PCR BASED IDENTIFICATION OF BILE SALT HYDROLASE POSITIVE LACTOBACILLI

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE
IN
DAIRYING
(DAIRY MICROBIOLOGY)

BY

NEELAKANTESHWARA. G

B.Tech (Dairy Technology)

DIVISION OF DAIRY MICROBIOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(I. C. A. R.)
KARNAL - 132001 (HARYANA), INDIA

2005

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DEDICATED

TO

MY BELOVED

PARENTS

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(DAIRY MICROBIOLOGY)

Approved by:

( )
EXTERNAL EXAMINER

(Dr. V.K. BATISH)
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CERTIFICATE

This is to certify that the thesis entitled 'PCR Based Identification of Bile Salt Hydrolase Positive Lactobacilli' submitted by Mr.*Neelakanteshwara. G* towards the partial fulfilment of the requirements for the award of the degree of **Master of Science** in Dairying (**Dairy Microbiology**) of the **National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

6.7.05

Dated: June, 2005

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(Neelakanteshwara. G)

ABSTRACT

Probiotics have recently been able to evoke lot of commercial interest in functional and health food market by virtue of expressing health-promoting functions in the gut. Among these, lactobacilli constitute one of the important members and several probiotic lactobacillus strains are currently being explored in the production of probiotic preparations particularly in the western world. However, due to the limited availability of probiotic lactobacilli cultures, there is a need for isolation and characterization of novel indigenous probiotic cultures. Hence, development of a reliable identification tool is an important issue for their characterization. Molecular based tools can provide a solution for proper identification of probiotic Lactobacilli. BSH seems to be a potential probiotic marker that can be explored for screening of new probiotic lactobacilli and short listed cultures then can be further characterized for the additional functional attributes, directly related to secure health progress .In this study, we have targeted BSH gene for development of PCR assays. During the course of this investigation, a total of 100 isolates recovered from human faecal samples, buffalo milk, probiotic preparations were tentatively identified on the basis of Gram staining and catalase test. Out of total of 100 isolates, 35 were identified as Lactobacilli based on genus specific PCR assay. All the 35 Lactobacilli isolates were subjected to pH tolerance, bile tolerance, antibiotic susceptibility, antimicrobial activity and hydrophobicity assays. Some of the isolates exhibited bile tolerance, antimicrobial activity and hydrophobicity.

In order to develop BSH base PCR assays, a total of 7 primer pairs including two for cloning BSH gene were designed based on the BSH sequences available in NCBI database. The primer pair LjBSHAF/AR AND LjBSHBF/BR gave amplification products of 171 and 168 bp respectively with only *L. jhonsonii* La1indicating their specificity. All the 35 isolates of Lactobacilli were tested with the 7 pairs of primers. However, amplification could be observed with only two of the primer pairs designed from BSH gene sequence of *L. plantarum* (Accession No. A24002). Ten of the isolates gave an amplification product of 230 bp with primer pair LpBSHF/R and 975 bp with cloning primer LpBSH1F/975R. All the primer pairs were also checked for their specificity against non-Lactobacilli particularly members belonging to Enterobacteriacae. However, amplification of 231 bp product could be observed with only one set of the primer i.e. LpBSHF/R with *E. coli*, Listeria and Bifidobacteria. The BSH gene (975 bp) from 3 cultures namely, *L. plantarum* 201, Lb9 and Lb10 was then cloned in *E. coli* and got custom sequenced. The sequence analysis indicated high degree of homology (99%) between *L. plantarum* sequence available at NCBI.

सारांश

मनुष्य के पाचन तन्त्र को स्वास्थ्यवर्धक गुणों में प्रदर्शित करने के कारण प्रतिजैविक जीवाणुओं का वाणिज्यिक स्तर बढ़ा है और कारक व स्वास्थ्यवर्धक खाद्य पदार्थो के बाजार में अनेकों अवसर भी उत्पन्न हुए हैं। इन प्रतिजैविक जीवाणुओं में लक्टोबैसीलाई, जो एक महत्वपूर्ण जीवाणु है, को आजकल विभिन्न प्रतिजैविक पदार्थो में मुख्य रूप से पश्चिमी देशों में उपयोग किया जाता है। परन्तु इन प्रतिजैविक लैक्टोबेसीलाई जीवाणुओं की अधिकता न होने के कारण इन जीवाणुओं के पृथकीकरण व पहचान आवश्यक है। इसलिए इन जीवाणुओं के मानकीकरण हेतु उपयुक्त व विश्वास योग्य पहचान करने हेतु विधि विकास एक महत्वपूर्ण विषय है। प्रतिजैविक लैक्टोबैसीलाई के उपयुक्त पहचान हेतु आणविक विधियां उत्तम समाधान हो सकती है। BSH क्रिया एक मुख्य प्रतिजैविक चिह्न जिसे नये प्रतिजैविक लैक्टोबैसीलाई को छांटने में प्रयोग किया जा सकता है और इस आधार पर छांटे गये जीवाणुओं को अतिरिक्त प्रतिजैविक क्रियाओं के लिए जांचा जा सकता है; जिसका सीधा सम्बंध मानव स्वास्थ्य से है। कुछ प्रयोग में हमने BSH जीन को आधार रखकर PCR विधि का विकास किया। इन प्रयोगों में कुल 100 पृथक जीवाणुओं को मानव मल, भैंस के दूध, व विभिन्न प्रतिजैविक पदार्थो से निकाला गया व ग्राम रंग विधि व कैटालेज़ किण्वन के आधार पर प्रारम्भ में लैक्टोबैसीलाई के रूप में पहचाना गया। इसके उपरांत कुल 35 जीवाणुओं को जारी पूरक PCR विधि द्वारा लैक्टोबैसीलाई जीवाणु के रूप में पहचाना गया। इन सभी 35 जीवाणुओं को अम्ल, बाईल, एंटीबॉयोटिक जीवाणु कारक क्षमता, हाईड्रोफोबीसीटी व कैको 2 कोशिका जाल के साथ प्रभाव देखा गया। कुछ जीवाणुओं में बाईल क्षमता, जीवाणु कारक क्षमता व हाईड्रोफोबिसिटी पाई गई।

BSH आधारित PCR विधि में विकास हेतु कुल 7 प्राईमर जोड़ें (जिनमें दो BSH जीन की कलोनिंग में लिए थे) को NCBI संग्रह में उपलब्ध BSH जीन में क्रम के आधार पर विकसित किये गये। प्राईमर जोड़े क्रमशः LjBSHAF/AR / व LjBSHBF/BR ने PCR के पश्चात 171 –168 bp के उत्पाद दिये जो केवल लैक्टोबैसीलस जौनसोनाई के लिए पर्याय थे। सभी 35 पृथक जीवाणुओं को 7 प्राईमर जोड़ों में साथ जांचा गया। परन्तु PCR उत्पाद केवल 2 प्राईमर जोड़ों में साथ ही पाये गये जो कि लैक्टोबैसीलस प्लेनटेरम के BSH जीन क्रम के प्रयाय थे। कुल 10 जीवाणुओं ने LpBSHF/R के साथ 231 bp के व क्लोनिंग प्राईमर LpBSH1F/ /975 R के साथ 975 bp के उत्पाद दिये। सभी प्राईमर जोड़ों को उनकी पर्यायता की जांच हेतु लैक्टोबेसीलाई के अतिरिक्त मुख्य रूप से फैमिली एन्टैरोबैक्टीरीऐसी के सदस्यों के साथ PCR कर देखा गया। केवल एक प्राइमर जोड़े (LpBSHF/R) ने ई. कोलाई, लीस्टेरिया व बाईफीडोबैक्टीरिया के साथ 231 bp का PCR उत्पाद दिया। विभिन्न 3 जीवाणुओं (एल प्लेनटेरम 201, Lb9 & Lb10) के BSH जीन को ई. कोलाई में क्लोन कर उसका क्रम जाना गया। इस क्रम की NCBI में उपलब्ध लैक्टोबैसीलस प्लैनटैरम के क्रम से उत्तम श्रेणी की (99 प्रतिशत) समानता पाई गई ।

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ABBREVIATIONS

ATCC	:	American Type Culture Collection
DGGE	:	Denaturing Gradient Gel Electrophoresis
DNA	:	Deoxyribonucleic Acid
EBI	:	European Bioinformatics Institute
FISH	:	Fluorescence *in situ* Hybridisation
G+C	:	guanine-plus-cytosine
GI	:	gastrointestinal
GRAS	:	Generally Recognized As Safe
IL	:	Interleukin
PCR	:	Polymerase Chain Reaction
RNA	:	Ribonucleic Acid
rDNA	:	Ribosomal DNA
rRNA	:	Ribosomal RNA
SSCP	:	Single-Strand Conformation Polymorphism
TGGE	:	Thermal Gradient Gel Electrophoresis
TRF	:	Terminal Restriction Fragment
TRFLP	:	Terminal Restriction Fragment Length Polymorphism
NCFM	:	National Collection of Food Microorganism
BSH	:	Bile Salt Hydrolase
NCDC	:	National Collection of Dairy Cultures
LAB	:	Lactic Acid Bacteria
ITS – PCR	:	Internal Transcribed Spacer PCR
APF	:	Aggregation-promoting Factor
RAPD	:	Randomly Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
OTU	:	Operational Taxonomic Units
ssDNA	:	single stranded DNA
dsDNA	:	double stranded DNA
bp	:	base pairs
PI-PCR	:	Prokaryotic *in situ* PCR
dNTPs	:	deoxyribonucleotides
EHC	:	Enterohepatic circulation
kb	:	Kilobase
mMol/h	:	milliMole per hour
LP	:	*Lactobacillus plantarum*
OD	:	Optical Density
GDCA	:	Glycodeoxycholic acid
TDCA	:	Taurodeoxycholic acid
CBSH	:	Conjugated Bile Salt hydrolase
CBST1	:	Conjugated Bile Salt Transporter
BHI	:	Brain heart infusion
MRS	:	De mann rogosa sharpe

1.0 INTRODUCTION

In recent times, there has been a wide spread change in consumer perception towards food. Food is being perceived not only as a source of nutrition but also as therapeutic agent. This shift has generated new concepts of functional foods and neutraceuticals. Lactic acid bacteria play a very important role as starters in production of such health foods as they are food grade organisms and are generally regarded as safe (GRAS). Included among them are a special class of beneficial organisms designated as probiotics which can promote the health of the consumers by expressing several desirable biological functions in the gut. There are numerous probiotic genera, species and strains that have been identified and characterized over the past one hundred years. Most notably, *Lactobacillus* and Bifidobacteria are most often considered in the probiotic category.

Probiotics are defined as “Live microbial food supplements beneficial to health and have a positive effect in the prevention and treatment of intestinal microbial balance (Fuller, 1989). A probiotic strain should be of host origin, non-pathogenic, technologically suitable for industrial processes, acid and bile-fast, adhere to the gut epithelial tissue, produce antimicrobial substances, modulate immune responses and influence the metabolic activities of the gut (Dunne *et al.,* 1999)**.**

Strains selected for use as probiotic bacteria should be able to tolerate acid for atleast 90 min, tolerate bile acids, attach to the epithelium and grow in the lower intestinal tract before they can start providing any health benefits (Lanszer *et al.* 1999). Cellular stress begins in the stomach, which has pH as low as 1.5. After the bacteria pass through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. After traveling through this harsh environment, the organism colonizes the epithelium of the lower intestinal tract.

Lactic acid bacteria traditionally used as starter cultures in the preparation of various fermented foods can also play a significant role as

major ingredients in functional foods and neutraceuticals because of expression of health promoting functions. They have potential significance in fermentation, bio- processing, agriculture and health benefiting foods.

The probiotic properties of lactic acid bacteria are considered to be strain specific. Hence, the identification of probiotic lactobacilli at strain level by exploring appropriate analytical techniques has become extremely important step and is the need of the hour to enhance their credibility and commercial value.

Conventional phenotypic methods, however, are not suitable for identification of LAB, and are often ambiguous. Moreover, differentiation of these organisms at strain level is a difficult task. Genotypic methods and genetic analysis of genome sequences have paved the way for recent molecular techniques to be used for identification. The commonly used molecular techniques for identification of these organisms include Polymerase Chain Reaction with group specific primers, Dot Blot Hybridization (Dorea *et al.,* 1998), FISH (Sghir *et al.,* 1998), Terminal Restriction Fragment Length Polymorphism (TRFLP), Density Gradient Get Electrophoresis (DGGE) (Zoentendal *et al.,* 1998) and so on.

Among the aforesaid techniques, PCR is an effortless and reliable method for detection by targeting the particular gene responsible for probiotic characteristics. The recent exploration of complete genome sequences of probiotic organisms has been of immense value to develop the primers for assaying strain specific probiotic organisms for their use in functional foods, pharmaceuticals and neutraceuticals.

Probiotic properties of the lactobacilli strains are supposed to be strain specific and these functional properties are encoded by the specific genes located in the genomes of particular strains of lactobacilli. The knowledge of genome maps will provide a solid platform for comparative genomic analysis of these organisms that survive and establish in the hostile environment of the gastrointestinal tract of humans and protect them against the invasion of undesirable organisms.

Genome sequences and bioinformatics present volumes of information for rational selection of genes for identification, confirmation and characterization of functional roles of lactobacilli. The currently presumed roles associated with probiotic attributes important for colonization, survival and functionality include acid tolerance, bile tolerance, surface proteins, lipoteichoic acid, adherence factors and aggregation proteins etc**.** Some of the important genes that are encoded in genome of *L. acidophilus* NCFM are S layer protein (SlpA and –B), Mub-9, (Mucus binding protein), BSH A and B (Bile salt hydrolases), PrLA-I and PrLA-II (phage ruminants), EPS cluster (endopolysaccharide) and Lactocin-B (bacteriocin). Out of these, BSH seems to be a potential probiotic marker that can be expressed for screening of new probiotic lactobacilli and the short listed cultures then can be further characterized for the additional functional attributes, directly related to secure human health progress.

Bile salt hydrolase activity has been reported to be expressed in several probiotic lactobacilli. The multiplicity of BSH encoding genes and bile transporters in *Lactobacillus johnsonii* NCC 533 and other lactobacilli species implies the potential importance of these gene sets for GIT survival and persistence.

By targeting the genes encoding BSH enzyme or targeting the genes responsible for other particular probiotic functions, appropriate specific primers can be designed to explore and develop suitable PCR techniques for identification of probiotic lactobacilli. This study is primarily focused to develop the primers for bile salt hydrolase genes in lactobacilli which will be helpful in mass screening of the probiotic lactobacilli with bile salt hydrolase activity. Incorporation of such genetic tools in probiotic research is expected to reveal the contribution of probiotics in general human health and well being and will explicitly identify the mechanisms and corresponding host responses that provide the basis for their positive roles.

Keeping all the relevant issues in mind, this project was undertaken with the following objectives:

1) To develop PCR based techniques targeted against BSH for identification of BSH positive lactobacilli.

2) To apply the aforesaid techniques for screening of indigenous probiotic lactobacilli.

3) To clone and sequence BSH gene from selected indigenous BSH positive lactobacilli isolates to explore the possibility of developing strain specific PCR.

2.0 REVIEW OF LITERATURE

The human gastrointestinal tract is inhabited by a diverse bacterial population that constitutes a complex ecosystem. More than 400 different bacterial species have been isolated and identified in faeces (Rolfe,1997).

In healthy individuals, the stomach and upper small intestine have relatively low number of microorganisms. The lower small intestine is a transition zone between the sparely populated upper gastrointestinal tract and the heavily bacterially populated colon. In the lower ileum, the number of bacteria increases to the level of 10^6 to 10^7 organisms per milliliters of contents. However in the colon, the bacterial concentration increases dramatically, reaches the staggering figures of 10^{11} and 10^{12} organism per milliliter of faecal material. Colonization of the organisms in the gastrointestinal tract of humans occurs within a few days after birth (Haenel, 1970). The course of colonization is influenced by gestational age, type of delivery, and dietary constituents. The initial phase of colonization occurs over approximately a two week period. During this period, the bacterial colonization is similar in breast and formula fed infants. In case of breast fed infants, the other bacterial reduction occurs with an increase in Bifidobacteria in the intestine and this type of decrease in bacterial population was not found in formula fed infants (Stand and Lec, 1982).

2.1 LACTIC ACID BACTERIA

The lactic acid bacteria which are abundantly found in intestinal tract are Lactobacilli, *Bifido*bac*terium, Enterococcus faecalis* and *faecium.* Among them, Lactobacilli are the most common and important member of lactic acid bacteria which comprise a diverse group of gram-positive food-grade bacteria used as starters in the preparation of fermented foods. Most lactobacilli are represented by non-sporulating, catalase-negative, non-aerobic, but aero tolerant, fastidious and acid tolerant rods devoid of cytochromes producing lactic acid as the major end product during the fermentation of carbohydrates

(Axelsson, 1998). Being fastidious in nature, lactobacilli and other lactic acid bacteria require a rich environment for their growth. Lactobacilli and Bifidobacteria are also the two key members of Probiotic group known for exhibiting their health promoting functions in man and animals and also in protecting the gut against foreign invasion.

2.2 THE GENUS *LACTOBACILLUS*

The genus *Lactobacillus* is extremely heterogeneous, containing species with 32-53% G + C content of the chromosomal DNA arranged into three groups based on differences in sugar metabolism caused by the presence or absence of fructose-1, 6-diphosphate aldolase and phosphoketolase (Axelsson, 1998).

To date, 56 species of the genus *Lactobacillus* have been identified. Different species of lactobacilli used as probiotics include *Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus cellobiosus, Lactobacillus crispatus, Lactobacillus curvatus, Lactobacillus fermentum, Lactobacillus GG (Lactobacillus rhamnosus* or *Lactobacillus casei* subspecies *rhamnosus), Lactobacillus gasseri, Lactobacillus johnsonii, and Lactobacillus salivarius. Lactobacillus plantarum* 299v strain was isolated from sour dough and *Lactobacillus plantarum* was of human origin. Other probiotic strains of *Lactobacillus* are *Lactobacillus acidophilus* BG2F04, *Lactobacillus acidophilus* INT-9, *Lactobacillus plantarum* ST31, *Lactobacillus reuteri, Lactobacillus johnsonii* LA1, *Lactobacillus acidophilus* NCFB 1748, *Lactobacillus casei* strain *shirota, Lactobacillus acidophilus* NCFM, *Lactobacillus acidophilus* DDS-1, *Lactobacillus delbrueckii* subspecies *delbrueckii, Lactobacillus delbrueckii* subspecies *bulgaricus* type 2038, *Lactobacillus* SBT-2062, *Lactobacillus brevis, Lactobacillus salivarius* UCC 118 and *Lactobacillus paracasei* subsp *paracasei* F19.

2.3 PROBIOTIC ATTRIBUTES OF LACTOBACILLI

Several health promoting functions have been found to be associated with probiotic lactobacilli. In one of the initial studies, administration of *L.*

rhamnosus GG was shown to enhance the production of IL-10, which acts as an anti-inflammatory mediator in atopic disease in children (Pessi *et al.,* 2000).

Alleviation of diarrhea is a well documented characteristic of some strains of probiotic lactobacilli; particularly the ability of *L. rhamnosus* GG to shorten the duration of acute rotavirus diarrhea has been established (de Roos and Katan *et al*., 2000). Similarly, administration of yoghurt with *Lactobacillus* GG was previously shown to reduce the harmful faecal enzyme activity (Ling *et al.,* 1994).

Several probiotic lactobacillus strains have the ability to lower the serum cholesterol as was revealed by different investigators. In one such studies, intake of BSH positive *L. plantarum* in yoghurt showed significant reduction in cholesterol (De Smet *et al.,* 1994). In a different study carried out by Toranto *et al* (2000), intake of *L. reuteri* also led to reduction in serum cholesterol up to 22-23%. Similarly, implantation of microencapsulated genetically engineered probiotic bacterium *L. plantarum* 80 where BSH was over expressed showed reduction in serum cholesterol (Jones *et al.,* 2004).

Beyond such implications on health, lactic acid bacteria are also vastly used in industrial fermentations, food industry, pharmaceuticals and neutraceuticals. Hence, there is lot of scope for isolation and identification of novel strains of lactobacilli and new methodologies which are rapid and specific for widespread applications in food and dairy industry. In view of high stakes involved in exploration of their commercial value particularly in the booming functional / health food market, the correct identification of Probiotic lactobacilli has become extremely important to rule out the possibility of false claims and to resolve disputes concerning their identity in Probiotic preparations.

2.4 IDENTIFICATION OF LACTIC ACID BACTERIA

2.4.1 Phenotypic identification

Traditionally, lactic acid bacteria have been classified on the basis of phenotypic properties e.g. morphology, mode of glucose fermentation, growth

at different temperatures, lactic acid configuration, fermentation of various carbohydrates, methyl esters of fatty acids (Decallone *et al.,* 1991) and pattern of proteins in the cell wall (Gatti *et al.,* 1999) or entire cell (Trakalidou *et al.,* 1994). Some of the phenotypic fingerprinting techniques based on phenotypic and genotypic characteristics are Polyacrylamide Gel Electrophoresis of soluble proteins, fatty acid analysis, bacteriophage typing and sero typing. The phenotypic fingerprints obtained, are usually less sensitive and changes in the fingerprint may not necessarily mean a different organism, but rather could be attributed to a change in expression of the particular phenotypic trait.

Some of the experiments are well documented to compare the phenotypic and genotypic studies. In a study to assess the methods like carbohydrate fermentation, partial 16S rDNA sequencing and cellular fatty acid methyl ester methods were used to determine the taxonomic relationship of the probiotic lactobacilli and Bifidobacteria. The variability among replicates of FAME analysis was so high that it was concluded that this approach was not useful for speciation of probiotic lactobacilli and also variation in fermentation profiles were observed in *L. Johnsonii* strains and this might lead to inaccurate speciation (Yeung *et al.,* 2002). However, the 16S rDNA sequencing results were highly reliable. The results suggest that the use of the first 500 bp of the 16S rDNA is effective for species identification.

Drawbacks of conventional methods are lack of reproducibility, type ability and discriminating power while analyzing the phenotype as whole information potential of a genome is never expressed i.e. gene expression is directly related to the environmental conditions (Farber, 1996). Also plate culturing techniques may not always reveal the true microbial populations because most of the GIT organisms are difficult to cultivate. It has been estimated that only less than 50% of species present in the gut micro flora have been cultured on existing microbial growth media (Wilson *et al.,* 1996). Polyphasic approaches combining biochemical, molecular and morphological data are important for accurate classification of LAB (Klein, *et al.,* 1999).

2.4.2 Genotypic Identification

The phylogenetic information encoded by 16S rDNA has enabled the development of molecular biology techniques, which allow the characterization of the whole human gut microbiota (Lawson, 1999). These technique have found application in tracking the pathogens in food industry and monitoring of the specific strains as they have high discriminating power. In studying the microbial ecology of human gut, molecular techniques have been found to be quite useful and effective in characterization of microbial community, composition, enumeration and monitoring of microbial population, tracking of specific strains of bacteria in the gut micro flora.

2.4.2.1 16S rRNA analysis

Accurate typing of unknown isolates is now achieved by sequence analysis of 16S ribosomal RNA (rRNA). This tool for classifying organisms and evaluating their evolutionary relatedness was first developed by Woese and coworkers (1987). This molecular phylogeny approach has revolutionized the field of microbial ecology and has allowed meaningful phylogenetic relationships between microbes in natural ecosystems to be discerned. Technically, this is quite feasible as the polymerase chain reaction (PCR) can be used to directly amplify the 16S rRNA gene directly from colonies using primers which are directed at universally conserved regions at both ends of the gene.

