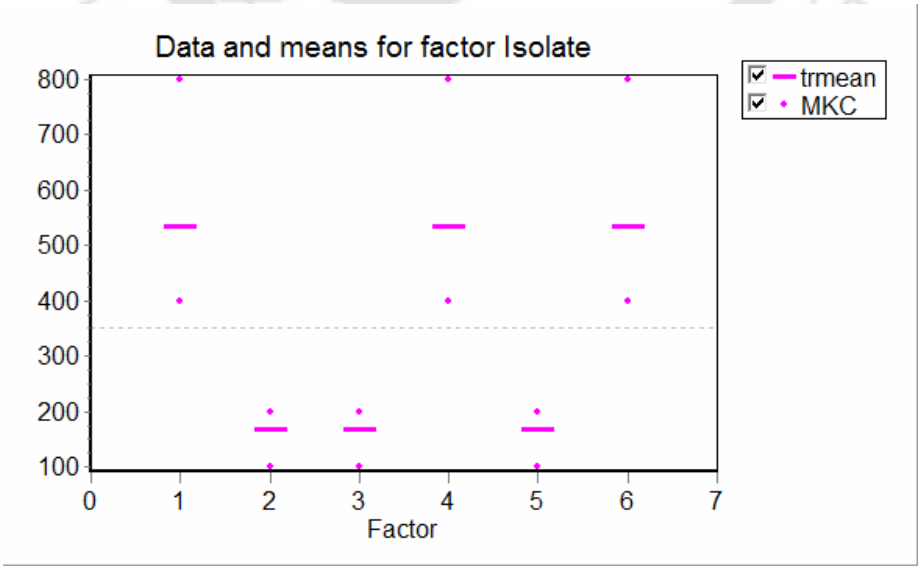
```
YVAr 'MIC'
: ONEway 'Isolate'

          ANOVA TABLE for MIC

Source   DF             SS             MS          F       Prob.
-------------------------------------------------------------------------
Isolate    5         151250          30250        4.3      0.018
Residual  12          85000         7083.3
-------------------------------------------------------------------------
Total     17         236250
-------------------------------------------------------------------------

Overall Mean = 175   s (Residual) = 84.16
Coefficient of Variation = 48.1 %

The following observations have large residuals
Observation   Value   Residual       se     Ratio     %RSS
      2         400      133.3     68.7      1.94      20.9
     12         400      133.3     68.7      1.94      20.9
     18         400      133.3     68.7      1.94      20.9

       MAIN EFFECTS
       ------------

         Isolate
    Level     Mean   Count   S.E.

     DF9      266.7     3   48.59 [1]
    DF14      83.33     3   48.59 [2]
   CRA21      83.33     3   48.59 [3]
   CRA28      266.7     3   48.59 [4]
   CRA51      83.33     3   48.59 [5]
   CRA61      266.7     3   48.59 [6]
```



2c. Statistical analyses (by an ANOVA test with the criterion of a P value of <0.05) of bacteriocins for its minimum killing concentration (MKC) against Listeria monocytogenes Scott A.

OneWay Analysis of Variance

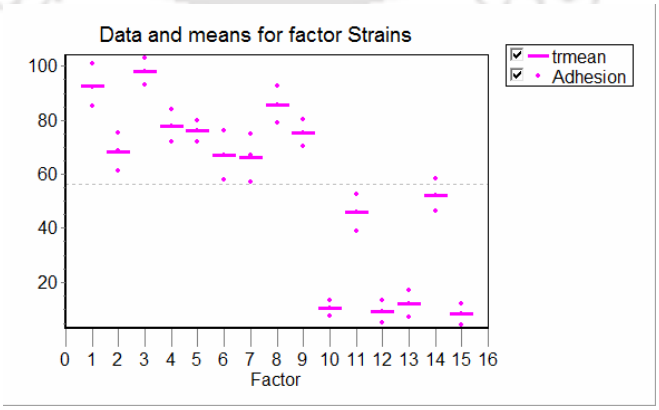
```
YVAr 'MKC'
: ONEway 'Isolate'

          ANOVA TABLE for MKC

Source   DF             SS             MS          F       Prob.
-----------------------------------------------------------------------
Isolate    5         605000         121000        4.3      0.018
Residual  12         340000          28333
-----------------------------------------------------------------------
Total     17         945000
-----------------------------------------------------------------------

Overall Mean = 350   s (Residual) = 168.3
Coefficient of Variation = 48.1 %

The following observations have large residuals
Observation    Value    Residual       se      Ratio      %RSS
      3          800       266.7      137       1.94       20.9
     10          800       266.7      137       1.94       20.9
     18          800       266.7      137       1.94       20.9

       MAIN EFFECTS
      ------------
        Isolate
    Level      Mean    Count    S.E.

     DF9       533.3      3    97.18 [1]
    DF14       166.7      3    97.18 [2]
   CRA21       166.7      3    97.18 [3]
   CRA28       533.3      3    97.18 [4]
   CRA51       166.7      3    97.18 [5]
   CRA61       533.3      3    97.18 [6]
```



2d. Statistical analyses (by an ANOVA test with the criterion of a P value of <0.05) of LAB strains used for in vitro adhesion assays.