To complement the rRNA sequence approach, analysis of another molecule, which is not as conserved as 16S rRNA but still retains the characteristics of a meaningful phylogenetic marker is required. Two important criteria for such a molecule are that it is universally present in bacteria and it has high sequence conservation, which illustrates that sequence changes are less influenced by temporary environmental changes.

2.4.2.2 Internal transcribed spacer (ITS –PCR)

The region between the 16S and 23S rRNA genes is termed as the internal transcribed spacer (ITS). However, these genes are technically

feasible to obtain with the help of PCR and can be used to amplify the molecule directly from colonies using primers directed at universally conserved regions within the bordering 16S and 23S rRNA genes. This approach has been explored in the genetic characterization of lactobacilli, Bifidobacteria and LAB. In one such study, Leblond-Bourget *et al*. (1996) had evaluated the sequence analysis of these genes for characterizing bifidobacteria and found that this technique was much more sensitive than the rRNA analysis.

In another study, Lucchini *et al* (1998) were able to characterize *Lactobacillus gasseri* 4B2 a human isolate by a strong auto aggregating phenotype mediated by APF (aggregation-promoting factor), and two primer pairs were developed for simultaneous amplification of a specific fragment of the APF gene and a highly conserved region of the 16S rRNA gene. The methodology allowed a fast and reliable identification of the target strain without any DNA extraction procedure.

2.4.2.3 Randomly amplified polymorphic DNA (RAPD)

RAPD is a very simple and rapid method. In the PCR reaction, short primers of random sequences are used under low stringency annealing conditions, which results in the amplification of randomly sized DNA fragments. The reproducibility of RAPD patterns, however, is occasionally poor and the method needs to be performed under carefully controlled conditions. RAPD profiling has been successfully applied to distinguish between strains of *Bifidobacterium* (Roy *et al.,* 1996) and strains of *L. acidophilus* group (Du Plessis *et al.,* 1995). A multiplex RAPD -PCR using a combination of two 10-mer primers in a single PCR reaction enabled differentiation of *Lactobacillus* strains from the gastrointestinal tract of mice.

2.4.2.4 Ribotyping

A ribotype is essentially an RFLP consisting of the restriction fragments from a particular genome which contain rRNA genes. The basis of the technique is that bacteria generally contain multiple copies (up to eight or more) of the rRNA genes throughout their genome, thus enabling the RFLP to

be obtained. Ribotyping shows high discriminating power at the species level rather on the strain level. However, to obtain a ribotype for an organism, it must first be cultured to obtain enough cells for the procedure. Total DNA is then isolated and is totally restricted into multiple fragments, of sizes ranging from < 1 kb to > 20 kb, using a restriction enzyme with a frequently occurring recognition sequences, generally a 6 bp recognizing enzyme. The restricted fragments are then separated by agarose gel electrophoresis and subsequently hybridized with a probe targeted to either the 16S, 23S or 5S rRNA genes. In practice, probes to the 16S rRNA are the most commonly used. (Kimura *et al*., 1997). In a recent study, Gurakan (2004) while exploring ribotyping, used four different restriction enzymes which had different recognition sites in the spacer region. However, no different digestion patterns were observed which showed that sequence variation in the spacer region among *Lactobacillus* strains had not been sufficient for specific identification of *L. plantarum* strains. Thus, PCR Ribotyping was determined as an inefficient method for identification of *L. plantarum* in strain level, however, selection of enzyme is of prime importance.

2.4.2.5 Restriction Fragment Length Polymorphism (RFLP)

This is a rapid technique, which involves amplifying the 16S rRNA gene using the PCR with primers targeted at universally conserved regions within this gene. The resulting amplicon is then restricted with an appropriate restriction enzyme and restriction fragments are size separated by agarose gel electrophoresis, forming a characteristic RFLP. The choice of restriction enzyme depends on the particular genus and must be experimentally determined. It can be carried out on very few cells, thus eliminating the need to culture colonies. This is a major advantage of all PCR based fingerprinting techniques. The discriminatory power of this technique is generally low because of the conserved nature of this gene. However, it has probably the highest reproducibility of all the PCR based fingerprinting techniques.

Nagashima *et al.,* (2002) explored the aforesaid RFLP and developed new primer-enzyme combinations for terminal restriction fragment length

polymorphism (T-RFLP) targeting of the 16S rRNA gene. The 16S rRNA gene was amplified from human faecal DNA. The resulting amplified product was digested with RsaI plus BfaI or with BslI enzymes. Operational Taxonomic Units (OTUs) were detected with RsaI and BfaI digestion and 14 predominant OTUs were detected with BslI digestion. This new T-RFLP method made easy to predict what kind of intestinal bacterial group corresponded to each OTU on the basis of the terminal restriction fragment length compared with the conventional T-RFLP. And moreover, it made possible to identify the bacterial species that an OTU represents by cloning and sequencing.

2.5 GENETIC FINGERPRINTING

A number of techniques based on DNA fingerprinting have been developed and used for identification of LAB including Probiotic strains and these will be reviewed here.

2.5.1 Denaturing Gradient Gel Electrophoresis (DGGE) and Temperautre Gradient Gel Electrophoresis (TGGE)

DGGE can separate individual rRNA genes from the universally amplified product. Although, all the individual rRNA genes within the amplicon are of the same length, electrophoresis through a linearly increasing gradient of denaturants can separate the products of different sequence (Fischer and Lerman, 1979). The principle is based on the melting of rRNA genes at specific denaturing points based on their sequence. Therefore, each individual sequence will begin to melt at a characteristic denaturing point. The melting changes the conformation of the DNA molecule, slowing its migration through the gel. Urea and formamide are generally used to form the denaturing gradient. However, temperature can also be used, thus creating a Temperature Gradient Gel Electrophoresis (TGGE). When an individual rRNA gene begins to melt, its migration slows and it becomes separated from the PCR amplicon. Further migration of the gene through the denaturing gradient, however, could result in the double-stranded DNA becoming denatured into ssDNA products. To prevent this to happen, a GC clamp consisting of 30 - 50 "G" and "C" bases attached to the 5' end of one of the primers are used to

amplify the rRNA product. As G/C rich DNA regions are resistant to melting, this tag can maintain the integrity of the double stranded rRNA genes.

In one of the investigation carried out out on these lines, probiotic microorganisms were identified by PCR-DGGE analysis from South African dairy products. The DGGE profiles obtained were compared to reference marker for five lactobacilli and the method proved to be culture independent approach for rapid and specific identification (Theunissen ***et al***., 2004).

2.5.2 Pulse Field Gel Electrophoresis (PFGE)

PFGE essentially means using an electrical pulse system to migrate very large fragments of DNA through an agarose gel. The fragments are obtained by digesting the genome with rare cutting restriction enzymes such as NotI, which generally have an 8 bp recognition site or a 6 bp recognition site which may be statistically rare for the particular genome. Coeuret ***et al.*** (2004) used PFGE to identify strains to asses the accuracy of labeling with regard to genus and species and found the method to be convenient for identifying probiotic lactobacilli in probiotic food and animal feed.

2.5.3 *In situ* Analysis of Intestinal Micro flora

The ability to obtain information on single cells *in situ* in faecal or intestinal samples is very intriguing. This event however is now feasible, primarily due to the development of sensitive fluorescent labels, which enable probes to be visualized by fluorescent microscopy. Visualization of specific strains at the single cell level *in situ* can be achieved by prokaryotic *in situ* PCR (PI-PCR) or fluorescent *in situ* hybridization (FISH).

2.5.4 Flourescent *in situ* Hybridization (FISH)

An alternative to *in situ* PCR amplification is to hybridize fluorescent labeled oligonucleotide probes directly to cells fixed on a glass slide. The fixing process permeates the cells to allow the short probes to access the nucleic acid inside the cell. The cells with the hybridized fluorescent probe can subsequently be visualized by fluorescent microscopy. This technique has been explored by several investigators in determining the load of viable

organisms in the faeces and gut. By applying this technique, the number of bacteria in human faecal samples was shown to be approximately ten-fold higher than number estimated through standard culture techniques, when non-specific probes to the 16S rRNA for FISH were used (Harmsen *et al.,* 2000; Langendijk *et al*., 1995).

This technique, in conjunction with PI-PCR, can potentially reveal which specific genes are expressed by the micro flora *in situ* in the human intestine.

2.6 POLYMERASE CHAIN REACTION (PCR)

Among all the techniques which are simple and can be performed at laboratory levels for identification purposes with minute amounts of DNA is PCR. It was developed in 1993 by Kary Mullis, a Nobel Prize recipient. PCR is one of the most useful molecular tools of modern time. In its simplest form, PCR is used to amplify a specific DNA sequence over a billion-fold from a single copy, using a thermo stable DNA polymerase (usually Taq DNA polymerase), deoxynucleotides (dNTP) and two primers, whose sequence is complementary to either end of the targeted sequence. The method is highly sensitive and reliable. The major advantages of PCR are the ability to utilize minute samples to produce a high yield of amplified target DNA, the specificity of the reaction, the flexibility of the method, and the simplicity and speed of the automated procedure.

Some of the molecular techniques used in identification of probiotics involve targeting some specific genes which are responsible for probiotic attributes. Hence, for targeting a particular gene in a strain, we require an efficient tool, which is easy to perform, cost effective, relatively rapid amenable to statistical analysis and reproducible. Nowadays, PCR is one of the most sensitive, specific and versatile laboratory method used because of its reliability, cost-effectiveness and possibility to obtain results within less than 8-24 hours of receipt of a specimen. Any microorganism alive or dead or even DNA debris can be readily detected with equal facility and regardless of the nutritional requirements of the organisms. The on going genome projects of *Lactobacillus s*pecies and others have paved way to identify specific genes

which are responsible for probiotic properties. These particular genes can be targeted for identification of particular strain or particular probiotic property that is present in variable regions of genes. A few attempts have been made in this regard to apply PCR based techniques for identification of Probiotic cultures by targeting genes associated with probiotic functions. These are reviewed as follows.

Tieking *et al., (*2003) used PCR primers derived from conserved amino acid sequences of bacterial levansucrase genes. It was shown that 6 of the 15 fructan-producing lactobacilli and none of 20 glucan producers or EPS-negative strains carried a levansucrase gene. It was remarkable that formation of glucan and fructan was most frequently found in intestinal isolates and strains of the species *Lactobacillus reuteri, Lactobacillus pontis*, and *Lactobacillus frumenti* from type II sourdoughs. Fructan-forming lactobacilli were detected by Lev PCR; this PCR is a useful tool for rapid screening of lactobacilli for EPS formation. The primer sequences were 5`-GA(CT) GTI TGG GA(CT) (AT)(GC)I TGG C-3` (LevV; forward primer) and 5`-TCI T(CT)(CT) TC(AG) TCI (GC)(AT)I (AG)(AC)C AT-3`(LevR; backward primer), where I stands for inosine).

In one of the initial studies carried out on these lines, Horie *et al*., (2002) explored primer set (CbsA2F - CbsA2R) for amplifying conserved regions of S-layer genes to identify *L. crispatus* and the specificity of this set was compared with that of another primer set (Cri 16SI - Cri 16SII) which has been reported as a species-specific primer set targeting the 16S rRNA gene. Among species in the *L. acidophilus* A1-A4 groups, when KOD polymerase was used for amplification, the primer set CbsA2F-CbsA2R gave PCR products with *L. crispatus* strains only. However, when Taq polymerase was used, this primer set gave products with one *L. amylovorus* strain as well as with *L. crispatus* strains. The primer set Cri 16SI-Cri 16SII gave PCR products with *L. crispatus* strains and two *L. acidophilus* strains, regardless of whether the polymerase used was KOD or Taq. A PCR targeting the S-layer gene and amplified with KOD polymerase can identify *L. crispatus* accurately and rapidly.

In a different but related study, Ventura *et al., (*2002) identified genes encoding the aggregation-promoting factor (APF) protein from six different strains of *Lactobacillus johnsonii* and *Lactobacillus gasseri*. Both species harbor two *apf* genes, *apf*1 and *apf*2. Multiple alignments of the deduced amino acid sequences of these *apf* genes demonstrate a very strong sequence conservation of all of the genes with the exception of their central regions. PCR was used to amplify both *apf* genes in all investigated strains. DNA fragments of ~1.0 to 1.3 kb corresponding to apf2 genes were amplified using the oligonucleotides C1 (5'-GGCAAACTAACGGTTGG-3') and C2 (5'-GAGCACCAGTCCATGAAC-3'), while apf1 genes were amplified by employing the oligonucleotides X-ONE (5'-GTAACTTGAACACGCTTTC-3') and X-TWO (5'-CATAAACTGTAACATAAGGC-3') or PROM-1 (5'-GACTGACAAATATGAAAGG-3') and PROM-2 (5'-CTATAACATAAATGCTACTAC-3').

2.61. Multiplex-PCR

In a multiplex PCR, more than one set of primers is used to enable the simultaneous amplification of a number of target DNA regions. More is the numbers of target regions amplified; the more reliable is the technique. A disadvantage of the technique is that prior sequence knowledge is required and it is technically challenging to design optimal reaction conditions. It was recently adapted for the reliable identification of human *Lactobacillus species* (Song *et al.,* 2000).

2.7 BILE SALT HYDROLASE (BSH) - AN IMPORTANT PROBIOTIC MARKER

Bile acids are produced *de novo* in the liver from cholesterol. The steroid nucleus is conjugated with an amide bond at the carboxyl C-24 position to one of two amino acids, glycine or taurine (Baron *et al.,* 1997). These conjugated bile acids are secreted along with the common bile directly into the duodenum. They then pass through the intestine, where the bile acids are deconjugated, dehydroxylated, dehydrogenated and desulfated by

microbial enzymes. These enzymes constitute a class collectively known as conjugated bile salt hydrolases.

The capacity to deconjugate bile acids by bile salt hydrolase (BSH) is widespread among members of the autocthonous gastrointestinal microflora. These BSHs are expressed by several gastrointestinal bacterial genera including *Bacteriodes*, *Clostridium, Enterococcus, Bifidobacterium* and *Lactobacillus* etc. However, BSH activity was detected only in stationary phase cells of *lactobacillus* isolates *L. salivarius* AK 21 and *L. aviarius* AK 113. It has also been shown in *L. Johnsonii* and *L. reuterii* (Lundeen *et al.,* 1990). Lactobacilli have been shown to be the predominant producers of BSH activity in the mouse gut .Gastric lactobacilli contribute approximately 86% of total BSH activity in the ileum and 74% in cecum of mice (Tannock *et al.,* 1989).

2.7.1 Bile Salt Hydrolase's (BSH) and its characteristization

Some of the bacterial species other than lactobacilli that are capable of deconjugating bile salts may use the amino acid taurine as an electron acceptor Tannock *et al.,*(1989). BSH is a detergent shock protein that protects the bacteria from the toxicity of bile acids in the gastrointestinal tract. BSH activity is important at some level for *Lactobacillus* colonization of the human intestine Moser *et al.,* (2001). Sauge *et al.* (1992) suggested that deconjugation might be a detoxification mechanism for survival of lactobacilli in the hostile environment in the gut. Also **CBSH** enables lactobacilli to survive intestinal stress. Tannock *et al.* (1997) demonstrated that growth of common intestinal bacteria *in vitro* was inhibited by free bile acids but not by conjugated bile acids.

Lundeen *et al.* (1992) characterized four isozymes of bile salt hydrolase (BSH) and purified from the cytosol extract of *Lactobacillus* sp. Strain 100-100. The four proteins were designated BSH A, B, C and D. These isoforms consists of one or two poly peptides. The peptides have molecular weights of 42,000 and 38,000 and are designated α and β respectively.

The approximate native molecular weights of BSH A, B, C and D are 115,000, 105,000, 95,000 and 80,000, respectively. The native proteins are trimers. The four isozymes are the array of possible subunit combinations α_3, $\alpha_2\beta_1$, $\alpha_1\beta_2$ and β_3 for A, B, C, & D respectively. This is the first report that any bacterial BSH A, B and C have similar catalytic properties and have α as their subunit and differ in molecular weight, subunit composition and behavior in anion exchange chromatography. Whereas, BSHD had a very low enzymatic activity and suggests that the α-peptide is the main catalytic subunit in these enzymes.

BSH activity was encoded by two separate loci on the genome of *L. Johnsonii* strains 100-100. One locus encodes cbsHβ and is a part of operon of three genes encoding two for bile acid hydrolysis and other for transport. This locus is conserved in other *Lactobacillus* species. The second locus encodes another BSH, cbsHα which is not arranged in tandem.

BSH operon of *L. Johnsonii* strain 100-100 contains two genes, $cbsT_1$, and $cbsT_2$ that share a high level of similarity in this DNA and predicted amino acid sequence. Functional studies have shown that $cbsT_2$ and partial $cbsT_1$, when expressed in *E.coli,* increased uptake of conjugated bile acids (Elkins *et al.,* 1998).

Earlier, Elkins *et al., (*2001) analyzed the DNA sequence of the locus and revealed a complete ORF for $cbsT_1$, and no other ORFs in tandem. The three genes $cbsT_1$, $cbsT_2$ and cbsHβ probably constituted on operon *in L. Johnsonii*-100-100. DNA sequence was obtained and analyzed from the chsHβ locus of the human isolate *L acidophilus* strain ks13 **.**This organism has $cbsT_1$, $cbnsT_2$, and cbsβ genes that are 84, 87 and 85% identical in DNA sequence. Second locus encodes another BSH, cbshα, which is not arranged in tandem.

Purified α and β hydrolase's have estimated molecular masses of 42 and 38 kDa respectively (Lundeen *et al.,* 1992). cbshα shares the highest

amino acid sequence similarity with the BSH from *L. plantaum* 80 (Christiaen's *et al.,* 1992).

Lactobacilli with in the species *L. acidophilus*, *L. brevis*, *L. buchnerii*, *L. fermentum*, *L. gasseri* and *L. plantarum* express a variable BSH phenotype. Phenotypic and genetic screen suggest that BSH activity was acquired horizontally in lactobacilli and that BSH activity is important at some level for lactobacilli to colonize the lower gastrointestinal tract.

In an experimental study previously conducted by Gilliland *et al.* (1977) it was found that all isolates of *L. acidophilus* from human feaces deconjugated taurocholate, whereas only one of six deconjugated glychocholate. Corzo, (1997) in a related study showed that *L. acidophilus* and *L. casei* deconjugate dbile salts during growth by producing the enzyme bile salt hydrolase .

BSH activity is enhanced considerably when assayed in cells that are suspended in the supernatant solutions from cultures incubated with TCA (conjugated bile salt). Therefore, an extra cellular factor present in the spent medium might in some way enhance the enzymatic activity. However, this needs to be precisely identified. On the other hand, neither cholic acid nor taurine stimulate BSH activity.

Continuing further on these lines, Buck *et al* (1994) reported that all *L. acidophilus* strains of human origin showed greater variation in bile tolerance. The difference in bile tolerance may be attributed to the difference in growth of the individual strains as was proposed by Pulasani *et al.* (1983). Later, Gopal *et al.,* (1996) reported that strains of *L. acidophilus* with low bile tolerance grew more slowly in a medium without bile.

2.7.2 Primers for Cloning BSH

In the above mentioned study, Elkins *et al*.,2001 had identified conserved amino acids motif in cloned BSHs by aligning predicted protein sequences. A Motif at the amino terminus and a Motif centered around residue 225 were selected and used to construct primers Hβ675 a and Hβ675

b. The primers were engineered to cbsHβ sequence in strain 100-100 and encompassed a region of approximately 675 bp. The primers were used in standard PCR reaction to amplify genomic DNA from other lactobacilli and resulted into the amplification of 675 bp product from the targeted gene in lactobacilli.

2.8 SIGNIFICANCE OF BSH ON HEALTH

Bile salt hydrolysis is the principle microbial reaction in that it performs a gate keeping function: Bile salts are the water soluble end products of cholesterol and are synthesized in the liver during normal enterohepatic circulation (EHC). The average bile salt pool of 4.0 g is secreted into the duodenum twice during each meal, or an average of 6-8 times/ day for the purpose of forming mixed micelles with the products of lipid digestion. Bile salts must be deconjugated before sterol transformation can occur (Batra *et al.* 1990). Because of the amphipathic nature, the conjugated bile salts form spontaneous micelles that trap dietary cholesterol and fats and facilitate their absorption by the intestinal epithelium. The bile acids are then actively transported by a sodium dependent transporter through the epithelium into the blood stream. Their return to the liver completes the entrohepatic cycle. During intestinal transit, 90% - 95% of secondary bile salts are absorbed in the terminal ileum and are returned to the liver via the portal vein. About 75% of bile acid secretion is reabsorbed in the conjugated from in an active sodium dependent way. The remaining 25% of these bile salts is hydrolyzed during intestinal transits and only 15% is reabsorbed in a passive way thus about 60% of deconjugated bile acids are reabsorbed. it has been shown that one mMol of deconjugated bile salt, formed through breakdown by microencapsulated Lp 80 (pCBH1) is responsible for a 0.4 mMol faecal excretion. The bile salt pool is replenished by hepatic synthesis of new bile from serum cholesterol. In this way interruption of EHC can result in an increased bile acid biosynthesis of up to 15-fold from 0.02 mMol/h to 0.3 mMol/h. However, intake of microencapsulated LP80 (pcBH^{1}) can reduce the serum cholesterol during interceptor of the EHC. Microencapsulated cells

diminish the bioavailability of BSH-deconjugated bile acids totally. Here for cholesterol therapy number of factors must be considered such as dosage, frequency and mode of therapeutic administration, composition of microcapsule membrane and potential effects of by products. The conjugated bile salts are stored and concentrated in the gall bladder during the fasting state, and after consumption of a fat containing meal these compounds are released into the duodenum, where they play a major role in the dispersion and absorption of fats including bacterial phospholipids and cell membranes (Tannock *et al.,* 1994).

2.8.1 Deconjugation of bile salts- a means to lower cholesterol by probiotic LAB

Some species of lactobacilli that are present in the intestinal tract can deconjugate both taurocholic and glycocholic acids in anaerobic conditions. This deconjugation activity is important because deconjugated bile acids do not function well in the intestinal absorption of cholesterol than conjugated bile acids (Eysen, 1973). Increased deconjugation of bile acids could also result in greater excretion of bile acids from the intestinal tract because free bile acids are less likely to be reabsorbed in the intestine (Chickai *et al.,* 1987). Increased excretion of bile acids stimulates the synthesis of replacement bile acids from cholesterol, thus providing the potential to reduce cholesterol levels in the body. The synthesis of bile acids is homeostatically regulated by the amount of bile acids returning to the liver (Danielloon, 1975). The solubility of cholic acid, a deconjugated bile acid decreases, as pH of the media decreases. This is because Cholic acid is insoluble at pH less than 5. As the pH drops because of acid production during bacterial growth, cholic acid precipitates from the broth and may also cause the cholesterol to precipitate, if the cholesterol micelles are disrupted. Deconjugation of bile salts and the incorporation of cholesterol into the cellular membrane have the potential to lower serum cholesterol concentration in human if it gets incorporated into cells of lactobacilli during growth in the small intestine **.**Absorption on the surface of cells makes cholesterol unavailable for absorption into the blood.

Lactobacillus acidophilus is an indigenous and dominant *Lactobacillus* species that is present in the gastrointestinal tract of most healthy adults (Tannock, 1995). The important factor that should be considered in selecting *L. acidophilus* as a probiotic culture or as a food adjunct is bile tolerance, which enables a selected strain to survive, grow and provide therapeutic benefits in the intestinal tract (Gilliland, 1989). One of the important beneficial health effects attributed to *Lactobacillus acidophilus* is its capability to reduce serum cholesterol, possibly through binding of the dietary cholesterol with the bacterial cells in the small intestine before cholesterol can be absorbed into the body. There is a great variation among *L. acidophilus* strains in their ability to bind cholesterol and this variation should be considered where culture is being selected for probiotic or food adjunct use.

Hill and Drasar (1968) observed that both strains of *L. casei* deconjugated sodium glycocholate better than sodium taurocholate. Sodium glycocholate predominates in the intestinal tract of adult humans. Bile salt deconjugation and cholesterol assimilation are concomitant reactions and have potential importance in exerting control of serum cholesterol concentrations in humans. To maintain the necessary levels of conjugated bile salts for the enterohepatic circulation, the excreted bile salts are replaced by synthesis of new ones in the body from cholesterol.

In a more recent study, Bron *et al.* (2004) examined the genetic responses of *L plantarum* $WCFS_1$ to bile salts. A stepwise increase in the porcine bile concentration resulted in a stepwise decrease in the maximal growth rate and the OD_{600} values of *L. plantarum*. The observed gradual decrease in the growth rate coincided with this gradually increasing severity of changes in morphology including bulky structure on the cell surface, the formation of membrane vesicles and clumping of the cells. Moreover, the formation of ghost cells was observed which suggested that cell wall integrity was lost after addition of bile, and this possibly led to leakage of intracellular material from cells and a disturbed energy balance.

Conjugated bile acid hydrolase gene from the silage isolate *L-plantaum* 80 was cloned and expressed in *E.coli* MC1061. Screening was done by direct plate assay. The obtained amino acid sequence was shown to have 52% and showed similarity with a penicillin V amidase from *Bacillus sphaericus*. Preliminary characterization of the gene product showed that it is a cholylglycine hydrolase (DC. 3.5.1.2.4) with only slight activity against taurine conjugates. The optimum pH was between 4.7 and 5.5 and optimum temperature ranged from 30 to 45^{o}C (Christians *et al.,* 1992).

After a meal, bile salt concentration sharply increases in the duodenum upto Ca 15 mMol/L and then progressively decreases to 5mMol/ L. In the Jejunum, the bile salt concentration falls below 4 mMol/L because of active ileal absorption (Heaton, 1985). Secondary bile salts form miscelles with phospholipids and therefore, have lower antibacterial activity than artificial solutions of pure bile salt. In case of *in vivo* studies, the successive stresses by gastric acid and bile can be expected to exert a stronger antimicrobial effect than either of the parameters alone (marteau *et al.,* 1997).

Foo *et al.* (2003) studied the effect of addition of *L. plantarum* 1 UL4 metabolite @ the rate of 35% and 70% in drinking water of rats. The rats with treatment of 70% UL4 showed a decrease in the total cholesterol concentration, increase in the faecal LAB counts, and reduced *enterobacteraciae* counts and feacal pH. The 35% UL4 rats had lower faecal pH than other treatment groups. It reduced the growth rate of rats.

In yet another study conducted by the same group i.e. Foo *et al.* (2003), it was observed that feeding of fermented fruits *with Lactobacillus* culture had no adverse effects on the growth performance but it decreased significantly faecal counts of *enterobactriacae* and increased significantly the concentrations of cholesterol in the blood.

Recently, Jones *et al.* (2004) clearly showed that microencapsulated *Lactobacillus plantarum* 80 (pcBH$_1$) was able to effectively break-down physiologically relevant concentrations of bile acids *in vitro*. The BSH activity result showed that 0.26 g CDW of microencapsulated LP80 (pcBH$_1$) can

break down 0.2 mMol of GDCA in a 4 h period and 0.1 mMol of TDCA in 5 hr period against an average bile salt secretion of 2 mMol 4 h (standard Hoffman, 1989).

From the foregoing review, it can be inferred that the knowledge regarding the gene sequences encoding probiotic functions in lactobacilli and their application as targets for developing molecular techniques for identification of probiotic lactobacilli is quite scanty. Hence, there is considerable scope to initiate work on these lines. The proposed project is one such attempt on these lines and was undertaken with the following work plan.

Work Plan

- Isolation of *lactobacilli* from mother's milk and human feacal samples and other niches
    - Growth medium (BCP-Lac-MRS Agar & MRS broth)
    - Microscopic examination
- Identification of isolates as *lactobacilli* by PCR using genus specific primers LbLMA1 and R-16-1
    - Target gene 16s- 23srRNA
- Screening of indigenous Lactobacillus isolates for probiotic functions
    - Resistance to Low pH
    - Bile tolerance
    - BSH activity
    - Hydrophobicity
    - Antibacterial activity
- Development of PCR assays for identification of BSH positive lactobacilli
- Designing and synthesis of primers targeted against BSH gene sequences available in NCBI database
- Extraction of genomic DNA from reference and indigenous probiotic *lactobacilli* strains as well as non probiotic lactobacilli strains i.e. BSH negative (Pospeich and Neikmann method)

- Standardization of PCR assays using the aforesaid primers for bile salt tolerance
    - Optimization of PCR conditions
    - Annealing temperature
    - Primer concentrations and ratios
    - No. of cycles
    - Magnesium chloride concentrations
- Monitoring of expected PCR amplified products on agarose gel
- Evaluation of BSH-PCR Assays for sensitivity and specificity with probiotic and non probiotic strains
- Combining BSH-PCR with genus specific PCR for development of multiplex PCR for direct screening of bsh positive *lactobacilli*
- Checking specificity against targeted and non targeted organisms
- Cloning and sequencing of *lactobacillus* BSH gene in *E.coli*
    - Designing of BSH primers for cloning BSH gene based on the available gene sequences in data base
    - PCR amplification of BSH gene.
    - Cloning the product in pGEMT vector
- Transformation of the ligation mix in *E. coli DH5α* & screening of ampr transformants for insert by mini preps
- Sequencing of the BSH clones
- Analysis of the above bsh sequence data for homology and variation at the nucleotide level.

3.0 MATERIALS AND METHODS

3.1 BACTERIAL CULTURES

The bacterial cultures used in this study are listed in **Table 1** The Lactobacillus strains, non-lactobacilli as well as other non-lactic acid bacteria were obtained from the repository of National Collection of Dairy Cultures (NCDC), NDRI, Karnal, Molecular Biology Unit (MBU), NDRI, Karnal and Dr. K. J. Heller, federal Research Centre for Nutrition and Food, Kiel, Germany.

Table 1 Bacterial cultures used in the investigation

Sl. No.	Reference strains	Source
A)	**Lactobacillus cultures :**	
1.	*Lactobacillus acidophilus* (La), 195	NCDC
2.	Lactobacillus plantarum (Lpl), 201	NCDC
3.	*Lactobacillus johnsonii* La1	Kiel, Germany
4.	*Lactobacillus acidophilus* LA7	Kiel, Germany
5.	*Lactobacillus acidophilus*	Kiel, Germany
6.	*Lactobacillus acidophilus*	AIIMS, New Delhi
B)	**Other non-lactobacilli cultures :**	
1.	*Lactococcus lactis* ssp. *lactis*, 60	NCDC
2.	*Lactococcus lactis* ssp. *cremoris*, 81	NCDC
3.	*Lactococcus lactis* ssp. *diacetylactis*, 61	NCDC
4.	*Pediococcus pentosaceus*, 35	NCDC
5.	Bifidobacteria	MBU
6.	*Propionibacterium freudenreichii* ssp.*shermanii*, 139	NCDC
7.	*Leuconostoc mesenteroides* ssp. *cremoris,* 29	NCDC
8.	*Leuconostoc mesenteroides* ssp. *lactis*, 200	NCDC

C)	**Other non-LAB cultures**	
1.	*Enterobacter aerogenes* 106	NCDC
2.	*Shigella dysentriae* 107	NCDC
3.	*Salmonella typhi 113*	NCDC
4.	*Enterococcus faecalis* 114	NCDC
5.	*Klebsiella pneumoniae 138*	NCDC
6.	*Enterobacter aerogenes 173*	NCDC
7.	*Enterococcus faecium 211*	NCDC
8.	*Enterobacter aerogenes 248*	NCDC
9.	*Escherichia coli 0157* :H7	MBU
10.	*Listeria monocytogenes*	MBU
11.	*Bifidobacterium bifidum 203*	MBU
12.	*Bifidobacterium bifidum 228*	MBU
13.	*Bifidobacterium bifidum 228*	MBU

3.1.1 Culture Purity

The purity of all bacterial cultures was always ascertained prior to use by Gram's staining and cell morphology.

3.1.2 Maintenance and Propagation of Cultures

The bacterial cultures of Lactobacillus strains were propagated in MRS broth at 37°C for 16 to 18 h. One set was maintained as glycerol stocks at –70°C by mixing equal volume (500 µl) each of overnight grown bacterial culture and sterilized 50 percent glycerol. The cultures were stored at –70°C in ultra low deep freezer (New Brunswick Scientific, USA), until further use. The cultures were always activated in MRS broth tubes prior to use by subculturing. Another set of cultures were propagated at 37°C and preserved in litmus milk tubes and stored in refrigerator. The genomic DNA from non-lactic acid bacteria (LAB) used in this study was obtained from MBU.

3.2 MOLECULAR WEIGHT MARKERS

The molecular weight markers, viz λ DNA/EcoR1 + HindIII double digest (Gibco BRL, USA, Bangalore Genei), 1kb ladder (Bangalore Genei) and 100 bp ladder were used in the study:

1. λ DNA / EcoR1 + HindIII double digest (0.125kb, .564kb, .831kb, .947kb, 1.275kb, 2.027kb, 3.530kb, 4.268kb, 4.973kb, 5.148kb, 21.226kb).
2. 1kb Ladder (1kb, 2kb, 3kb, 4kb, 5kb, 6kb, 7kb, 8kb, 9kb, 10kb).
3. 100bp ladder (0.1 to 1.0 kb)

3.3 ISOLATION OF LACTOBACILLI

3.3.1 Collection of Samples

Lactobacilli cultures were isolated from mother's milk, human faecal samples, buffalo milk and probiotics capsules. The samples collected were enriched in MRS broth. For human faecal samples, sterile swabs were used and put in MRS broth for enrichment. Incubation was carried out at 37^{o}C/2-3 hrs and streaking was done on BCP-Lac-MRS Agar and typical Yellowish colonies were selected for morphological examination under microscope. Also pour plating was done with the dilutions 10^{7} and 10^{8} and the submerged colonies were selected for morphological examinations.

3.3.2 Microscopic Examination

Gram staining and spore staining were performed by following standard methods. Then, the selected colonies were transferred into MRS broth and incubated for over night at 37^{o}C.

3.3.3 Catalase Test

Catalase test was performed by growing the culture in MRS broth overnight at 37^{o}C followed by addition of a drop of hydrogen peroxide. The production of effervescence indicated catalase positive reaction.

3.4 ISOLATION OF GENOMIC DNA

The genomic DNA from all the cultures used in this study grown for 16 to 18 h in MRS broth at 37^{o}C was extracted by the above method (Pospiech and Neikmann, 1996).

3.4.1 Procedure

The cells were harvested from 1.5 ml of overnight grown culture of Lactobacilli in a microcentrifuge for 10 min at 3000 g. The supernatant was discarded carefully without disturbing the pellet taking all necessary precautions. The pellet was resuspended in 0.5 ml of SET buffer (pH 7.5). Lysozyme was added to the above cell suspension at a concentration of 1 mg/ml and incubated at 37^{o}C/1 hr. The above step was followed by addition of 1/10 (one-tenth) volume of 10% SDS and further incubated at 37^{o}C with occasional inversion for 30 min. One third volume of 5 M NaCl and equal volume of chloroform: isoamylalcohol (24:1) were then added to the above mix and incubated at room temperature for 30 min with frequent inversions for 30 min.

The samples were centrifuged at 4,000 rpm/15 min and the aqueous phase was transferred to a new tube. The DNA was precipitated by adding one and half volume of isopropanol and the tubes were inverted gently to mix and kept for 30 min at -20^{o}C or kept at -20^{o}C for overnight. The DNA pellet thus obtained was recovered after centrifugation at 12,000 rpm and washed with 70% ethanol and dried under vacuum in a speed vac system (Martin Christ, Germany) for 10 min. and finally dissolved in 50 μl TE buffer (pH 8.0). The extracted genomic DNA along with tracking dye were run on one percent agarose gel .electrophoresis (mini/max submarine), (Hoeffer, USA) at 80 V for 30 min using 1 x TAE buffer. The gels were monitored on UV transilluminator (Fotodyne, USA) after staining with ethidium bromide (0.5 (μg/ml). The gels were subsequently photographed (MP-4 system, Fotodyne, USA).

3.5. DETERMINATION OF DNA CONCENTRATION

3.5.1 Spectrophotometric Analysis

The concentration of DNA samples used in the study were determined by the measurement of optical density. (OD) in a UV spectrophotometer (DU 640, Beckman, USA) at 260 and 280 nm. The purity of DNA was ascertained

by measuring as ratios at 260 to 280 nm. The concentration and quality of DNA was calculated by the following equivalents as suggested by Sambrock et al. (1989).

DNA mg/ml = A_{260} x dilution x 50.0

A_{260} = Absorbance in OD at 260 nm.

10D = 50 μg/ml (double standard DNA) and

10D = 30 μg/ml (Single standard DNA)

3.6. IDENTIFICATION OF ISOLATES AS LACTOBACILLI BY PCR

The lactobacillus isolates were identified by PCR using primers LbLMA1 and R16-1.

3.6.1 PCR Reaction

Before setting up of PCR, all the reagents were thawed except Taq DNA polymerase, mixed and spinned. Reagents were always kept on ice bath during the period of setting up of the reaction. The reaction mix comprising of 10x PCR buffer (containing $MgCL_2$), dNTPs and primers was prepared and distributed to reaction tubes according to the requirements. The final volume of PCR mix was adjusted to 25μl.

Reagents	Volume	Conc.
Sterile milliqwater	Var	--
Reaction buffer, 10x with $MgCL_2$	2.5 μl	1x
dntp mix	2 μl	0.2 mM
Primer forward	1 μl	0.1 – 1 mM
Primer reverse	1 μl	0.1 – 1 mM
Taq DNA polymerase	0.5 – 1 μl	0.5 – 30
Template DNA	1-10 μl	100 ng – 1 μg

3.6.2 PCR Cycling Steps

Template DNA was initially denatured at 95°C for 2 to 5 min. Next 30 to 45 cycles were programmed based on annealing temperatures of primers used. The respective denaturation, annealing and extension temperatures

used in this study for different sets of primers targeted against different Lactobacillus species have been explained under Results and Discussion.

Finally, an additional extension was given at 72°C for 5 to 10 min. After the run was over, the amplified PCR products were held at 4°C until further use.

3.6.3 Analysis of PCR Products by Agarose Gel Electrophoresis

PCR amplified products obtained with different templates were electrophoresed on the agarose gels (1.0 to 2.0%) by following the standard procedure as given by (Sambrook *et al.,* 1989).

3.6.3.1 Agarose gel

Agarose of 1 to 2 percent concentration was prepared by dissolving the appropriate quantities of agarose in IX TAE buffer (pH 8.0) in a microwave oven or by keeping in boiling water bath. Ethidium bromide stock solution was added directly to molten agarose solution at the rate of 0.5 µg/ml before casting the gel (mini / midi / maxi).

3.6.3.1.1 Procedure

Molten agarose was cooled to 50°C and poured into respective moulds of minigel (50 ml) and midigel (100 ml) using appropriate comb (8 to 20). The surface was levelled before pouring the gel. After complete setting of the gel, the comb was removed carefully and the gel plate was mounted on respective electrophoresis tanks (submarine mini, Hoeffer); Horizontal (midi, Bio-Rad). The respective electrophoresis tanks were filled with IX TAE electrophoresis buffer to cover the gel to a depth of about 1 mm. The DNA samples were mixed with 5 µl of tracking dye and were loaded slowly into the slots of submarine gel using micropipette. Electrophoresis was carried out at 100 V (60 mA current) for one hour in mini gel electrophoresis apparatus and 1½ h in case of maxi gel system. After completion of electrophoresis, the gels were taken out of the chamber and examined under UV transilluminator (302 nm, Fotodyne) and photographed using a Polaroid camera with Polaroid type 55 sheet film (MP4 system).

3.7 SUGAR FERMENTATION PATTERNS OF *LACTOBACILLUS* ISOLATES

The composition of the medium used for sugar fermentation is given below.

3.7.1 CHL Medium:

Component		Wt.
Polypeptone	:	10.0 gm
Yeast extract	:	15.0 gm
Tween 80	:	1.0 ml
K_2HPO_4	:	2.0 gm
Sodium acetate	:	5.0 gm
Dipotassium Phosphate	:	2.0 gm
$MgSO_4$, $7H_2O$	:	0.2 gm
$MnSO_4$, $4H_2O$	:	0.05 gm
Bromocresol purple	:	0.17 gm
Distilled Water	:	1000 ml
$_PH$	:	6.9

3.7.2 Procedure

Small test tubes (12x75 mm) were used for production of acid from different sugars. CHL medium was used as the basal medium. Four ml of the medium was taken in each tube and sterilized by autoclaving. One sugar disc (Hi-Media) was aseptically added to each tube. Each sugar tube was inoculated with 0.1 ml of inoculum and the tubes were incubated at 37^0C for 24- 48hrs and the results of colour change was recorded as positive and a control was used to compare the colour change.

3.8 SCREENING OF *LACTOBACILLUS* ISOLATES FOR PROBIOTIC ATTRIBUTES

For selection of probiotic lactobacilli, the isolates were subjected to a battery of tests recommended as per WHO standards (FAO/WHO, 2002) as given below.

3.8.1 Acid Tolerance

MRS broth was used to stimulate acidic conditions of gut. The broth was adjusted to pH 3.0, 2.5, 2.0 and 1.5 with 5 N HCl along with broth adjusted to neutral pH as a control. Overnight grown culture in MRS of *Lactobacillus* at 37^{o}C was inoculated at different pH values.

One ml of culture was taken from each tube immediately (0 hrs) and 10 fold dilutions were prepared in 0.1% peptone water. Pour plating was done using MRS agar. Then, one ml of culture was taken from each of these tubes after 1, 2, and 3 hrs respectively and plating procedure was repeated. The plates were incubated at 37^{o}C for 24 to 48 hrs and the results were recorded. Similarly, the optical density was measured at 600 nm. First the cell concentration was adjusted to 0.3 OD, then they were added to the pH adjusted tubes and OD was taken at 0 hr, 1hr, 2 hr and 3 hr.

3.8.2 Bile Tolerance

The cultures were grown on MRS agar and single colony was inoculated into broth after 18 hr growth. The pH was adjusted to 4.5 with sterile 0.1 N HCl or 0.1 N NaOH. Bile salt solutions at 1.0%, 1.5% and 2.0% concentrations were prepared under sterile conditions with one control in MRS broth.

Overnight grown cultures were inoculated at different concentration of bile and immediately 1ml of culture was taken and 10 fold serial dilutions were prepared with 0.1% peptone water. Then the cultures were further incubated at 37^{o}C and pour plating was carried out using BCP-Lac MRS agar. The plates were incubated at 37^{o}C/24-48 hrs and colonies were counted. The procedure was repeated at 1, 2 and 3 hrs respectively. Similarly, OD at 600 nm was measured at different bile concentrations by taking bile solution in MRS broth without culture as blank. The reading was taken at 0, 1,2and 3 hrs.

3.8.3 Antibacterial Activity

3.8.3.1 Spot on lawn method

Cultures were grown in MRS broth for 18 hrs at 37^{o}C and a fresh culture (24 hr old) of indicator bacteria (0.04 ml) grown for 4 hrs at their

optimum growth temperature was added to 5 ml of nutrient agar (0.85%) and over laid on MRS agar (basal plate (1.8%) which had been laid one day before and incubated at 37^{o}C).

The soft agar was allowed to solidify thus generating a potential mat of the indicator bacteria. After overlaying, an aliquot of 5μl of the culture was directly applied onto the indicator lawn and examined for the presence or absence of zones of inhibition after 24 hrs at this optimum growth temperature.

3.8.3.2 Antimicrobial activity by agar well assay method

The cultures were grown In MRS broth for 18 to 24 hrs at 37^{0}C. The cells were removed by centrifugation at 12,000rpm for 20 min at 5^{0}C. The culture supernatant thus obtained was sterilized by passing through a 0.22μm filter and used in the assays

A fresh culture of indicator bacteria 40μl grown for 16 to 18hrs in BHI at their optimum growth temperature was mixed with 5ml of soft agar melted and cooled to 45^{0}C and poured into a Petri dish containing 15-20ml MRS agar supplemented with β-glycerophosphate. The soft agar was allowed to solidify. The plates were refrigerated at 5^{0}C for 1 hr before several wells were punched out of the agar with the sterile glass borer. The wells were filled with 100μl of supernatant. The plates were once again refrigerated 5^{0}C for 3-4hr to facilitate the diffusion of supernatant and were incubated at 37^{o}C for 24-48hrs. A clear zone of 1mm or more was considered as positive inhibition.

3.8.4 Antibiotic Susceptibility

For determining the antibiotic susceptibility / resistance of *Lactobacillus* isolates against different antibiotics, the protocol as recommended by HiMedia was used. The cultures were screened for antibiotic susceptibility by disc diffusion method. 15 ml of MRS agar was poured in petriplate and allowed to solidify. Then, it was a over laid with 4 ml of soft agar seeded with 200 μl of active culture at 45^{o}C. Petriplates were allowed to stand at room temperature for 15 min before dispensing the antibiotic discs. The Himedia antibiotic discs

were dispensed onto agar by Forceps under aseptic conditions. Plates were incubated at 37^{o}C aerobically for 24 hr without pre-diffusion or pre-incubation. Diameter (mm) of zone of inhibition diameter was measured. using antibiotic zone scale and results were expressed in terms of resistance ,moderate susceptibility or susceptibility as indicated in the standard table given below.

Antimicrobial agents and associated interperative zone diameters for disc diffusion antibiotic susceptibility testing (Charteris *et al.,* 1998).

Group	Antibiotic		Interperative zone diameters (mm)		
	Name	Disc conc. (µg)	R	MS	S
Glycopeptides	Vancomycin	30	≤ 14	15-16	≥ 17
Aminoglycosides	Gentamicin	10	≤ 12	-	≥ 13
	Streptomycin	10	≤ 11	12-14	≥ 15
Tetracyclines	Tetracycline	30	≤ 14	15-18	≥ 19
Single antibiotics	Chloramphenicol	30	≤ 13	14-17	≥ 18
Macrolides	Erythromycin	15	≤ 13	14-17	≥ 18
Quinolones	Ciprofloxacin	5	≤ 13	14-18	≥ 19

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

3.8.5 BSH Assays

The isolated cultures were screened for bile salt hydrolase activity on MRS agar plates supplemented with 0.5%sodiumglycocholate. The cultures grown for over night were inoculated onto the agar medium which was then incubated for 5 days. BSH activity was present when cholic acid precipitated in the agar medium below and around a colony.