OneWay Analysis of Variance

```
YVAr 'Adhesion'
: ONEway 'Strains'

          ANOVA TABLE for Adhesion

Source   DF             SS             MS           F       Prob.
-----------------------------------------------------------------------
Strains   14          42779         3055.7        79.2     0.000
Residual  30        1156.9           38.565
-----------------------------------------------------------------------
Total     44          43936
-----------------------------------------------------------------------

Overall Mean = 56.3   s (Residual) = 6.21
Coefficient of Variation = 11.0 %

      MAIN EFFECTS
      ------------


        Strains
    Level     Mean   Count   S.E.

   B4495      92.77     3   3.585 [1]
   B2178      68.33     3   3.585 [2]
      GG        98     3   3.585 [3]
   N2126       77.8     3   3.585 [4]
   B4240       75.9     3   3.585 [5]
     DF3       66.9     3   3.585 [6]
     DF9      66.23     3   3.585 [7]
    DF14       85.8     3   3.585 [8]
    DF45       75.3     3   3.585 [9]
  CDRA60       10.4     3   3.585 [10]
   CRA21       45.7     3   3.585 [11]
   CRA28        9.1     3   3.585 [12]
   CRA38       11.9     3   3.585 [13]
   CRA39       52.3     3   3.585 [14]
   CRA51        8.1     3   3.585 [15]
```



Appendix

Table A1

Gene sequence deposition in NCBI GenBank

S. No.	Gene[a]	Length	Gene product	Accession No.
1.	16S rRNA partial seq.	656 bp	Non-coding sequence	EF636660
2.	16S rRNA partial seq.	689 bp	Non-coding sequence	EF636661
3.	16S rRNA partial seq.	696 bp	Non-coding sequence	EF636662
4.	16S rRNA partial seq.	793 bp	Non-coding sequence	FJ424061
5.	16S rRNA partial seq.	684 bp	Non-coding sequence	FJ424059
6.	16S rRNA partial seq.	665 bp	Non-coding sequence	FJ424060
7.	plantaricin A partial cds	52 bp	Plantaricin A peptide	FJ424063
8.	pediocin partial cds	110 bp	Pediocin peptide	EU616745
9.	enterocin A partial cds	106 bp	Enterocin A peptide	FJ424062

[a] Seq: Sequence; cds: Coding sequence

Publications from Ph.D. research work

(A) Publications in refereed international journals:

1. Singh, A.K., Ramesh, A. (2008). Succession of dominant and antagonistic lactic acid bacteria in fermented cucumber: Insights from a PCR-based approach. *Food Microbiology* **25**, 278-287.
2. Singh, A.K., Ramesh, A. (2009). Evaluation of a facile method of template DNA preparation for PCR-based detection and typing of lactic acid bacteria. *Food Microbiology* **26**, 504-513.
3. Singh, A.K., Adhikari, M.D., Ramesh, A. (2009). Anti-listerial bacteriocin from lactic acid bacteria: Isolation from indigenous samples, assessment of bacteriocin activity and food application potential. *International Journal of Food Microbiology*. Manuscript No. FOOD-D-09-01058 **(Under Review)**.
4. Singh, A.K., Ramesh, A. (2009). A novel primer targeting a 16S rDNA conserved region generates discriminatory patterns in AP-PCR and facilitates strain tracking and molecular fingerprinting of bacteriocin producing lactic acid bacteria. *International Journal of Food Microbiology*. **(To be submitted)**.
5. Singh, A.K., Adhikari, M.D., Ramesh, A. (2009). Assessment of the probiotic traits of antagonistic lactic acid bacteria based on functional assay for bacteriocin activity and *in vitro* adhesion to intestinal cells. **(To be submitted)**.

(B) Conference Presentations:

1. Singh, A. K., Singh, S. and Ramesh, A. (2006) A molecular approach for detection and typing of lactic acid bacteria from fermented samples. Presented in 18th Indian Convention of Food Scientists & Technologists (ICFOST), Acharya N.G. Ranga Agricultural University (ANGRAU), Nov. 16-17, Hyderabad, India.
2. Singh, A.K., Ramesh, A. (2008). Application of molecular fingerprinting techniques to study genetic diversity of antagonistic lactic acid bacteria. Presented in 19th Indian Convention of Food Scientists & Technologists (ICFOST), Indian Institute of Technology Kharagpur, 31 December 2007-2 January 2008. **(Received the best poster award in the area of Food Microbiology)**.

Contributions in other collaborative research projects:

During the Ph.D. research program, comprehensive research skills were acquired in the area of molecular microbiology with special reference to molecular strain detection and design of antimicrobial assays. The developed research skills were also utilized towards significant contributions in collaborative interdisciplinary research projects dealing with biomineralization using lactic acid bacteria, fluorescent nanoparticle based rapid estimation of bacterial cells and enzyme-mediated nanoparticle synthesis. The outcome of the contributions is as follows:

1. Borah. B.M., Singh. A. K., Ramesh. A., Das. G. (2009). Lactic acid bacterial extract as a biogenic mineral growth modifier. *Journal of Crystal Growth* **311**, 2664-2672.
2. Panda, B.R., Singh, A.K., Ramesh, A., Chattopadhyay, A. (2008). Rapid estimation of bacteria by a fluorescent Au-nanoparticle-polythiophene composite. *Langmuir* **24**, 11995-12000.
3. Rangnekar, A., Sarma, T. K., Singh, A. K., Deka, J., Ramesh, A., Chattopadhyay, A. (2007). Retention of enzymatic activity of α-amylase in the reductive synthesis of gold nanoparticles. *Langmuir* **23**, 5700-5706.