3.8.6 Cell Surface Hydrophobicity

Adhesion to n-hexadecane was carried out to assess the cell surface hydrophobicity.

3.8.6.1 Adhesion to hexadecane

The method of Rosenberg *et al.* (1980) with slight modification was adopted to measure the cell surface hydrophobicity.

Phosphate urea magnesium sulphate (PUM) buffer :

Ingredients		***g/litre***
$K_2HPO_4.3H_2O$		22.2
KH_2PO_4	:	7.26
Urea	:	1.8
$MgSO_4$	:	0.2
pH	:	7.1
Distilled Water	:	1000ml

3.8.6.2 Protocol

The bacterial cells grown in MRS broth at 37^0C for 18 hrs were Harvested and the bacterial cell pellet was washed twice with PUM buffer and resuspended in PUM buffer.The cells absorbance was Adjusted to 0.8-0.9 at 610 nm using spectrophotometer. Then add 2.4 ml of turbid bacterial suspension in acid washed test tubes followed by addition of 0.4 ml of n – Hexadecane and incubated at 37^0C for 10 min. The two phases were separated under controlled conditions for 2 minutes at full speed. The mixed phases were then kept at 37^oC for 1 h for phase separation. Separate the aqueous phase with the help of a micropipette and measure the light absorbance of aqueous phase at 610 nm. The fraction of adherent cells was taken as percent decrease in absorbance of the aqueous phase after mixing and phase separation as compared to that of original suspension.

$$\text{Percent cell surface hydrophobicity} = \frac{\text{Initial O.D. - Final O.D.}}{\text{Initial O.D.}} \text{ X } 100$$

3.9. IDENTIFICATION OF BSH POSITIVE LACTOBACILLI BY PCR

3.9.1 Designing of Primers

The BSH gene sequences of Lactobacilli were retrieved from NCBI nucleotide database and aligned using DNA star program as well as Clustal

W (1.82) Multiple Alignment Programme. The accession numbers of BSH gene sequences of lactobacillus are given in **Table 2.** By analyzing the conserved regions of the aligned sequences, primers were designed and got custom synthesized from Imperial Biomed.

Table 2 Accession numbers of BSH gene sequences of Lactobacilli

S.No	Lactobacillus Species	Accession. No	Reference
1.	*Lactobacillus johnsonii*	AF297873	Elkins *et al*, 2001
2.	*Lactobacillus gasseri*	AF305888	Russel and Klaenhammer, 2001
3.	*Lactobacillus johnsonii*	AF054971	Elkins and Savage, 1998
4.	*Lactobacillus acidophilus*	AF091248	Savage and Moser, 1999
5.	*Lactobacillus plantarum*	A24002	Patent

3.9.2 Sequences of BSH primers

The primers specific for BSH gene from different organisms were got custom synthesized and the sequences are given in **Table 3**.

Table 3 List of Primers for BSH based PCR assays and cloning of BSH

S. No.	Primer	Sequence of primers	Reference
1.	LbLMA1 R-161	5’ ctc aaa act aaa caa agt ttc 3’ 5’ ctt gta cac acc gcc cgt tca 3’ (250 bp)	Dubernet *et al*, 2002
2.	LaBSH1F LaBSH951R	5’ atg tgt act ggt tta aga ttc 3’ 5’ tta ata agt aat tag ctt atc 3’ (951 bp)	This study
3.	LjBSHAF LjBSHAR	5’ ata gtc gcg ggt tag gga ct 3’ 5’ cat ctg ttc cct ttg gct gt 3’ (171 bp)	This study
4.	LgBSHF LgBSHR	5’ tcc atc cct ttt gct tgt tc 3’ 5’ gtt cca ggc gaa cct gat aa 3’ (220)	This study
5.	Lj1BSHBF Lj1BSHBR	5’ tcc ttg ggg tgt agg aac tg 3’ 5’ cct ttg atc atg gca aca ga 3’	This study

		(168 bp)	
6.	LaBSHF LaBSHR	5’ ttc atc gtt tgc agt tgc tc 3’ 5’ gag ctg tag cgt cat gtg ga 3’ (196 bp)	This study
7.	LpBSHF LpBSHR	5’ atc acc gct aca ttg gtt gg 3’ 5’ agt ccg ccc att cct cta ct 3’ (231 bp)	This study
8.	Lp1F Lp975R	5’ atg tgt act gcc ata act tat 3’ 5’ tta gtt aac tgc ata gta ttg 3’ (975 bp)	This study
9.	BbBSH1F BbBSH951R	5’ atg tgc act ggt gtt cgt ttc 3’ 5’ tca atc ggc ggt gat cag ctc 3’ (951 bp)	This study
10.	BbBSHF BbBSHR	5’ ata tgg aaa acc tgc gca ac 3’ 5’ ttg ttc tgc tgc gga tag tg 3’ (124 bp)	This study
11.	BlBSHF BlBSHR	5’ agg ccg atg tat ttc gac tg 3’ 5’ tga acc agt gca gca gag ac 3’ (243 bp)	This study
12.	BbBSHAF BbBSHAR	5’ ctc gac tgg agc ttc tcc tac 3’ 5’ gcc gga tcc tca tacgtg ttc 3’ (830 bp)	This study

3.9.3 BSH based PCR Assays

PCR was carried out as described earlier under section (3.6.1).

3.10 CLONING OF BSH GENE FROM LACTOBACILLI

3.10.1 Vector for Cloning of BSH The PCR product cloning vector pGEMT-easy from Promega was used for cloning of PCR product of BSH gene. The map of the vector is shown in **Fig. 1.**

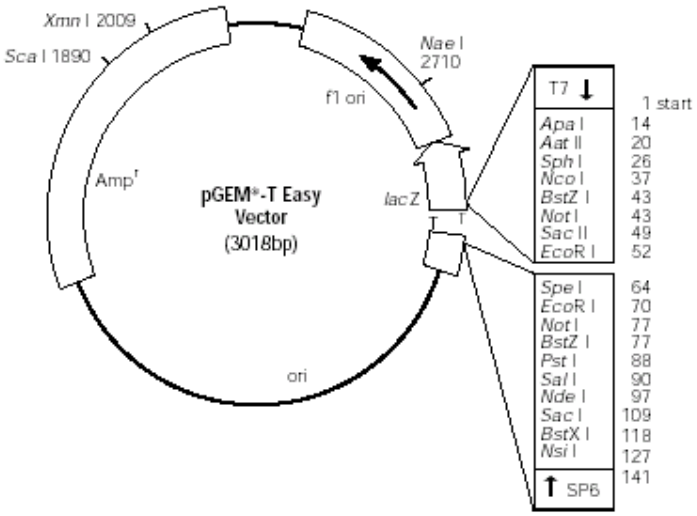

Figure 2. pGEM®-T and pGEM®-T Easy Vector circle maps.

3.10.2 PCR amplification of BSH gene from BSH positive *Lactobacillus* isolates

The BSH gene from two of the *Lactobacillus* isolates as well as *Lactobacillus plantarum* 201 was amplified using primer pair Lp1F and Lp975R and High Fidelity Taq Polymerase.

3.11 PCR PRODUCT PURIFICATION:

The PCR products obtained in all the above reactions were purified using the following kit.

3.11.1 MILLIPORE KIT:

PCR product was transferred to the upper reservoir of the column fitted with collection tube and spinned for 15 min at 4,000 rpm. DNA binds to the membrane and other components come in flow through. The collection tube with flow through was discarded and the spin column was placed in an inverted position in a new collection tube. To this, 25 μl of TE buffer/ Nuclease free water was added and spinned for 2 min at 4,000 rpm. The

bound DNA was thus eluted into TE buffer. The DNA eluted was run on 1% native agarose gel along with molecular size marker for size conformation.

3.12 LIGATION OF PCR product of BSH gene:

The DNA purified from reaction mixture was ligated into pGEM-T easy vector (Promega) by using pGEMT-easy kit (Promega). The reaction mixture was prepared as follows:

pGEM-T vector (50 ng/ul)	:	0.5 μl
MilliQ water	:	x μl
2X ligation buffer	:	5.0 μl
Ligase (T4 DNA ligase, 3 U/ul)	:	1.0 μl
Insert DNA	:	100-150 ng/1-2 μl (Depending on concentration)
Total ligation mixture	:	10.0 μl

The ligation reaction was performed at refrigeration temperature for overnight before using it for transformation.

3.13. TRANSFORMATION

3.13.1 Preparation of competent cells:

- Single colony from a plate of freshly grown E. coli DH5α (16-20 hrs at 37°C) was picked and transferred to 100 ml LB broth in a 1l flask. The culture was incubated for 3 hrs at 37°C with vigorous shaking 1300 cycles/min in a rotary shaker until OD at 600 nm reached 0.3-0.5.
- The cells were then transferred aseptically to a sterile ice-cold 50 ml polypropylene tubes, and the cultures were kept at 0°C for 10 min. All the procedure was carried out aseptically on ice. The cells were recovered by centrifugation at 4,000 rpm for 10 min at 4°C. The media was decanted and the pellet was resuspended in 10 ml of ice-cold 0.1M $CaCl_2$ and stored on ice.
- The cells were recovered by centrifugation at 4,000rpm for 10min. The excess fluid was decanted and the pellet was resuspended in 2 ml of ice cold 0.1M $CaCl_2$ for each 50 ml of original culture.

- Finally, 166 μl of the competent cells prepared were transferred to sterile falcon culture tubes followed by addition of 34 μl of sterile 100% glycerol giving final concentration of 17% glycerol. The competent cells thus prepared were stored in aliquots at -70°C for further use.

3.13.2 Transformation Using Competent Cells

- An aliquot of 5 μl of the ligated DNA was added to 100 μl of competent cells and mixed gently by flicking. Positive control was also set up to check for transformation using pPUC19 DNA. The mix was incubated on ice for about 30-45 min.
- The tubes were then transferred to water bath maintained at 42°C and held for exactly 90 secs. The tubes were rapidly transferred to ice box and allowed to chill for 1-2 min.
- To the above mix, 900 μl of Luria Bertani (LB) broth was added and mixed. The tubes were incubated at 37°C/1 hr in a rotary shaker to allow the bacteria to recover and express the antibiotic resistance. After incubation, 200 μl of transformed mix were spread evenly on LB agar plates with ampicillin 100 μg/ml) and incubated at 37°C/16-20 hrs.
- The transformants obtained on LB agar plates with ampicillin were inoculated into LB broth containing ampicillin and incubated in shaking incubator at 37°C/12-16 hrs after which the cells were pelleted and used for Plasmid isolation.

3.14 PLASMID ISOLATION

Three different protocols were followed in the present investigation to isolate recombinant plasmids from *E. coli* transformants.

3.14.1 Alkali lysis method:

3.14.1.1 Reagents

- Solution I (GTE)
  50 mM Glucose
  25 mM Tris HCl
  10 mM EDTA

pH 8.0

- Solution II (Freshly prepared)
  0.2M Sodium hydroxide
  1% SDS
- Solution III
  5M Potassium acetate pH 4.8
- RNase in TE Buffer (10 mg/ml)
- 3M Sodium Acetate
- Phenol
- Chloroform
- Chloroform:Isoamylalcohol mix (24:1)
- 70% ethanol

3.14.1.2 Procedure

- The cell pellet obtained was washed with sterile water to remove any traces of medium. The pellet was resuspended in 100 µl of ice-cold solution I by vigorous shaking.
- An aliquot of 200 µl of freshly prepared solution II was added and mixed by inverting rapidly for five times and incubated at 5-10 min on ice / Room temperature. To the above mixture 150 µl of ice-cold solution III was added and vortexed to disperse solution III through viscous layer. The tubes were stored on ice for 3-5 min.
- The mixture was centrifuged at 12,000 rpm for 5 min at 4°C in a microfuge. The supernatant obtained was transferred to a fresh tube.
- DNA was precipitated by adding one volume of absolute alcohol at room temperature and mixed by vortexing. The mixture was allowed to stand for 30 min at -20°C and centrifuged for 5 min at 12,000 rpm at 4°C and the supernatant was discarded.
- The pellet was dissolved in 100 µl RNase solution in TE buffer and incubated at room temperature for 1hr. To this, $1/10^{th}$ volume of 3M sodium acetate (pH 5.2) was added and Plasmid DNA was reextracted with equal volume of 1:1 phenol: chloroform. The mixture was

centrifuged (1200rpm/5min) and the upper aqueous layer was taken into a new eppendorf.

- One volume of 24:1chloroform:isoamylalcohol mix was added and centrifuged. The upper aqueous layer was transferred into a new tube. To this two volumes of chilled ethanol was added and kept at -70°C for 10 min and centrifuged 12000 rpm for 10 min. Supernatant obtained was discarded.
- The pellet was rinsed with 70% ethanol (100 μl) and the pellet was air dried for 5-10 min.

3.14.2 PLASMID ISOLATION (SIGMA KIT)

3.14.2.1 Reagents

- Resuspension solution
- Lysis solution
- Neutralization/binding solution
- Column preparation solution
- Wash solution
- Elution solution

3.14.2.2 Procedure

- E. coli cells were harvested by centrifugation of overnight grown culture at 12,000 rpm for 1 min. The cell pellet was resuspended in 200 μl of resuspension solution and mixed by vortexing. The cells in suspension solution were lysed by adding 200 μl of lysis solution. The contents were mixed by gentle inversion until the mixture becomes clear and viscous.
- The cell debris were precipitated by adding 350 μl of the neutralization/binding solution and mixed by inversion and centrifuged at 12,000 rpm for 10 min.
- The column was assembled as per manufacturer's instructions and washed with 500 μl of column preparation solution and centrifuged at 12,000 rpm for 1 min. The flow through was discarded.

- The cleared lysate obtained above was transferred to the column prepared and centrifuged at 12,000 rpm for 1 min and flow through was discarded.
- An aliquot of 750 μl of the diluted wash solution was added to the column and centrifuged at 12,000 rpm for 1 min. The column wash step removed residual salts and other contaminants introduced during the column load. The flow through was discarded and the column was again centrifuged at 12,000 rpm for 1-2 min without any additional wash solution to remove excess ethanol.
- The column was transferred to a fresh collection tube and added with 100 μl of elution solution and centrifuged at 12,000 rpm for 1 min to elute the DNA.
- The eluted DNA was run on 1% native agarose gel for confirmation.

3.15 PCR AMPLIFICATION

The plasmid DNA obtained was subjected to amplification reaction by using BSH primers Lp1F and Lp951R. The reaction mix was prepared as follows:

Milli Q	17.0 μl
10x buffer	2.5 μl
dNTPs(10 mM)	2.0 μl
Forward primer	1.0 μl
Reverse primer	1.0 μl
Taq polymerase	0.5 μl
Template	1.0 μl (1:10 diluted miniprep DNA)
Total reaction mix	25.0 μl

PCR conditions:

Initial denaturation:	94°C/4 min
Denaturation:	94°C/30 sec
Annealing:	56°C/1 min.
Extension:	72°C/1 min
Final extension:	72°C/5 min
Number of cycles:	35

The PCR product obtained was run on 1% native agarose gel for information long with molecular size marker.

3.16 RESTIRCTION ENDONUCLEASE DIGESTION:

Plasmid DNA obtained was used for restriction digestion with EcoRI.

3.16.1 Procedure:

Restriction digestion of minipreps obtained from different transformants were set up using EcoRI in a 20 μl reaction mix.

- The reaction mix prepared as follows:

Components	EcoRI
MilliQ	x μl
10X Buffer	2.0 μl
BSA (10X)	2.0 μl
Enzyme	1.0 μl
DNA	5.0 μl
Total reaction mix:	20.0 μl

- The mix was quickly spinned and incubated at 37°C/2 hrs. The digested samples were then run on 1% native agaorse gel along with undigested sample and marker for size determination.

3.17 SELECTION OF CLONES FOR CUSTOM SEQUENCING

Based on PCR amplification and restriction digestion, the best clones were selected for custom sequencing. The minipreps were sent to Bangalore Genei for getting the sequencing data using ABI Prism.

3.18 ANALYSIS OF SEQUENCE DATA

The sequences obtained were assembled using DNA Star programme and aligned using CLUSTAL W (1.82) multiple sequence alignment editor.

4.0 RESULTS AND DISCUSSION

The main purpose of this investigation was to isolate indigenous and novel lactobacilli of human origin from the gut, having distinct probiotic functions and their subsequent characterization by developing appropriate PCR techniques based on selected probiotic markers. To achieve these objectives, a sizeable number of representative lactobacilli isolates were recovered on MRS agar from human faecal samples, probiotic preparations and raw buffalo milk. Based on the preliminary screening by colony characteristics and morphological factors on microscopic examination after Gram and spore staining as well as catalase test, a total of 100 isolates were selected as the subject for further studies. The results pertaining to isolation of typical lactobacilli of human origin from different niches are presented below.

4.1. ISOLATION OF LACTOBACILLI FROM DIFFERENT NICHES

In the present investigation, a total of 25 human faecal samples from the subjects belonging to age group 23 – 30 including infant faecal samples, 5 human milk samples, 5 buffalo milk samples and two probiotic preparations (Prolac and Yakult) were used for the isolation of lactobacilli using BCP-Lac MRS agar as the growth medium. After plating the appropriate dilutions of the pre-enriched samples, (MRS broth) on BCP-MRS agar plates, typical yellow coloured colonies developed on the agar plates after 24 hrs. Initially, a sizable number of typical representative colonies from each sample were randomly picked from the agar plates and transferred to MRS broth and incubated at 37oC for overnight. The isolates grown in MRS broth were checked for purity under a microscope (Leica, Germany) after Gram and spore staining and the pure cultures appearing as typical thin, small and large rods and exhibiting Gram positive reaction on staining were subjected to catalase test. Based on these results, a total of 100 isolates typical of lactobacilli and catalase negative were selected as subject for further studies. A set of these isolates was preserved and maintained in litmus milk as well as glycerol stocks stored at -80^{o}C in a deep freezer (New Bruinswick, USA).

4.2 IDENTIFICATION OF ISOLATES AS LACTOBACILLI BY GENUS SPECIFIC PCR

In order to ascertain the true identity of the aforesaid isolates as lactobacilli, all of them were subjected to PCR assay based on genus specific primers LbLMA1/R161 targeted against 16SrRNA developed previously in our lab. The PCR assay conducted with the template DNA obtained from the standard lactobacillus cultures and the lactobacillus isolates resulted into the amplification of a 250 bp PCR products on the agarose gel which was specific for lactobacilli only. On the basis of our PCR results, 35 isolates out of 100 colonies along with three standard probiotic cultures namely *Lb. johnsonii La1, Lb. acidophilus LA7 and Lb. acidophilus P* showed the positive signal in the form of a distinct 250 bp band on the gel as indicated in **Fig. 2**, thereby, establishing their identity as lactobacilli. Our results with this regard are in complete agreement with these of Dubernet *et al*, 2002; Suja, 2003 and Neha, 2003 who also demonstrated amplification of 250 bp product in the PCR assay with all the standard cultures as well as wild isolates of lactobacilli used in their study.

The break up of these 35 isolates indicating the source of their recovery / origin has been recorded in **Table 4.** As can be evidenced from the table, majority of these isolates (24) were recovered from human faecal samples, 8 from raw buffalo milk, two from probiotic preparations and one from human milk.

Table 4 Break up of the isolates confirmed as Lactobacilli by genus specific PCR

S. No.	Isolate	Source	Morphology	Catalase Reaction	Polymerase Chain Reaction
1	Lb1	Capsule	Gram +ve rods	-ve	+ve for Lb
2	Lb2	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
3	Lb3	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
4	Lb4	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
5	Lb5	Feacal	Gram +ve rods	-ve	+ve for Lb

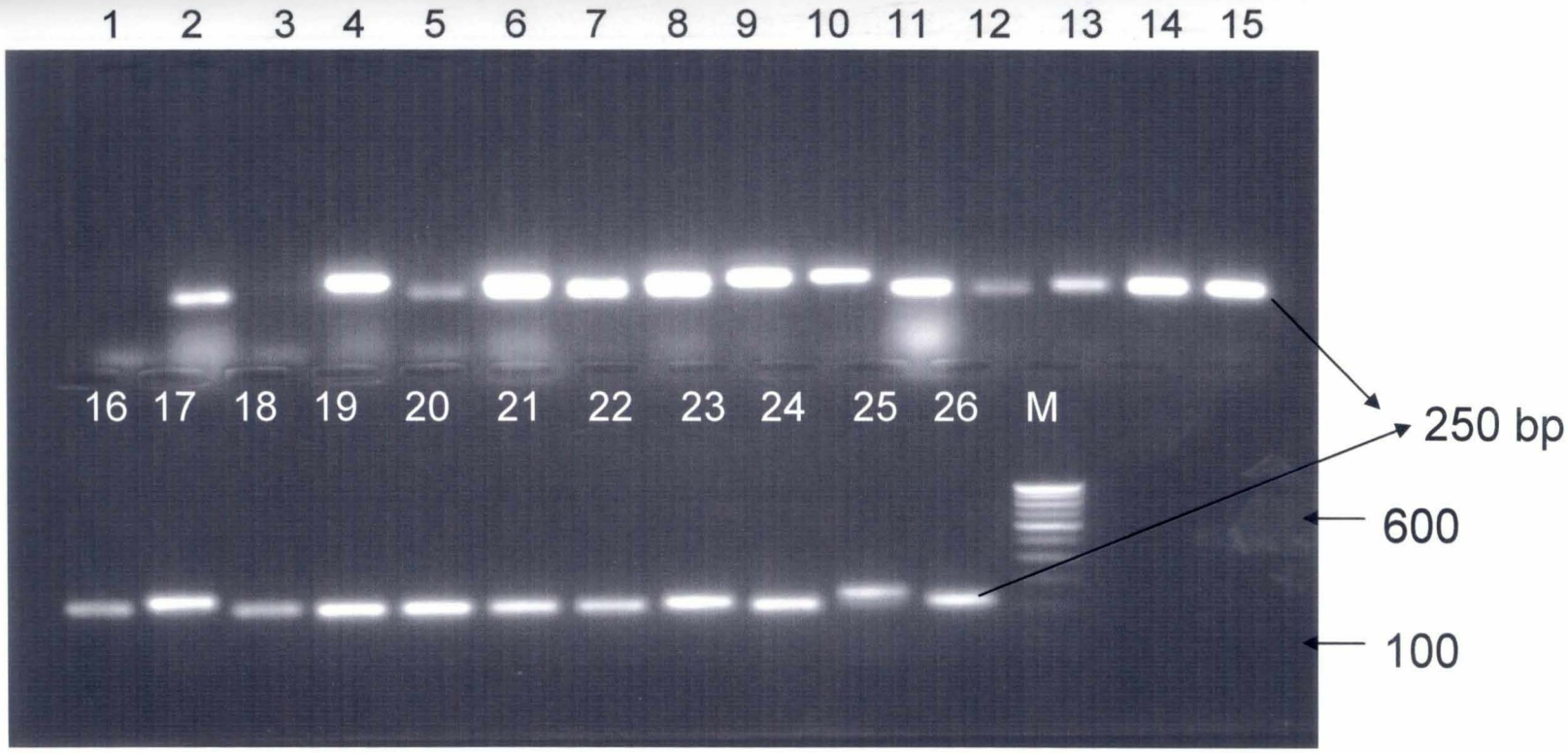

Fig. 2. Identification of Lactobacillus isolates with PCR using LbLMA-1/R16-1 genus specific primers

Upper Lanes : 1 – 15 (Lactobacillus isolates)

Lower Lanes : 16 – 23 (Lactobacillus isolates); **24,** *Lactobacillus johnsonii* La1; **25,** *Lactobacillus acidophilus* LA7; 26, *Lactobacillus acidophilus* P; **M,** 100bp

6	Lb6	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
7	Lb7	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
8	Lb8	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
9	Lb9	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
10	Lb10	Buffalo Milk	Gram +ve rods	-ve	+ve for Lb
11	Lb11	Feacal	Gram +ve rods	-ve	+ve for Lb
12	Lb12	Feacal	Gram +ve rods	-ve	+ve for Lb
13	Lb13	Feacal	Gram +ve rods	-ve	+ve for Lb
14	Lb14	Capsules	Gram +ve rods	-ve	+ve for Lb
15	Lb15	Feacal	Gram +ve rods	-ve	+ve for Lb
16	Lb16	Feacal	Gram +ve rods	-ve	+ve for Lb
17	Lb17	Feacal	Gram +ve rods	-ve	+ve for Lb
18	Lb18	Feacal	Gram +ve rods	-ve	+ve for Lb
19	Lb19	Human milk	Short rods	-ve	+ve for Lb
20	Lb20	Feacal	Gram +ve rods	-ve	+ve for Lb
21	Lb21	Feacal	Gram +ve rods	-ve	+ve for Lb
22	Lb22	Feacal	Gram +ve rods	-ve	+ve for Lb
23	Lb23	Feacal	Gram +ve rods	-ve	+ve for Lb
24	Lb24	Feacal	Gram +ve rods	-ve	+ve for Lb
25	Lb25	Feacal	Gram +ve rods	-ve	+ve for Lb
26	Lb26	Feacal	Gram +ve rods	-ve	+ve for Lb
27	Lb27	Feacal	Gram +ve rods	-ve	+ve for Lb
28	Lb28	Feacal	Gram +ve rods	-ve	+ve for Lb
29	Lb29	Feacal	Gram +ve rods	-ve	+ve for Lb
30	Lb30	Feacal	Gram +ve rods	-ve	+ve for Lb
31	Lb31	Feacal	Gram +ve rods	-ve	+ve for Lb
32	Lb32	Feacal	Gram +ve rods	-ve	+ve for Lb
33	Lb33	Feacal	Gram +ve rods	-ve	+ve for Lb
34	Lb34	Feacal	Gram +ve rods	-ve	+ve for Lb
35	Lb35	Feacal	Gram +ve rods	-ve	+ve for Lb

4.3 SUGAR FERMENTATION PROFILE OF LACTOBACILLUS ISOLATES

In this investigation, an attempt was also made to tentatively give the species status to the *Lactobacillus* isolates confirmed by genus specific PCR on the basis of their sugar fermentation characteristics. The data pertaining to sugar fermentation profiles of 14 selected cultures has been presented in **Table 5** and **Fig. 3**.

Table 5. Sugar Fermentation Profile of Selected Lactobacillus Isolates

Isolates	Arabi-nose	Mannitol	NAGA	Cello-biose	Melli-biose	Sucrose	Treha-lose	Tentative identify-cation at species level
Lb1	–	+	+	+	+	+	+	Lp
Lb2	–	+	+	+	–	+	+	Lc
Lb3	–	+	+	+	+	+	+	Lp
Lb4	–	+	+	+	+	+	+	Lp
Lb5	–	+	+	+	+	+	+	Lp
Lb6	–	+	+	+	+	+	+	Lp
Lb7	–	+	–	+	+	+	+	Lf
Lb8	–	+	+	+	+	+	+	Lp
Lb10	–	+	+	+	–	+	+	Lp
Lb12	–	+	+	+	+	+	+	La
Lb13	–	+	+	+	+	+	–	Lf
Lb14	–	+	+	+	+	+	+	Lp
Lb16	–	+	+	+	+	+	+	Lp
Lb18	–	+	+	+	+	+	+	Lp

Lp – *Lb. plantarum*; Lc – *Lb. casei*; Lf - *Lb. fermentum*; La – *Lb. acidophilus*

As is quite evident form the data presented therein, all the isolates were able to ferment Mannitol, Cellobiose and Sucrose, However, arabinose could not be fermented by any of the isolates. On the other hand, N-acetyl-glucosamine and melibiose were not fermented by only one isolate (Lb7) and

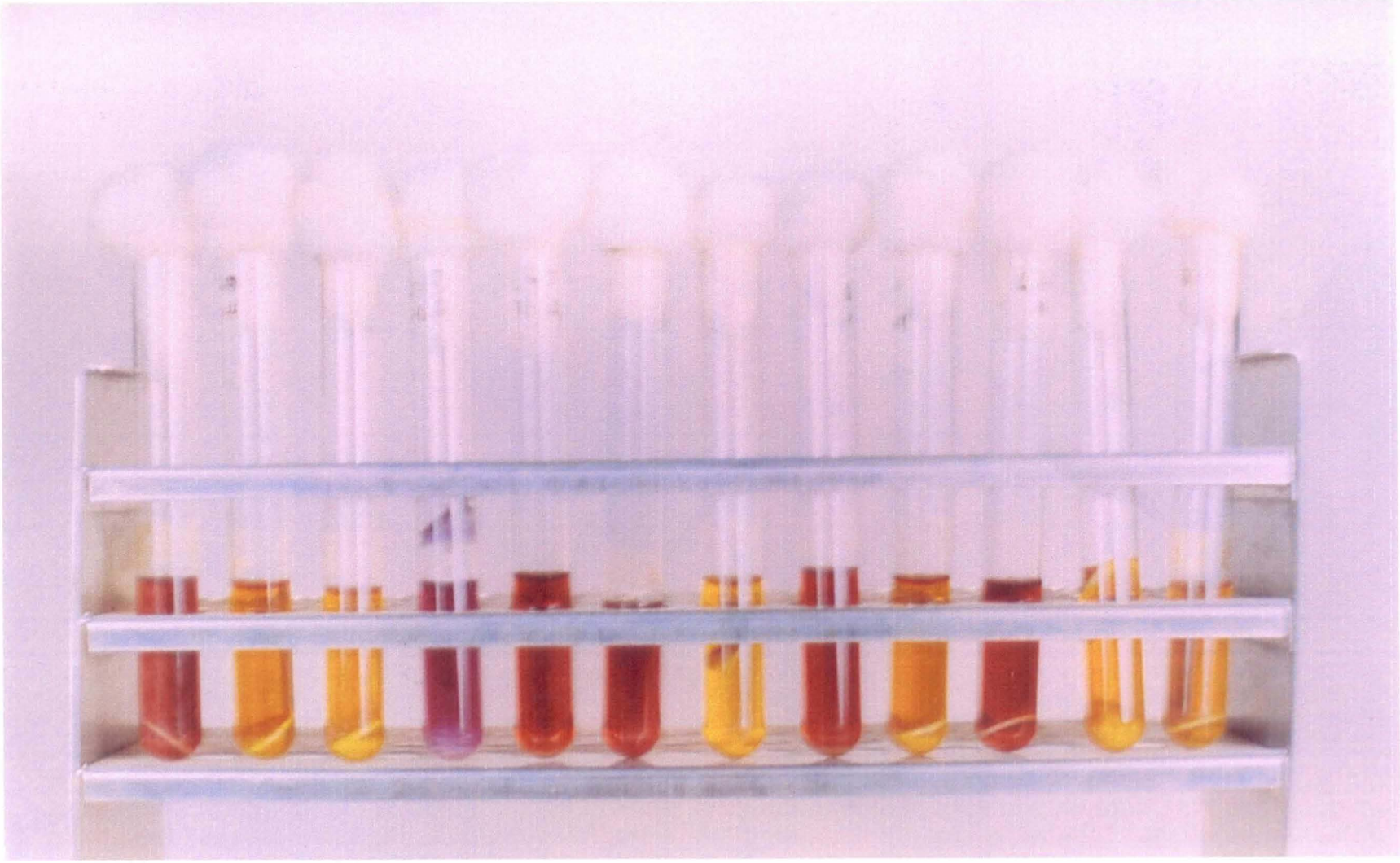

Fig : 3 Sugar fermentation pattern of Lactobacillus spp

two isolates (Lb2 and Lb10) respectively. Based on the typical sugar fermentation patterns in respect of our isolates vis-à-vis the standard fermentation profiles of different lactobacillus species as given in Bergey's Manual, 10 of the isolates namely Lb1, Lb3, Lb4,Lb5, Lb6,Lb8, Lb10, Lb14, Lb16 and Lb18 were tentatively identified as *Lb. plantarum*, one as *Lb. casei* (Lb2), two as *Lb. fermentum* (Lb7, Lb13) and one as *Lb. acidophilus* (Lb12). However, these results are just tentative and hence needs further substantiation by including some additional sugars such as Ribose, Dulcitol, Rhamnose, Xylose, Sorbitol etc. in the experiment to make them more realistic and conclusive. Our results in this regard are consistent with similar observations made by Mishra (2001). However, the latter investigator also used API system for identification of species of *Lactobacillus* isolates used in his study.

4. 4. SCREENING OF INDIGENOUS LACTOBACILLUS ISOLATES FOR PROBIOTIC ATTRIBUTES

Since, the long term target of the present investigation was to find promising and novel indigenous probiotic lactobacilli for commercial application, all the 35 PCR positive Lactobacillus isolates that emerged from this study were subjected to a battery of standard tests recommended for determining their probiotic attributes as per WHO guidelines. The results pertaining to each of these tests will now be presented separately.

4.4.1 Acid Tolerance

Acid tolerance is perhaps one of the most important pre-requisites for the selection of probiotic lactobacilli as they must survive the harsh acidic environment in the gut to remain there for a while in good number and express their health promoting functions (Conway *et al*, 1987 and WHO guidelines, 2002). In the present investigation, the Lactobacillus isolates were subjected to *in vitro* tolerance to different acid levels of pH 1.0, 2.0 and 3.0 for various time intervals at 37^{o}C to simulate the conditions prevalent in the human gut. The results pertaining to acid resistance of our isolates have been recorded in **Table 6** and **Figures 4 and 5.**

pH tolerance

Fig : 4

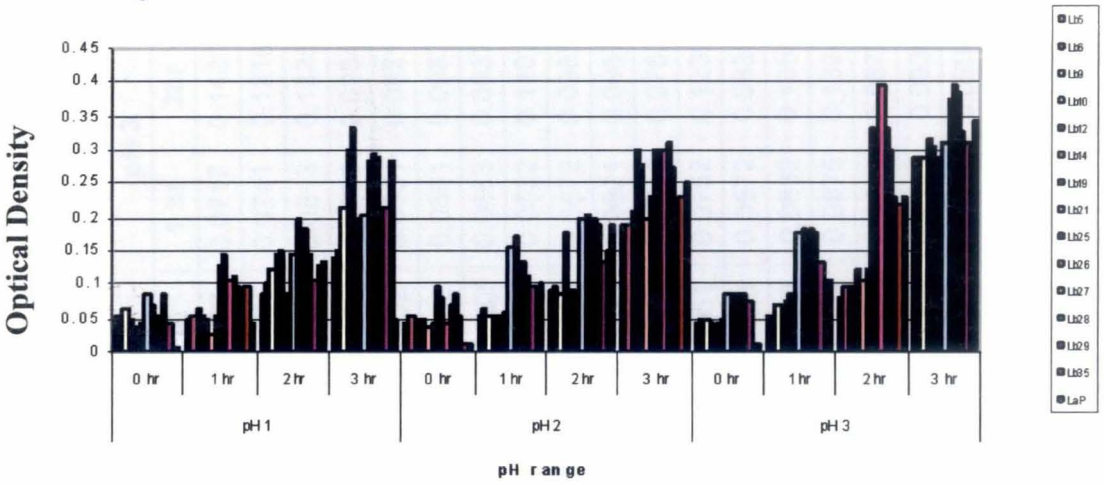

Fig : 5

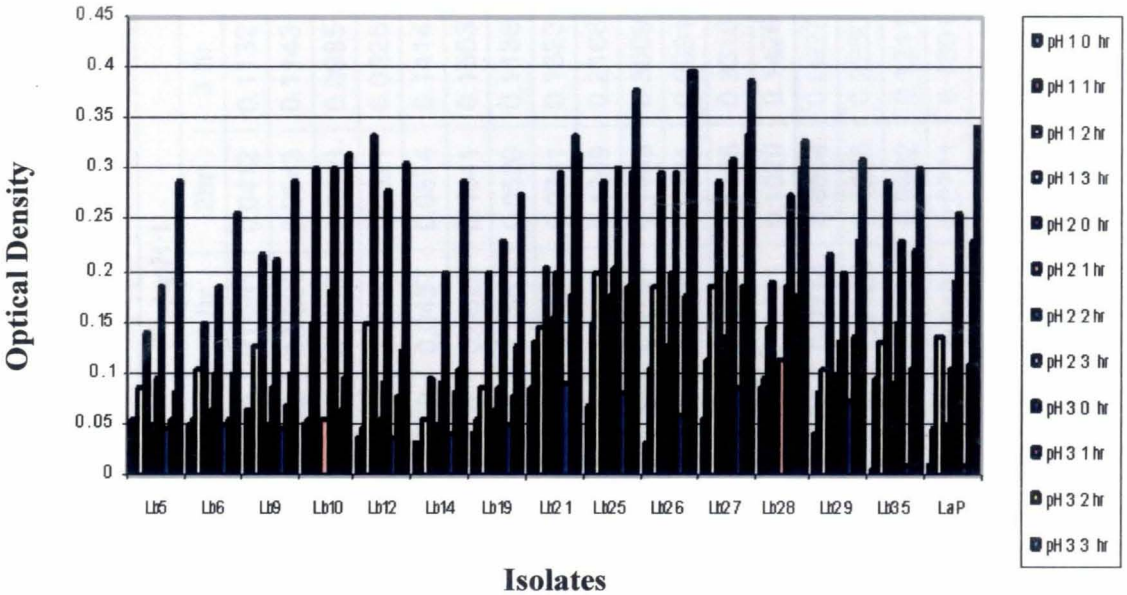

Table 6: Acid Tolerance of Lactobacillus Isolates

Culture	Initial O.D.	pH 1				pH 2				pH 3			
		0 hr	1 hr	2hr	3 hr	0 hr	1 hr	2hr	3 hr	0 hr	1 hr	2hr	3 hr
Lb1	0.3-0.4	0.0312	0.0381	0.0412	0.1132	0.0342	0.0392	0.0862	0.1035	0.0431	0.0717	0.1421	0.1573
Lb2		0.0362	0.0396	0.0543	0.1143	0.0311	0.0371	0.0753	0.0982	0.0371	0.0741	0.1316	0.1824
Lb3		0.0263	0.0293	0.0313	0.0985	0.0301	0.0342	0.0612	0.0952	0.0342	0.0813	0.1825	0.2010
Lb4		0.0275	0.0281	0.0521	0.0826	0.0253	0.0302	0.0567	0.0882	0.0381	0.0673	0.0954	0.1654
Lb5		0.0560	0.0457	0.0874	0.1414	0.0402	0.0511	0.0926	0.1852	0.0452	0.0531	0.0821	0.2876
Lb6		0.0501	0.0528	0.1041	0.1503	0.0532	0.0621	0.0987	0.1858	0.0492	0.0561	0.0983	0.2562
Lb7		0.0275	0.0553	0.0520	0.1136	0.0312	0.0589	0.0713	0.1324	0.0362	0.0653	0.0932	0.2031
Lb8		0.0304	0.0405	0.0711	0.1323	0.0357	0.0652	0.0748	0.1835	0.0395	0.0532	0.1102	0.1986
Lb9		0.0625	0.0628	0.1249	0.2168	0.0431	0.0516	0.0873	0.2104	0.0456	0.0672	0.0985	0.2899
Lb10		0.0488	0.0541	0.1463	0.3009	0.0461	0.0548	0.1782	0.2995	0.0443	0.0621	0.0967	0.3152
Lb11		0.0283	0.0291	0.0481	0.0931	0.0351	0.0396	0.0723	0.1536	0.0396	0.0542	0.0783	0.1724
Lb12		0.0363	0.0461	0.1485	0.3316	0.0477	0.0562	0.0915	0.2785	0.0351	0.0752	0.1232	0.3041
Lb13		0.0261	0.0371	0.1020	0.1476	0.0355	0.0382	0.0659	0.1836	0.0386	0.0572	0.0835	0.1863
Lb14		0.0305	0.0274	0.0554	0.0927	0.0378	0.0495	0.0892	0.1987	0.0415	0.0832	0.1053	0.2873
Lb15		0.0421	0.0426	0.1223	0.2250	0.0461	0.0536	0.0935	0.2015	0.0402	0.0875	0.1324	0.2134
Lb16		0.0312	0.0311	0.0602	0.1211	0.0432	0.0446	0.0559	0.0936	0.0415	0.0531	0.0872	0.1652
Lb17		0.0416	0.0425	0.0771	0.1201	0.0334	0.0387	0.0752	0.0983	0.0389	0.0544	0.0936	0.1935
Lb18		0.0351	0.0326	0.0653	0.1142	0.0385	0.0562	0.0873	0.1532	0.0352	0.0615	0.0994	0.1857
Lb19		0.0418	0.0531	0.0856	0.1988	0.0453	0.0613	0.0858	0.2317	0.0493	0.0752	0.1258	0.2734
Lb20		0.0365	0.0394	0.0583	0.0935	0.0318	0.0045	0.0732	0.1548	0.0436	0.0538	0.0833	0.1844

Table 6 cont…….

Culture	Initial O.D.	pH 1				pH 2				pH 3			
		0 hr	1 hr	2hr	3 hr	0 hr	1 hr	2hr	3 hr	0 hr	1 hr	2hr	3 hr
Lb21	0.4-0.5	0.0874	0.1283	0.1452	0.2035	0.0981	0.1541	0.1989	0.2983	0.0882	0.1766	0.3312	0.3129
Lb22		0.0813	0.1365	0.1798	0.2153	0.0873	0.1638	0.1932	0.2343	0.0875	0.1700	0.2131	0.2755
Lb23		0.0451	0.0982	0.1654	0.1987	0.0564	0.0985	0.1126	0.1324	0.0613	0.0975	0.1356	0.1877
Lb24		0.0424	0.0856	0.1542	0.2003	0.0617	0.0963	0.1132	0.1465	0.0754	0.0943	0.1178	0.1654
Lb25		0.0673	0.1467	0.1985	0.2864	0.0789	0.1734	0.2014	0.3011	0.0831	0.1832	0.2987	0.3766
Lb26		0.0316	0.1057	0.1835	0.2963	0.0422	0.1242	0.1973	0.2988	0.0566	0.1737	0.3957	0.3951
Lb27		0.0544	0.1123	0.1824	0.2883	0.0676	0.1351	0.1999	0.3112	0.0875	0.1834	0.3327	0.3874
Lb28		0.0843	0.0965	0.1432	0.1878	0.0854	0.1123	0.1865	0.2753	0.0851	0.1755	0.3015	0.3275
Lb29		0.0423	0.0793	0.1053	0.2157	0.0532	0.0978	0.1324	0.1988	0.0733	0.1342	0.2297	0.3103
Lb30		0.0775	0.0954	0.1352	0.1954	0.0833	0.0955	0.1176	0.1732	0.0871	0.1462	0.1985	0.2977
Lb31		0.0031	0.0517	0.0972	0.1236	0.0053	0.0631	0.0853	0.0976	0.0078	0.0931	0.1827	0.2013
Lb32		0.0036	0.0632	0.1033	0.1153	0.0038	0.0544	0.1213	0.1835	0.0075	0.0754	0.1065	0.2112
Lb33		0.0054	0.0873	0.1016	0.1213	0.0077	0.0751	0.1034	0.1765	0.0083	0.0862	0.1325	0.2437
Lb34		0.0044	0.0752	0.1051	0.1136	0.0052	0.0765	0.1362	0.1897	0.0052	0.0855	0.1301	0.2037
Lb35		0.0067	0.0962	0.1287	0.2865	0.0087	0.0893	0.1488	0.2304	0.0092	0.1032	0.2183	0.3012
LaP		0.0071	0.0453	0.1356	0.0476	0.0088	0.1013	0.1876	0.2543	0.0098	0.1092	0.2302	0.3420

As is quite evident from the data presented therein, some of the isolates namely Lb10, Lb12 and Lb26 were able to survive even at pH 1.0 for 1-3 hrs and their acid tolerance was comparable to that of standard probiotic cultures used in this study as indicated by relatively high OD values (0.3009, 0.3316 and 0.2963 for Lb10, Lb12 And Lb26 respectively) obtained at aforesaid pH after three hrs. Similarly, at pH 2.0 and 3.0, the aforesaid cultures particularly Lb10, the corresponding OD values were fairly high i.e. 0.2995 and 0.3152 after three hrs. Almost a similar trend in OD values was observed withteh remaining two cultures. Our results in this regard appear to be comparable with that of Mishra (2001) who also recorded high degree of acid tolerance in case of all the seven strains except NCDC-19 examined in his study even after 3 hrs of incubation at pH 1.0, 2.0 and 3.0, although there were considerable variations in acid tolerance between strains. Almost similar observations were made by (Goldin *et al* .,1992) and (Jacobsen *et al.,* 1999) who also recorded fairly high acid tolerance of their probiotic *Lactobacillus* cultures such as *Lactobacillus* GG, C1 and Y strains respectively.

Our results on acid tolerance of Lactobacillus isolates are, however, inconsistent with the findings of Lankaputhra and Shah (1995) who in general recorded a decrease in the number of survivors of *Lb. acidophilus* starins during 3 hrs of incubations at all the pH conditions used in their study. This contradiction in results on acid tolerance could be attributed to varied tolerance of different Lactobacillus spp. and strains towards acidic conditions.

4.4.2 Bile Tolerance

Bile tolerance is another important property used for selection of probiotic lactobacilli since human gastrointestinal tract (GIT) is studded with high concentrations of bile secretion in view of their potential role in food digestion. However, the rate of secretion of bile and the concentrations of bile in different regions of the GIT tract vary depending mainly on the type of food consumed. Bile concentrations in GIT can range from 0.5 to 2% at different regions and hence create an unfavourable environment in the gut for survival of micro-organisms due to toxicity of these salts. Hence, probiotic lactobacilli

are required to be bile tolerant to get implanted and survive in the gut in presence of such a high concentration of bile salts prevalent therein. In the present study, the *Lactobacillus* isolates were subjected to *in vitro* bile tolerance test by examining effect of different levels of bile salts 1.0, 1.5 and 2.0 % on the growth and survival of *Lactobacillus* isolates used in the study. The data pertaining to the same have been presented in **Table 7 and Figs. 6 and 7.**

As can be evidenced from these results, there was considerable variation in the bile tolerance of the *Lactobacillus* isolates examined in this investigation. However, some of these isolates exhibited fairly high bile tolerance at all three levels of bile concentration. In general, majority the isolates were able to survive at 1.0 %concentration of bile even after 3 hrs as can be revealed by fairly high OD_{600} values (more than 0.2) in almost all the cases. The survival however was relatively much lower at higher bile concentrations i.e. 1.5 and 2.0% during the corresponding period (3 hrs). The maximum bile tolerance at 1% level (OD values 0.5287 and 0.5130) was recorded with Lb27 and Lb26 respectively after 3 hrs of treatment. The corresponding values with 1.5 and 2.0 % bile concentrations with Lb27 were 0.3647 and 0.3126 which were comparable with those of the standard probiotic cultures used in the study as a positive control. The most bile sensitive isolate in this study was found to be Lb7 which could produce an OD_{600} value of only 0.0541. Our results in this regard are consistent with eth observations of several other investigators who also reported sizable variations in the bile tolerance among their probiotic strains after different exposure times (Gilliland and Speck, 1977; Klaenhammer and Kleemon, 1981; Gilliland and Walker, 1990; Lankaputhra and Shah, 1995 and Mishra, 2001).

4.4.3 Hydrophobicity of Lactobacillus Isolates

Another important *in vitro* test for studying the probiotic nature of lactobacilli is the hydrophobicity test based on the nature of their cell surface involved in interaction with phagocytes, adherence to non-wetable solid surfaces,

Bile tolerance

Fig : 6

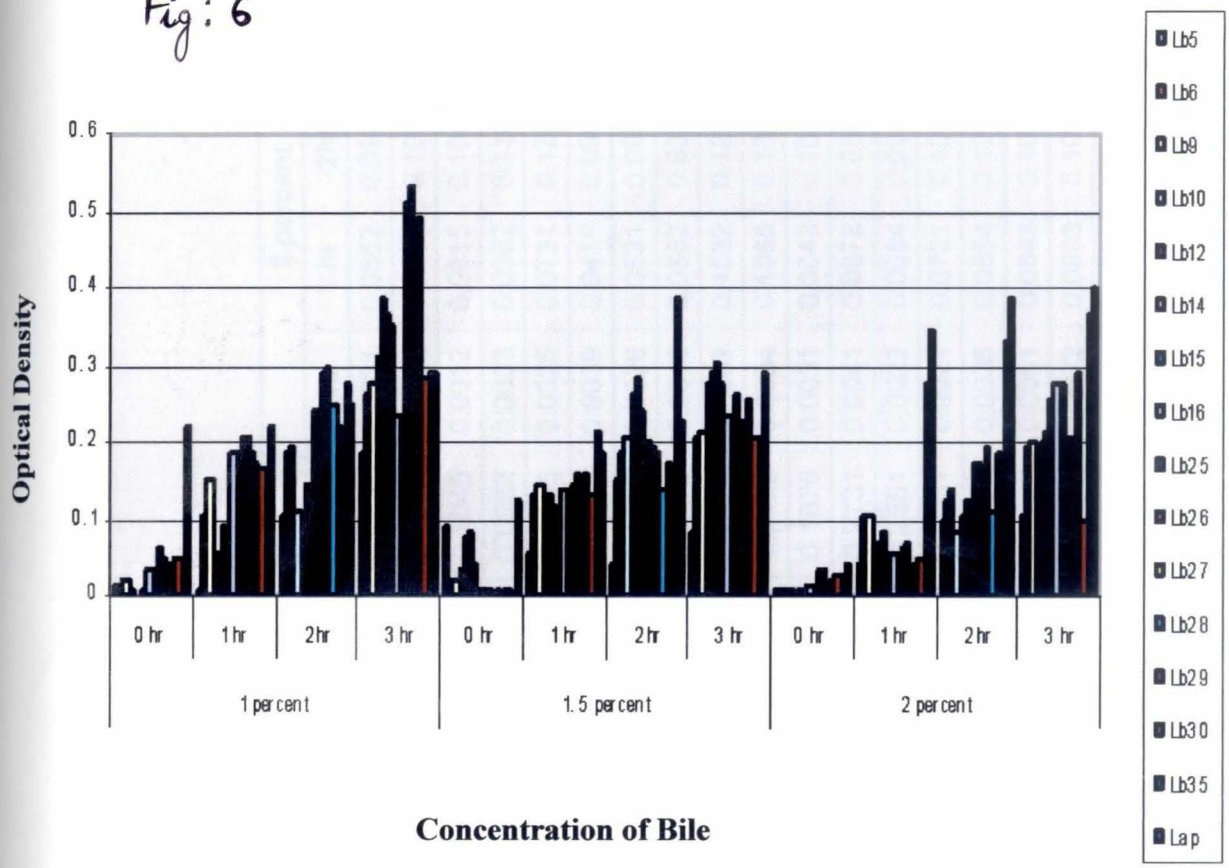

Fig : 7

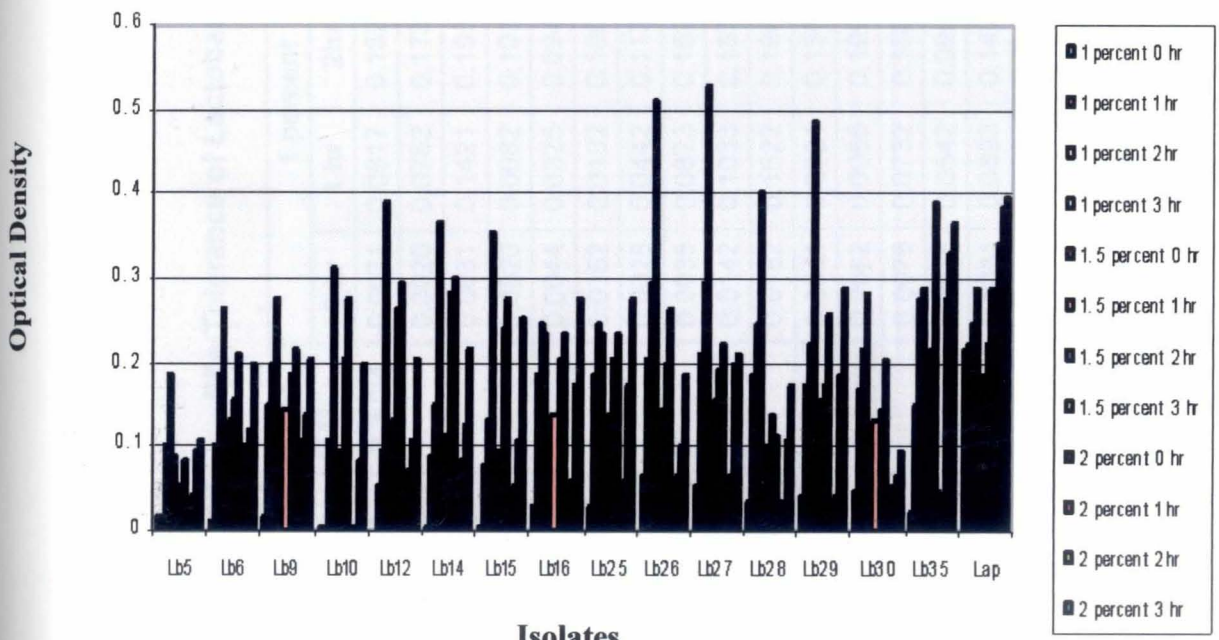

Table 7: Bile Tolerance of Lactobacillus Isolates

Culture	Initial O.D.	1 percent				1.5 percent				2 percent			
		0 hr	1 hr	2hr	3 hr	0 hr	1 hr	2hr	3 hr	0 hr	1 hr	2hr	3 hr
Lb1	0.3-0.4	0.0021	0.0817	0.1526	0.2013	0.0061	0.0712	0.0621	0.0531	0.0031	0.0852	0.0917	0.1288
Lb2		0.0020	0.0752	0.1730	0.2130	0.0042	0.0056	0.0762	0.1283	0.0042	0.0931	0.1036	0.1314
Lb3		0.0031	0.1421	0.1955	0.2541	0.0033	0.0732	0.1032	0.1045	0.0012	0.0815	0.1041	0.1260
Lb4		0.0020	0.0082	0.1034	0.2038	0.0029	0.0855	0.1076	0.0982	0.0033	0.0982	0.1133	0.1450
Lb5		0.0044	0.0325	0.0943	0.2096	0.0075	0.0413	0.0987	0.1445	0.0025	0.0731	0.1201	0.0972
Lb6		0.0162	0.0102	0.1001	0.1854	0.0897	0.0563	0.0415	0.0823	0.0039	0.0416	0.0987	0.1063
Lb7		0.0028	0.0442	0.1133	0.1957	0.0042	0.0317	0.1038	0.1128	0.0076	0.0531	0.0952	0.0541
Lb8		0.0035	0.0873	0.1526	0.1803	0.0071	0.0442	0.0972	0.1066	0.0018	0.0862	0.0326	0.0732
Lb9		0.0142	0.1033	0.1873	0.2655	0.0212	0.1320	0.1538	0.2071	0.0082	0.1032	0.1224	0.1951
Lb10		0.0182	0.1522	0.1964	0.2778	0.0192	0.1443	0.1876	0.2136	0.0094	0.1065	0.1367	0.2032
Lb11		0.0011	0.0921	0.1362	0.2134	0.0211	0.0021	0.1031	0.1876	0.0031	0.0043	0.1030	0.1141
Lb12		0.0082	0.0086	0.1095	0.3102	0.0334	0.0962	0.2062	0.2731	0.0041	0.0078	0.0826	0.1962
Lb13		0.0076	0.0732	0.1087	0.3520	0.0542	0.0988	0.2112	0.2851	0.0023	0.0084	0.0915	0.1854
Lb14		0.0015	0.0542	0.0982	0.3876	0.0788	0.1302	0.2652	0.2931	0.0021	0.0721	0.1052	0.2012
Lb15		0.0031	0.0893	0.1478	0.3670	0.0862	0.1162	0.2818	0.3021	0.0036	0.0854	0.1237	0.2136
Lb16		0.0052	0.0777	0.1322	0.3544	0.043	0.0954	0.2416	0.2752	0.0051	0.0543	0.1085	0.2544
Lb17		0.0078	0.0612	0.1856	0.3786	0.0552	0.0976	0.2032	0.2162	0.0072	0.0893	0.1003	0.2178
Lb18		0.0022	0.0312	0.0983	0.3302	0.0331	0.0835	0.2517	0.2031	0.0031	0.0963	0.1231	0.2312
Lb19		0.0173	0.0752	0.1302	0.3896	0.0897	0.1452	0.2983	0.3126	0.0044	0.0987	0.1432	0.2818
Lb20		0.0421	0.0317	0.0852	0.3012	0.0541	0.1063	0.2041	0.2513	0.0036	0.0044	0.1052	0.2043

Table 7 contd…….

Culture	Initial O.D.	1 percent				1.5 percent				2 percent			
		0 hr	1 hr	2hr	3 hr	0 hr	1 hr	2hr	3 hr	0 hr	1 hr	2hr	3 hr
Lb21	0.4-0.5	0.0316	0.1952	0.2734	0.3463	0.0062	0.1652	0.1788	0.2106	0.0118	0.0376	0.0832	0.1037
Lb22		0.0375	0.1724	0.2146	0.3752	0.0045	0.1300	0.1562	0.1877	0.0170	0.0367	0.0994	0.1967
Lb23		0.0345	0.1986	0.2324	0.4185	0.0032	0.1082	0.1635	0.1602	0.0103	0.0421	0.0958	0.1989
Lb24		0.0531	0.1763	0.2234	0.3978	0.0067	0.1132	0.1871	0.2052	0.0314	0.0736	0.0933	0.1603
Lb25		0.0326	0.1853	0.2431	0.2362	0.0075	0.1386	0.2012	0.2326	0.0112	0.0571	0.1751	0.2751
Lb26		0.0653	0.2051	0.2917	0.5130	0.0078	0.1442	0.1965	0.2637	0.0365	0.0642	0.1044	0.1875
Lb27		0.0512	0.2103	0.2936	0.5287	0.0032	0.1553	0.1896	0.2242	0.0357	0.0657	0.1953	0.2077
Lb28		0.0385	0.1856	0.2488	0.4006	0.0066	0.1008	0.1364	0.1123	0.0153	0.0372	0.1072	0.1752
Lb29		0.0436	0.1752	0.2215	0.4876	0.0034	0.1556	0.1725	0.2567	0.0178	0.0448	0.1832	0.2877
Lb30		0.0452	0.1654	0.2136	0.2837	0.0049	0.1341	0.1436	0.2042	0.0253	0.0513	0.0637	0.0956
Lb31		0.2143	0.2185	0.2345	0.2863	0.0043	0.2284	0.2342	0.2136	0.0071	0.2657	0.2478	0.2251
Lb32		0.1891	0.2303	0.2196	0.2547	0.0088	0.2982	0.2538	0.2216	0.0141	0.2753	0.2618	0.2731
Lb33		0.2899	0.2321	0.2085	0.2493	0.0094	0.2651	0.2983	0.2751	0.0705	0.2321	0.2517	0.2462
Lb34		0.2314	0.2484	0.2386	0.2381	0.0218	0.2342	0.2695	0.2113	0.0500	0.2436	0.2321	0.2003
Lb35		0.2660	0.1478	0.2742	0.2891	0.0023	0.2166	0.3871	0.0484	0.0305	0.2738	0.3312	0.3647
LaP		0.2176	0.2231	0.2462	0.2897	0.1255	0.1876	0.2245	0.2896	0.0413	0.3449	0.3851	0.3966

partitioning at liquid:liquid and liquid:air interfaces. The hydrophobicity to hydrocarbons is an important feature of probiotic Lactobacillus cell surface. In this investigation, the hydrophobicity of our Lactobacillus isolates was determined with one of the common hydrocarbons namely hexadecane. The results concerning the hydrophobicity of the test Lactobacillus cultures, the subject of this study are presented in **Table 8.**

Table 8: Cell surface hydrophobicity of Lactobacillus isolates

Culture No.	Initial O.D.	Final O.D.	% Hydrophobicity
Lb3	0.7815	0.5254	32
Lb10	0.8134	0.4522	44.4
Lb19	0.5431	0.1712	68.4
Lb23	0.7652	0.5515	27.9
Lb25	0.6608	0.4256	35.5
Lb26	0.5654	0.4165	51.8
Lb27	0.8916	0.3265	63.0
Lb29	0.7851	0.4691	40.2
Lb.casei shirota	0.8608	0.3431	60.1
. La P	0.7815	0.2163	72.3

From the data presented therein, it can be evidenced that maximum hydrophobicity (68.4%) towards hexadecane was recorded with Lb19 followed by Lb27 (63.0%) which were almost comparable to the values obtained with standard probiotic cultures *Lb. casei* Shirota and LaP (60.1 and 72.3%) respectively. Our results in this regards are in agreement with those of Morata De Ambrosini *et al* (1998) who also observed very high hydrophobicity of *Pediococcus pentosaceus* and *Propionibacterium acidopropinici* and *Lb. casei*. However, Conway and Reginold (1989) reported lack of correlation between capacity for adhesion and hydrophobicity. Hence, utmost care needs to be taken to draw any conclusive inference from such studies.

4.4.4 Antibiotic Resistance / Susceptibility of Lactobacillus Isolates

Antibiotic resistance / susceptibility of probiotic cultures can also influence their survival in the human gut since antibiotic therapy use dto protect the GI tract can not only disturb the normal gut flora but also the probiotic cultures implanted therein. This issue has generated lot of interest and debate in determining the antibiotic resistance of probiotic cultures including lactobacilli. To keep our interest alive in this direction, an attempt was made in this investigation also to study the antibiotic resistance / susceptibility of our lactobacillus isolates against some commonly used rugs in gut therapy. The results in regard to this aspect have been recorded in **Table 9.**

Table 9. Antibiotic resistance / susceptibility of Lactobacillus isolates

Isolates	Erythro-mycin	Tetracyc-line	Strepto-mycin	Genta-mycin	Vanco-mycin
	Zone of inhibition (mm)				
Lb1	27	40	15	20	30
Lb2	28	35	20	11	31
Lb3	23	29	18	14	R
Lb4	21	30	15	12	R
Lb5	20	25	7	10	8
Lb6	23	24	12	12	R
Lb7	20	35	8	11	7
Lb8	20	38	15	17	R
Lb9	18	35	14	12	R
Lb10	14	38	15	11	R
Lb 11	18	31	13	12	R
Lb12	13	22	12	14	R
Lb13	15	30	11	13	R
Lb14	25	32	13	15	R
Lb15	24	35	12	10	R
Lb16	20	31	13	12	R
Lb17	23	31	12	15	R
Lb18	24	30	15	17	R

Lb19	15	27	11	10	R
Lb20	23	28	14	12	R
Lb21	15	21	8	11	R
Lb22	11	26	9	13	R
Lb23	17	26	13	15	R
Lb24	18	22	10	9	R
Lb25	18	21	13	11	R
Lb26	13	25	11	12	R
Lb27	19	25	9	11	R
Lb28	10	24	8	10	R
Lb29	17	19	8	9	R
Lb30	18	24	15	11	R
Lb31	16	23	8	11	R
Lb32	20	27	11	13	R
Lb33	15	21	11	9	R
Lb34	19	25	9	13	R
Lb35	14	24	10	9	R

A critical appraisal of the results presented therein clearly indicates that almost all the cultures were sensitive to both erythromycin and tetracycline whereas streptomycin and gentamycin were weakly to moderately effective against these cultures. However, the most interesting observation was recorded with vancomycin as majority of the isolates except Lb2, Lb3 and Lb5 were resistant to this antibiotic. Our results in this regard are in agreement with those of Mishra (2001) who also recorded vancomycin resistance against all the Lactobacillus and Bifiodbacterial cultures examined in his study. Based on our results and similar observations made by other investigators, it can be stated that vancomycin resistance is a widespread phenomenon among lactobacilli as was reported earlier also (Ruoff et al, 1988 and Holliman and Bone, 1988).

4.4.4 Antibacterial activity of Lactobacillus isolates

Antibacterial activity is yet another desirable potential attribute used for the selection of probiotic cultures. The ability to produce certain proteinaceous

antimicrobial substances is an extremely beneficial property and hence can be used as a bonus to the probiotic culture to enhance its commcercial value sinc eteh production of the antimicrobial factors by the probiotic culture in the gut can not only provide a competitive edge to these beneficial cultures to survive and proliferate there but also help in the eradication of undesirable high risk pathogens and other infectious agents, the causative organisms of disease in human beings. Hence, in this investigation, the antibacterial activity of selected lactobacillus isolates against two high risk pathogens namely *E. coli* 0157:H7 and *L. monocytogenes* was determined. The results pertaining to the same have been presented in **Table 10.**

Table 10. Antibacterial activity of few selected Lactobacillus isolates

Isolates	*E. coli* 0157: H7	*L. monocytogenes*
Lb1	--	20mm
Lb3	--	13mm
Lb5	--	20mm
Lb7	--	23mm
Lb10	--	28mm
Lb13	--	12mm
Lb24	--	13mm
Lb26	13mm	14mm
Lb27	13mm	12mm
Lb29	12mm	--
Lb32	--	11mm
Lb34	--	12mm
Lb38	--	10mm
Lb39	--	13mm
LaP	14mm	21mm

As can be inferred from these results, almost all the selected Lactobacillus isolates examined in this study except one (Lb29) exhibited a moderate antibacterial activity against *L. monocytogenes*. However, only four of the cultures namely Lb26, Lb27, Lb29 and LaP were able to show weak

inhibitory activity against *E. coli* 0157: H7. The maximum antibacterial activity against *L. monocytogenes* was recorded with Lb10 as revealed by big inhibition zone (28 mm) in the cut well assay. Our results in this regard are comparable to those of several other investigators who also recorded strong antibacterial activity of probiotic lactobacilli against Gram positive and gram negative bacteria (Reid *et al,* 1998; Kabir *et al,* 1997; Maciel *et al*, 2003).

4.5 DEVELOPMENT OF PCR ASSAYS FOR SCREENING OF BILE SALT HYDROLASE POSITIVE LACTOBACILLI

Since one of the important genetic markers for selection of probiotic lactobacilli is bile salt hydrolase activity that confers bile tolerance to the organisms, we targeted the 'bsh' gene sequences available in the NCBI gene bank database for developing bsh-PCR assays for short listing of bsh positive lactobacilli in mass probiotic screening programmes. With this objective in mind, a total of seven pairs of primers namely LaBSH1F/951R; LjBSHAF/AR; LjBSHBF/BR; LgBSHF/R; LaBSHF/R; LpBSHF/R and Lp1F/975R based on the available lactobacillus bsh gene sequences were designed using primer3 designing software from the internet and got them custom synthesized. The detailed description of these primers along with the expected amplified products are given in Table **3.3.** The results pertaining to the use of these primers in the development of PCR assays for specific for bsh positive lactobacilli have been presented below.

4.5.1 Evaluation of BSH Primers Against Standard Probiotic Lactobacilli

To begin with, an attempt was made in this investigation to evaluate the suitability of the seven bsh primers designed in this study in their respective PCR assays against three known probiotic lactobacillus cultures namely *Lb. johnsonii La1, Lb. acidophilus LA7 and Lb. acidophilus P*. The PCR cycling conditions used in this study included 35 cycles each of denaturation at 94^{o}C for 30 sec. followed by annealing at 56^{o}C for 1 min and extension at 72^{o}C for 1 min. The PCR results pertaining to the same have been recorded in **Fig. 8.** As it is quite evident from the gel picture given in **Fig. 8**, no amplification was observed when primer pairs La BSH1F/951R;

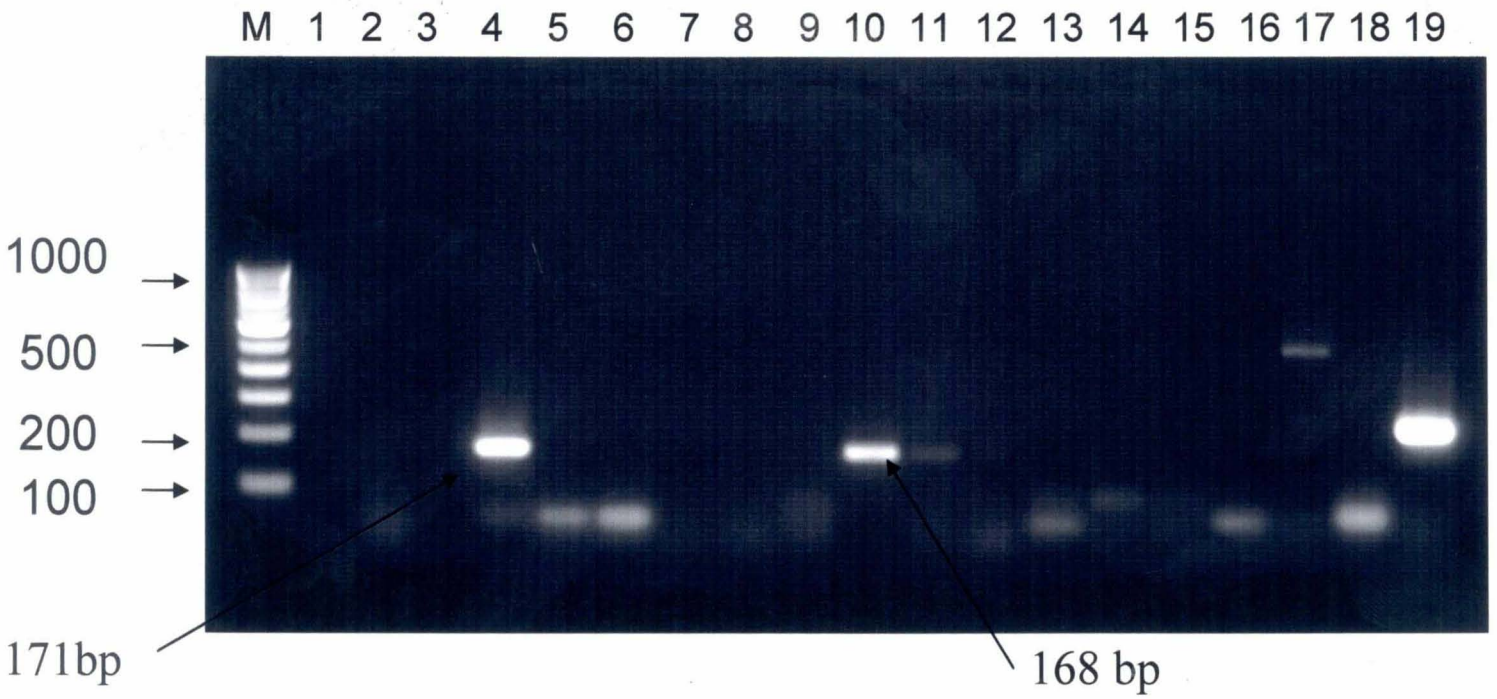

Fig. 8. PCR amplification of Probiotic Lactobacilli using BSH primer pairs

Lanes : M, 100 bp; **1 – 3 (LaBSH1F/951R);** 1, LjLA1; 2, LaLA7; 3, LaP; **4 - 6 (LjBSH553F/723R); 4, LjLA1;** 5, LaLA7; 6, LaP; **7 – 10 (LgBSH129F/349R);** 7, LjLA1; 8, LaLA7; 9 LaP; **10 - 12 (LjBSH567F/734R); 10, LjLA1;** 11, LaLA7; 12, LaP; **13 – 15 (LaBSH392F/588R);** 13, LjLA1; 14, LaLA7; 15, LaP; **16 –18 (LpBSH345F/575R);** 16, LjLA1; 17, LaLA7; 18, LaP; 19, La195

LgBSHF/R; LaBSHF/R and LpBSHF/R were used in their respective PCR assays with any of the template DNA from the three probiotic cultures used in the study, thereby, indicating that these primers were not suitable for the identification of the three selected probiotic strains.

However, the primer pairs LjBSHAF/AR and LjBSHAF/AR gave amplified products of 171 bp **(Lane 4)** and 168 bp **(Lane 10)** with *Lb. johnsonii* La1 only, thereby, suggesting that these primer pairs were specific for *Lb. johnsonii.* However, to confirm their specificity, these primers are required to be tested against some additional strains of targeted and non targeted probiotic lactobacilli to unequivocal prove their suitability for identification of *Lb. johnsonii.* However, the literature pertaining to designing of primers targeted against bsh gene from probiotic lactobacilli and their exploitation in PCR based identification of such organisms draws almost a blank. To the best of our knowledge, there is only a single report by Elkins *et al*, 2001 wherein the primers namely Hβ675a and Hβ675a were specifically designed against cbsHβ locus from *Lb. johnsonii* 100-100 and were used for survey of 50 Lactobacillus isolates. With the help of PCR assays using these primers, ten of the isolates were found positive for cbsHβ. However, nine of these isolates cultured from human sources and one dairy isolate was also found positive for cbsHβ. Contrary to our findings, their primers Hβ675a and Hβ675a could also amplify cbsHβ from *Lb. acidophilus*, *Lb. gasseri* and *Lb. johnsonii*.

4.5.2 Specificity of BSH Primers Against Non-Lactobacillus Cultures

In order to rule out the possibility of false positive results, all the bsh primers in their respective PCR assays were subjected to specificity test against some non targeted bacterial cultures belonging to enterobacteriacae family. The results pertaining to the same have been recorded in **Figs. 9 and 10.** As is quite evident from Fig. 9 that bsh PCR assay using primer pair LjBSHAF/AR did not result into amplification of the expected PCR product i.e. 171 bp band with any of the non-targeted cultures such as *E. coli* 0157:H7, *Enterobacter aerogenes*. *Salmonella*, *Shigella, Klebsiella pneuminae*, *Enterococcus faecalis*, *E. faecium, L. monocytogenes*, Bifidobacteria etc.,

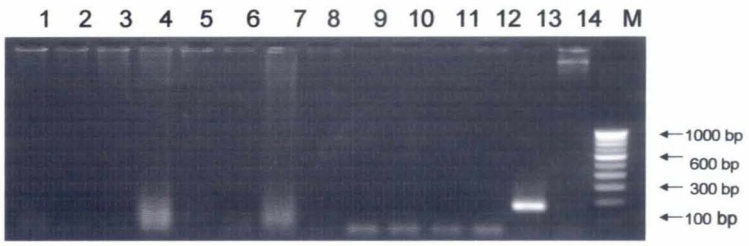

Fig. 9. Specificity of PCR assay against non-lactobacilli with primers LjBSHAF/AR

Lanes: 1, *Enterobacter aerogenes* 106; **2,** *Shigella dysentriae* 107; **3,** *Salmonella typhi* 113; **4,** *Enterococcus faecalis* 114; **5,** *Klebsiella pneumoniae* 138; **6,** *Enterobacter aerogenes* 173; **7,** *Enterococcucs faecium* 211; **8,** *Enterobacter aerogenes* 248; **9,** *Escherichia coli* 0157:H7 ; **10,** *Listeria monocytogenes* ; **11,** *Bifidobacterium bifidum* 203 ; **12,** *Bifidobacterium bifidum* 228 ; **13,** LjLA1; **14,** *Bifidobacterium bifidum* 228 ; **M,**100 bp marker

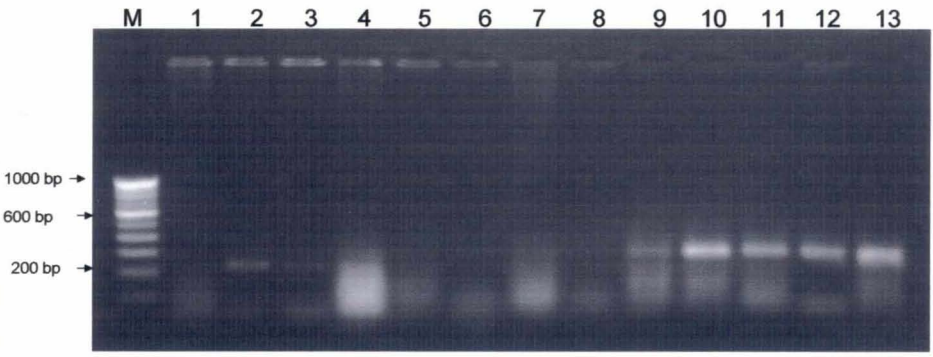

Fig. 10. Specificity of PCR assay against non-lactobacilli with primers LpBSHF/R

Lanes: M, 100 bp, **1,** *Enterobacter aerogenes* 106; **2,** *Shigella dysentriae* 107; **3,** *Salmonella typhi* 113; **4,** *Enterococcus faecalis* 114; **5,** *Klebsiella pneumoniae* 138; **6,** *Enterobacter aerogenes* 173; **7,** *Enterococcucs faecium* 211; **8,** *Enterobacter aerogenes* 248; **9,** *Escherichia coli* 0157:H7 ; **10,** *Listeria monocytogenes* ; **11,** *Bifidobacterium bifidum* 203 ; **12,** *Bifidobacterium bifidum* 228 ; **13,** Lp201

thereby, indicating that this bsh PCR assay was quite specific for the targeted culture only i.e. bsh positive L*b. johnsonii.* Similarly, other bsh PCR assays using the remaining primer pairs except LpBSHF/LpR based on lactobacillus bsh gene were also quite specific (data not shown). The bsh PCR exploring the primer pair LpBSHF/R however gave cross reactivisty with some non-targeted cultures such as *Bifidobacterium bifidum*, *E. coli* 0157:H7 and *L. monocytogenes* etc. **(Fig. 10, Lanes 9-12)** also thereby, suggesting conserved sequences at these regions in the BSH genes sequences of these cultures. Our results in this regard are consistent with the observations of Elkins *et al* (2001) who also could demonstrate positive signal of their bsh PCR assay developed for *Lb. johnsonii* with *Lb. acidophilus* and Lb. gasseri also although they did not check their PCR assays with members of enterobacteriacae family.

4.5.3 Multiplexing of BSH-PCR with genes specific PCR for direct identification of bsh positive lactobacilli

In order to explore the possibility of developing a multiplex PCR for direct screening of the isolates for bsh positive lactobacilli, we made an attempt to combine LjBSHAF/AR and LjBSHBF/BR with that of genus specific primers LbLMA1/pH-1 to evaluate the efficacy of these PCR assays with three standard probiotic cultures used in our study. The results pertaining to the same have been recorded to **Fig. 11.** A critical appraisal of the gel picture presented therein clearly demonstrates the concurrent amplification of 171, 250 bp and 168, 250 bp products respectively with template DNA from *Lb. johnsonii* La1 only **(Lane 13, 16)** indicating that the combination of the two primer pairs was compatible and did not interfere in the amplification of the PCR products specific for genus lactobacillus (250 bp) and bsh (168/171 bp) respectively. However, only 250 bp product **(Lanes 15, 17, 18)** and a non specific products **(Lane 14)** were detected with *Lb. acidophilus* La7 and *Lb. acidophilus* P. These results further substantiate our previous findings that the primer pairs LjBSHAF/AR and LjBSHBF/BR used in the multiplex PCR were specific for *Lb. johnsonii* and hence could be used for identification of bsh

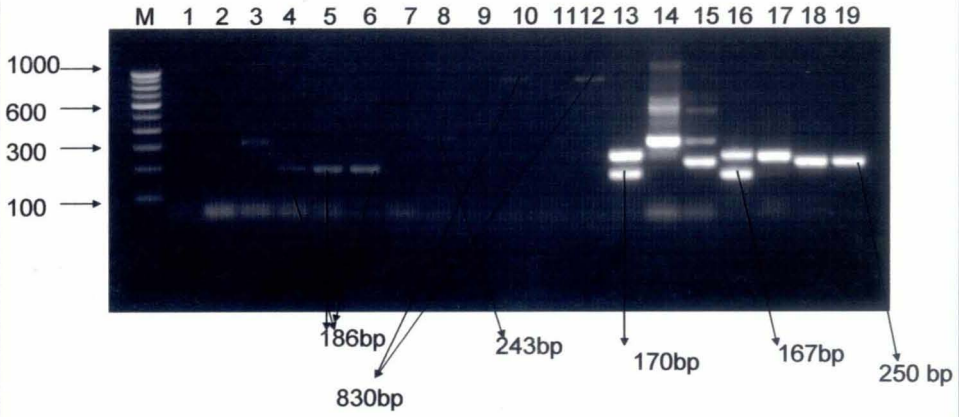

Fig. 11. PCR amplification of Probiotic Lactobacilli using bsh primer pairs targeted against Bifidobacterium and Lactobacillus spp.
Lanes : M, 100 bp; **1 – 3 (BbBSH1F/951R);** 1, LjLa1; 2, LaLA7; 3 LaP; **4 - 6 (BbBSHF/R);** 4, LjLa1; 5, LaLA7; 6, LaP; **7 – 10 (BlBSHF/R);** 7, LjLa1; 8, LaLA7; 9 LaP; **10 - 12 (BbBSHAF/AR);** 10, LjLa1; 11, LaLA7; 12, LaP; **13 – 15 (LjBSHAF/AR+ LbLMA-1/R16-1);** 13, LjLa1; 14, LaLA7; 15, LaP; **16 –18 (LjBSHBF/BR + LbLMA-1/R16-1);** 16, LjLa1; 17, LaLA7; 18, La; 19, La

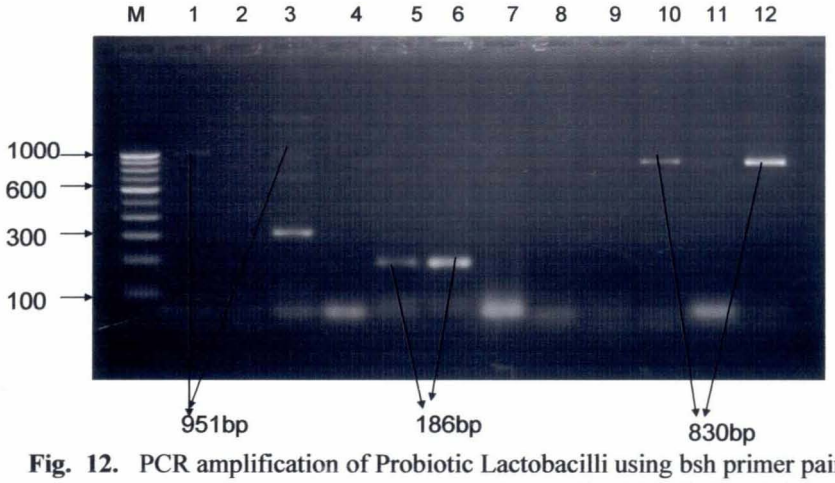

Fig. 12. PCR amplification of Probiotic Lactobacilli using bsh primer pairs targeted against Bifidobacterium along with LbLMA-1/R16-1
Lanes : M, 100 bp; **1 – 3 (BbBSH1F/951R);** 1, LjLa1; 2, LaLA7; 3 LaP; **4 - 6 (BbBSHF/R);** 4, LjLa1; 5, LaLA7; 6, LaP; **7 – 10 (BlBSHF/R);** 7, LjLa1; 8, LaLA7; 9 LaP; **10 - 12 (BbBSHAF/AR);** 10, LjLa1; 11, LaLA7; 12, LaP;

positive *Lb. johnsonii.* The aforesaid multiplex PCR can also find application in direct screening of isolates, libraries, probiotic preparations for bsh positive *Lb. johnsonii*. However, the validity of our results on suitability of bsh targeted primers and their multiplexing with genus specific primers used in this study can not be substantiated. Ours is perhaps the first report wherein the specific primers have been designed and used in conjunction with genus specific primes developing bsh PCR assays for screening of indigenous lactobacillus isolates from different niches. This strategy could be extremely valuable for direct isolation of bsh positive lactobacilli in one reaction since the bsh gene sequences are highly conserved at specific locations and hence the simplex PCR based on these regions may not be able to determine the identify of such isolates at genus level.

During the course of this study, some additional primers namely Bb BSH1F/951R; BbBSHF/R; BIBSHF/R and BbHSHAF/AR targeted against bsh positive gene sequences of bifidobacterial strains from NCBI gene bank available in our lab were also tested in their respective PCR assays against three probiotic lactobacillus strains to check whether the targeted gene sequence were conserved in lactobacilli also. The data concerning the same have been comprehensively presented in **Figs. 11 & 12.** It is quite evident from the gel picture that the primer pair viz. BLBSHF/ R was able to give positive signal with all the three probiotic lactobacillus cultures in the PCR assay used in this study as indicated by the primers of 186 bp band on the gel **(Figs. 11 and 12)**. These results clearly demonstrate that the targeted region for the BSH gene was conserved in both the probiotic genera i.e. lactobacilli and Bifidobacteria and hence the primer pair could be quite effective in the identification of bsh positive isolates belonging to both the genera. Similarly, another bifidobacterial bsh based primer pair BbBSHAF/AR could produce the desired PCR amplified product of expected size i.e. 830 bp with two of the probiotic lactobacillus cultures namely *Lb johnsonii* La1, *Lb. acidophilus* P. Both these cultures were found to be positive and produced 830 bp amplified product on the gel **(Fig. 11, Lane 10 and 12; Fig. 12, Lane 10 and 12)**. However, the third probiotic lactobacillus culture LA7 failed to produce 830 bp

product in the aforesaid PCR assay. The discrepancy could be attributed to either human error or false negative reaction. However, to resolve this issue for good, it is imperative that the PCR assay based on the aforesaid Bifidobacterial bsh primers are evaluated and checked against as many probiotic lactobacilli cultures as possible before applying these PCR assays for simultaneous identification of bsh positive probiotic lactobacilli and Bifidobacteria isolated from their natural niches. However, to further substantiate these findings, more extensive investigations are needed to determine the homology of bsh gene sequences of the two genera so that specific primers could be designed for simultaneous identification of both.

4.6 APPLICATION OF BSH-PCR ASSAYS IN IDENTIFICATION

After standardization of the PCR assays developed with the help of six primer pairs targeted against different regions of lactobacillus bsh gene sequences used in this study, they were applied on all the PCR positive lactobacillus isolates recovered from different sources. The results pertaining to the same in their respective PCR assays using specific primer pair and template from all the lactobacillus isolates have been presented in **Figs. 13 to 16.** A critical appraisal of these results clearly revealed that neither the primer pair LjBSHAF/ AR nor LjBSHBF/BR based on *Lb. johnsonii* could amplify the 171 or 168 bp product in their respective PCR with any of the 35 isolates, thereby, indicating that none of these isolates belongs to probiotic *Lb. johnsonii*. The only positive signal in the PCR was obtained with the template from standard probiotic culture *Lb. johnsonii* La1 as indicated by the appearance of 168 bp band on the gel **(Fig. 13, Lane 19).**

Similarly, when the primer pair namely LgBSHF/R used in the PCR assay was applied on the lactobacillus isolates obtained in this study, the expected 220 bp PCR amplified product could not be detected with any of the isolates suggesting that none of them belongs to bsh positive *Lb. gasseri* **(Fig. 14).** However, a non-specific spurious band (>700bp) was invariably detected with some isolates **(Fig. 14, Lane, 3, 4, 5, 6, 7, & 9),** thereby, suggesting the need for further standardization from the PCR assay for selective amplification of 220 bp product only.

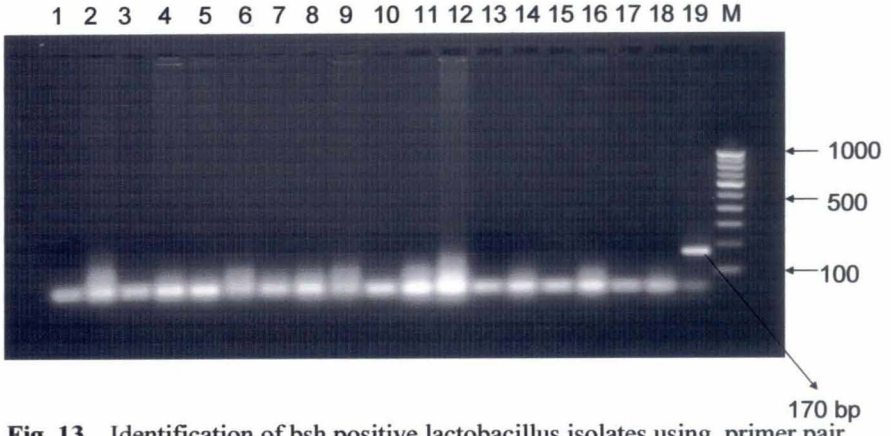

Fig. 13. Identification of bsh positive lactobacillus isolates using primer pair LjBSHAF/AR

Lanes : 1, Lb1; 2, Lb2; 3, Lb3; 4, Lb4; 5, Lb5; 6, Lb6; 7, Lb7; 8, Lb8; ,9, Lb9; 10, Lb10; 11, Lb11; 12, Lb12; 13, Lb13; 14, Lb14; 15, Lb15; 16, Lb16; 17, Lb17; 18, Lb18; 19, LjLA1, M, 100bp

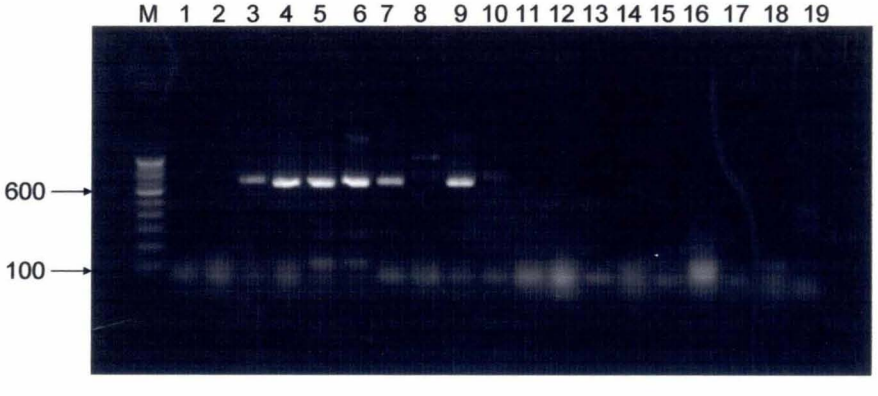

Fig. 14. Identification of bsh positive lactobacillus isolates using primer pair Lg BSHF/R

Lanes : M, 100bp; 1, Lb1; 2, Lb2; 3, Lb3; 4, Lb4; 5, Lb5; 6, Lb6; 7, Lb7; 8, Lb8; 9, Lb9; 10, Lb10; 11, Lb11; 12, Lb12; 13, Lb13; 14, Lb14; 15, Lb15; 16, Lb16; 17, Lb17; 18, Lb18; 19, LjLa1,

In an effort to find the applicability of the remaining two lactobacillus based bsh primers for identification of probiotic *Lb. acidophilus* and *Lb. plantarum* respectively, the PCR assays developed thereof were also subjected to all the lactobacillus isolates. However, the PCR assay developed in conjunction with La BSHF/R (*Lb. acidophilus* specific) failed to produce the expected amplified product of size 196 bp with any of the isolates **(Fig. 15).** This suggests that either the lactobacillus isolates did not belong to *Lb. acidophilus* or the primer sequences were not good enough from selective amplification of the expecting product with the PCR cycling condition used in this study. The sixth primer pair designated as LpBSHF/R specific for bsh of *Lb. plantarum* when used in the PCR assay was able to amplify the 230 bp product with at least ten of the isolates as shown in **Fig. 16 (Lanes 4, 5, 6, 8, 9, 10, 12, 15, 17 and 18)** without any ambiguity and hence all of them were identified as bsh positive *Lb. plantarum.*

From the foregoing results, it can be concluded that the primers targeted against bsh genes at different regions from different lactobacillus cultures behaved differently in their respective PCR assays developed in this study when applied on lactobacillus isolates recovered from different environments. Some of these primer pairs like LjBSHAF/ AR and LjBSHBF/BR were more specific and hence could not identify the Lactobacillus isolates while others such as LpBSHF/R were more conserved and hence were able to identify the isolates at high frequency. On the basis of these results, it can be inferred that the incidence of bsh positive *Lb. plantarum* among the Lactobacillus isolates examined in this study was much higher than other lactobacilli and majority of them came form human origin. Our results in this regard are in agreement with those of Elkins *et al* (2001) who also reported the majority of bsh positive isolates examined in their study on the basis of cbsHβ based PCR assay of human origin.

4.7 EVALUATION OF BSH CLONING PRIMER PAIR LP1F/975R FOR IDENTIFICATION OF BSH POSITIVE LACTOBACILLI

Prompted by the success of our previous *Lb. plantarum* based bsh primers in the PCR assay for bsh positive Lactobacilli, we tested the primer pair Lp1F/975R designed for cloning of bsh gene from *Lb. plantarum* based

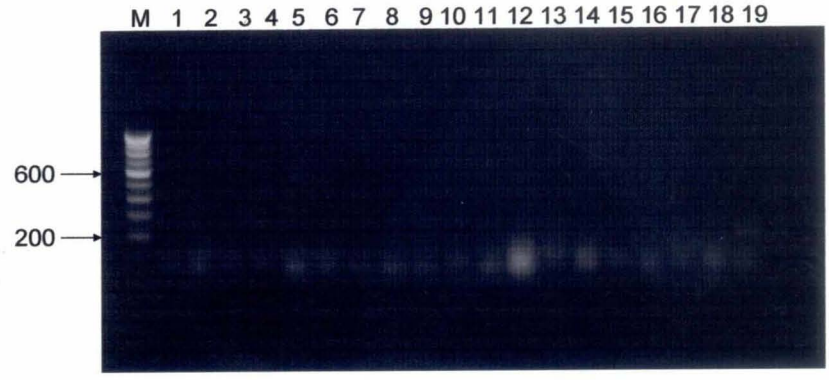

Fig. 15. Identification of bsh positive lactobacillus isolates using primer pair LaBSHF/R

Lanes : M, 100bp; 1, Lb1; 2, Lb2; 3, Lb3; 4, Lb4; 5, Lb5; 6, Lb6; 7, Lb7; 8, Lb8; ,9, Lb9; 10, Lb10; 11, Lb11; 12, Lb12; 13, Lb13; 14, Lb14; 15, Lb15; 16, Lb16; 17, Lb17; 18, Lb18; 19, LaLA7

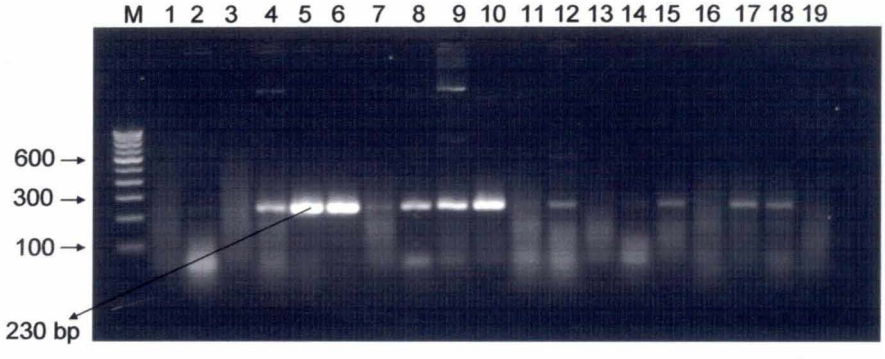

Fig. 16. Identification of bsh positive lactobacillus isolates using primer pair Lp BSHF/R

Lanes : M, 100bp; 1, Lb1; 2, Lb2; 3, Lb3; 4, Lb4; 5, Lb5; 6, Lb6; 7, Lb7; 8, Lb8; ,9, Lb9; 10, Lb10; 11, Lb11; 12, Lb12; 13, Lb13; 14, Lb14; 15, Lb15; 16, Lb16; 17, Lb17; 18, Lb18; 19, LjLa1

on the complete bsh gene sequence of *Lb. plantarum* available in the NCBI database for cloning the full length BSH gene sequence that spans around 975 bp. However, before attempting cloning of the entire bsh nucleotide sequence of the target organism, the aforesaid cloning primer pair was also explored in the study to develop a bsh PCR assay for identification of bsh positive lactobacilli. The bsh PCR assay developed with this particular cloning primer pair resulted into the amplification of 975 bp product representing the entire bsh gene sequence with template DNA from a standard *Lb. plantarum* Lp201 as shown from the gel picture presented in **Fig. 17 (Lane 3)**. The above PCR assay was then applied to all the lactobacillus isolates and the results pertaining to the same have been recorded in **Fig. 18.** As can be evidenced from the gel picture presented therein, the 975 bp amplified product was obtained in the PCR assay with at least seven of the isolates namely Lb5, Lb6, Lb8, Lb9, Lb10, Lb16 and Lb17 which were eventually identified as bsh positive *Lb. plantarum.* Further attempts were then made to develop a multiplex PCR by combining the lactobacillus specific primers LbLAM1/R16-1 in the aforesaid bsh PCR and testing the same against the selected lactobacillus isolates. The data concerning the same has been presented in **Fig. 19.** From the gel pictures presented therein it is quite clear that both the primer pairs used in the multiplex PCR were able to amplify the irrespective PCR products specifically and clearly with at least seven isolates as indicated by the formation of distinct bands of expected sizes 250 bp (Lactobacillus specific) and 975 bp (bsh specific) on the gel **(Fig. 19, Lanes, 5, 7 8, 9, 10, 16 and 17).** However, non-specific spurious and fuzzy bands were also detected with some of the isolates as show in **Fig. 19.** Our results in this regard using the *Lb. plantarum* bsh cloning primer corroborate our previous findings wherein the prevalence of bsh positive *Lb. plantarum* among the isolates from human faecal samples was established.

4.8 Cloning of bsh gene from lactobacillus

The second part of this investigation pertained to the cloning of the complete bsh gene from potential lactobacillus cultures selected on the basis of bsh activity with the objective of sequencing the entire gene for exploring its possible application in the identification of specific bsh psoitive lactobacillus at

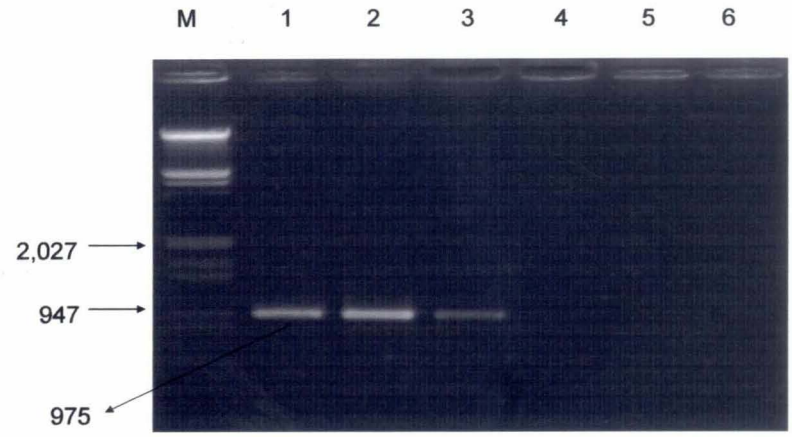

Fig. 17. PCR amplification of lactobacillus isolates using primer pairs Lp1F/975R
Lanes : M, λEcoR1/Hind III; **1,** Lb9; **2,** Lb10; **3,** Lp201; **4,** LjLA1SG1; **5,** LaLA7 **6,** LaP

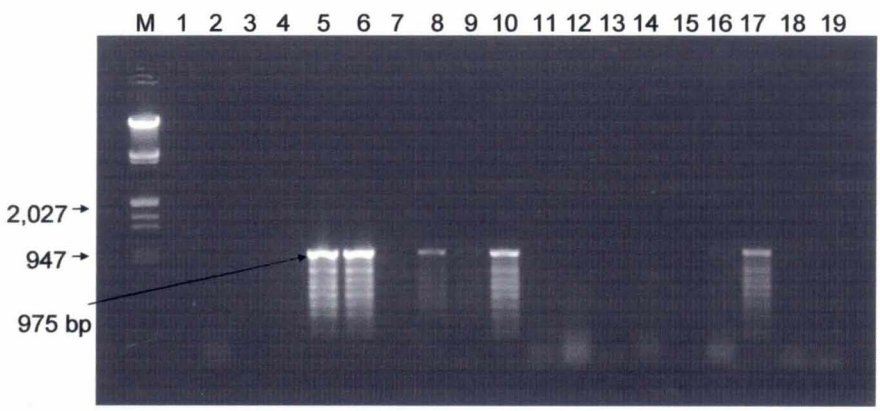

Fig. 18. Identification of BSH positive lactobacillus isolates using primer pair Lp 1F/975R
Lanes : 1, Lb1; 2, Lb2; 3, Lb3; 4, Lb4; 5, Lb5; 6, Lb6; 7, Lb7; 8, Lb8; ,9, Lb9; 10, Lb10; 11, Lb11; 12, Lb12; 13, Lb13; 14, Lb14; 15, Lb15; 16, Lb16; 17, Lb17; 18, Lb18; 19, LjLA1, M, 100bp

species or strain level. To achieve this, the bsh gene from one reference strain of *Lb. plantarum 201* and two of the bsh psoitive lactobacillus isolates was amplified using cloning primer pair Lp BSH 1F/ 975 R. The PCR reaction conducted with the template from these cultures resulted into the amplification of 975 bp product as has been shown in the gel picture presented in **Fig. 17.** The 975 bp PCR product representing the bsh gene in the targeted bsh psoitive lactobacillus cultures was purified using Millipore PCR product purification kit. The purified PCR product was used as an insert and directly ligated into pGEMT -easy vector (Promega). The ligated mix was then introduced into *E. coli.* DH5α by calcium chloride induced transformation that resulted into a sizable number of ampicillin resistant transformants on LB agar plates containing amplicillin (100 µg/ml). Screening of some randomly selected recombinant clones by plasmid minipreps revealed the presence of approx. 4 kb recombinant plasmid on the gel. The presence of insert in the recombinant vector was further ascertained by digestion with EcoRI. EcoRI digestion of the recombinant plasmid isolated from different clones resulted into cleavage of the 975 bp insert. The recombinant clones were also subjected to PCR assay using Lp1F and 975R primer pair which resulted into an amplified product of 975 bp as revealed in **Fig. 20,** thereby, further confirming the genuineness of the cloned product gene.

4.8.1 Nucleotide Sequencing of Cloned BSH Gene

Final confirmation of the identity of the cloned bsh gene in the recombinant plasmids from selected clones selected after ensuring the correct size of the insert in the vector by plasmid minipreps, PCR and digestion with EcoRI was done by determining the complete nucleotide sequence of the insert and its analysis. The nucleotide sequencing was carried out with the minipreps of the recombinant clones using T7 and SP6 sequencing primers for sequencing from both the strands. The sequences were then assembled using SeqMan (DNA star). The complete bsh nucleotide sequence comprising 975 bp in respect of one reference culture i.e. *Lb. plantarum* 201 and two lactobacillus isolates Lb9 and Lb10 have been deciphered in **Figs. 21, 22 and 23.**

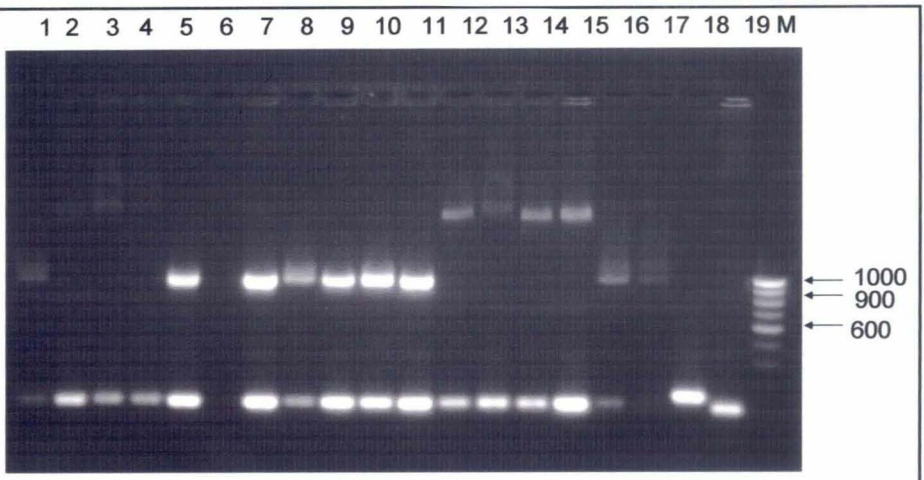

Fig. 19. Multiplex PCR assay using two primers pairs viz:LbLMA1/R16-1 and LP1F/975R

Lane : 1,Lb19; 2, Lb 20; 3, Lb 21;4, Lb 22; 5, Lb 23; 6, Lb 24;7, Lb 25;8, Lb 26; 9, Lb 27; 10, Lb 28; 11, Lb 29; 12, Lb 30; 13, Lb 31; 14, Lb 32;15, Lb 36; 16, Lb 37; 17, Lb 38; 18, Lb 39; 19,+ve 36; M,100 bp marker

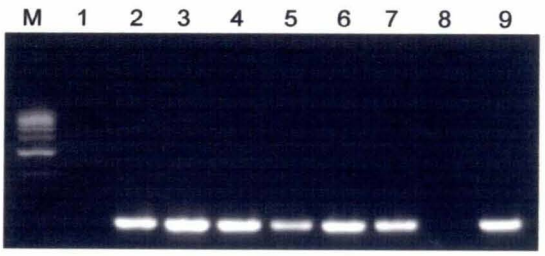

Fig. 20 Identification of BSH positive *E. coli* transformants using primers Lp1F/Lp975R
Lanes : M, λEcoR1/Hind III; **1,** Lb9(1); **2,** Lb9(4); **3,** Lb10(1); **4,** Lb10(2); **5,** Lp201(1) **6,** Lp201(2);

Fig. 21a. Nucleotide sequence of bsh from *L. plantarum* 201

ATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCGGTAGAAAT
TTCGATTATGAAATTTCATACAATGAAATGGTTACGATTACGCCTAGAAAA
TATCCACTAGTATTTCGTAAGGTGGAGAACTTAGATCACCATTATGCAATA
ATTGGAATTACTGCTGATGTAGAAAGCTACCCACTTTACTACGATGCGAT
GAATGAAAAAGGATTGTGTATTGCGGGATTAAATTTTGCAGGTTATGCTG
ATTATAAAAAATATGATGCTGATAAAGTTAATATCACACCATTTGAATTAAT
TCCTTGGCTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGAACA
TACAAAAACTAAACTTGGTTAATATTAATTTTAGTGAACAATTACCATTATC
ACCGCTACATTGGTTGGTTGCTGATAAACAGGAATCGATAGTTATTGAAA
GTGTTAAAGAAGGACTAAAAATTTACGACAATCCAGTAGGTGTGTTAACA
AACAATCCTAATTTTGACTACCAATTATTTAATTTGAACAACTATCGTGCC
TTATCAAATAGCACACCCCAAAATAGTTTTTCGGAAAAaGTGGATTTAGAT
AGTTATAGTAGAGGAATGGGCGGACTAGGATTACCTGGAGACTTGTCCT
CAATGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACTCGTTGCCG
ATGCAGACAGAGAGTGGCAGTGTTAGTCAGTTTTTCCATATACTAGGGTC
TGTAGAACAACAAAAAGGGCTATGTGAAGTTACTGACGGAAAGTACGAAT
ATACAATCTATTCTTCTTGTTGTGATATGGACAAGGGAGTTTATTACTATA
GAACTTATGACAATAGTCAAATTAACAGTGTCAGTTTAAACCATGAGCACT
TGGATACGACTGAATTAATTTCTTATCCATTACGATCAGAAGCACAATACT
ATGCAGTTAACTAA

Fig. 21b. Amino-acid sequence of bsh from *L. plantarum* 201

MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGI
TADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLL
GQFSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKI
YDNPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMG
GLGLPGDLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLC
EVTDGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVSLNHEHLDTTELISYP
LRSEAQYYAVN.

Fig. 22a. Nucleotide sequence of bsh of Lb9

ATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCGGTAGAAAT
TTCGATTATGAAATTTCATACAATGAAATGGTTACGATTACGCCTAGAAAA
TATCCACTAGTATTTCGTAAGGTGGAGAACTTAGATCACCATTATGCAATA
ATTGGAATTACTGCTGATGTAGAAAGCTACCCACTTTACTACGATGCGAT
GAATGAAAAAGGATTGTGTATTGCGGGATTAAATTTTGCAGGTTATGCTG
ATTATAAAAAATATGATGCTGATAAAGTTAATATCACACCATTTGAATTAAT
TCCTTGGTTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGAACA
TACAGAAACTAAACTTGGTTAATATTAATTTTAGTGAACAATTACCATTATC
ACCGCTACATTGGTTGGTTGCTGATAAACAGGAATCGATAGTTATTGAAA
GTGTTAAAGAAGGACTAAAAATTTACGACAATCCAGTAGGTGTGTTAACA
AACAATCCTAATTTTGACTACCAATTATTTAATTTGAACAACTATCGTGCC
TTATCAAATAGCACACCCCAAAATAGTTTTTCGGAAAAaGTGGATTTAGAT
AGTTATAGTAGAGGAATGGGCGGACTAGGATTACCTGGAGACTTGTCCT
CAATGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACTCGTTGCCG
ATGCAGACAGAGAGTGGCAGTGTTAGTCAGTTTTTCCATATACTAGGGTC
TGTAGAACAACAAAAAGGGCTATGTGAAGTTACTGACGGAAAGTACGAAT
ATACAATCTATTCTTCTTGTTGTGATATGGACAAGGGAGTTTATTACTATA
GAACTTATGACAATAGTCAAATTAACAGTGTCAGTTTAAACCATGAGCACT
TGGATACGACTGAATTAATTTCTTATCCATTACGATCAGAAGCACAATACT
ATGCAGTTAACTAA

Fig. 22b. Amino-acid sequence of bsh of Lb9

MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGI
TADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLL
GQFSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKI
YDNPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMG
GLGLPGDLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLC
EVTDGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVSLNHEHLDTTELISYP
LRSEAQYYAVN

Fig. 23a. Nucleotide sequence of bsh of Lb10

ATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCTTTAGAAATT
TCGATTATGAAATTTCATACAATGAAATGGTTACGATTACGCCTAGAAAAT
ATCCACTAGTATTTCGTAAGGTGGAGAACTTAGATCACCATTATGCAATA
ATTGGAATTACTGCTGATGTAGAAAGCTACCCACTTTACTACGATGCGAT
GAATGAAAAAGGATTGTGTATTGCGGGATTAAATTTTGCAGGTTATGCTG
ATTATAAAAAATATGATGCTGATAAAGTTAATATCACACCATTTGAATTAAT
TCCTTGGTTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGAACA
TACAAAAACTAAACtTGGTTAATATTAATTTTAGTGAACAATTACCATTATC
ACCGCTACATTGGTTGGTTGCTGATAAACAGGAATCGATAGTTATTGAAA
GTGTTAAAGAAGGACTAAAAATTTACGACAATCCAGTAGGTGTGTTAACA
AACAATCCTAATTTTGACTACCAATTATTTAATTTGAACAACTATCGTGCC
TTATCAAATAGCACACCCCAAAATAGTTTTTCGGAAAAAGTGGATTTAGAT
AGTTATAGTAGAGGAATGGGCGGACTAGGATTACCTGGAGACTTGTCCT
CAATGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACTCGTTGCCG
ATGCAGACAGAGAGTGGCAGTGTTAGTCAGTTTTTCCATATACTAGGGTC
TGTAGAACAACAAAAAGGGCTATGTGAAGTTACTGACGGAAAGTACGAAT
ATACAATCTATTCTTCTTGTTGTGATATGGACAAGGGAGTTTATTACTATA
GAACTTATGACAATAGTCAAATTAACAGTGTCAGTTTAAACCATGAGCACT
TGGATACGACTGAATTAATTTCTTATCCATTACGATCAGAAGCACAATACT
ATGCAGTTAACTAA

Fig. 23b. Amino-acid sequence of bsh of Lb10

MCTAITYQSYNNYFFRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGI
TADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLL
GQFSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKI
YDNPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMG
GLGLPGDLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLC
EVTDGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVSLNHEHLDTTELISYP
LRSEAQYYAVN.

Our cloning results clearly indicate the 975 bp PCR product cloned in *E. coli* in this study represented the entire ORF of the locus BSHα in *Lb. plantarum* and the two lactobacillus isolates Lb9 and Lb10. To the best of our knowledge, this is the first report wherein BSHα gene from a probiotic *Lb. plantarum* has been cloned and sequenced. However, complete bsh encoded on two separate loci from another probiotic Lactobacillus spp. namely *Lb. johnsonii* 100-100 has already been cloned and expressed in *E. coli* by Elkins *et al* (2001). *Lb. johnsonii* 100-100 produces two distinct bile salt hydrolases α and β that combine to form homo and hetero trimeric complexes. The results of Elkins *et al* (2001) indicated that the three genes cbsT1, cbsT2 and cbsh β constituted an operon in one of the bsh loci in *Lb. johnsonii* 100-100, while the second locus cbshα of *Lb. johnsonii* appears to be located in a different operon. The cbshα of *Lb. johnsonii* 100-100 appears to be comparable to the bshα gene (975 bp) from *Lb. plantarum* 201 which was the subject of our study. The occurrence of two distinct bsh moieties in probiotic lactobacilli is novel since other enteric bacteria are known to encode only one bsh (Coleman and Hudson, 1995; Grill *et al*, 1995; Tanaka *et al,* 2000)

4.8.2 Nucelotide Sequence Analysis of bsh Sequences

The sequences from *Lb. planatrum*, Lb9 and Lb10 were aligned with the sequences of *Lb. plantarum* available in the NCBI data base (Acc. NO. A24002) using ClustalW multiple sequence alignment programme. The data pertaining to the nucleotide sequence alignment has been recorded in **Fig. 24**. As is clearly evident from the sequence alignment data presented therein, all the three aforesaid nucleotides sequences exhibited a very high (99%) degree of homology between each other and with that of *Lb. plantarum* bsh sequence available in the NCBI data base (Acc. No. A24002). However, base alterations were recorded at 8 different locations at position 43, 44, 183, 216, 313, 360, 576 and 893. On the other hand, when amino-acid sequences deduced from the aforesaid bsh nucleotide sequences **(Fig. 25)** were aligned with each other and with that of NCBI sequence of *Lb. plantarum*, there was a difference of only two amino-acids at locations 15 and 288 bases, thereby,

Fig. 24. CLUSTAL W (1.82) multiple nucleotide sequence alignment

```
Lp9         ATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCGGTAGAAATTTCGATTAT 60
Lp10        ATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCTTTAGAAATTTCGATTAT 60
Lp201       ATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCGGTAGAAATTTCGATTAT 60
A24002      ATGTGTACTGCCATAACTTATCAATCTTATAATAATTACTTCGGTAGAAATTTCGATTAT 60
            ************************************************ ***************

Lp9         GAAATTTCATACAATGAAATGGTTACGATTACGCCTAGAAAATATCCACTAGTATTTCGT 120
Lp10        GAAATTTCATACAATGAAATGGTTACGATTACGCCTAGAAAATATCCACTAGTATTTCGT 120
Lp201       GAAATTTCATACAATGAAATGGTTACGATTACGCCTAGAAAATATCCACTAGTATTTCGT 120
A24002      GAAATTTCATACAATGAAATGGTTACGATTACGCCTAGAAAATATCCACTAGTATTTCGT 120
            ************************************************************

Lp9         AAGGTGGAGAACTTAGATCACCATTATGCAATAATTGGAATTACTGCTGATGTAGAAAGC 180
Lp10        AAGGTGGAGAACTTAGATCACCATTATGCAATAATTGGAATTACTGCTGATGTAGAAAGC 180
Lp201       AAGGTGGAGAACTTAGATCACCATTATGCAATAATTGGAATTACTGCTGATGTAGAAAGC 180
A24002      AAGGTGGAGAACTTAGATCACCATTATGCAATAATTGGAATTACTGCTGATGTAGAAAGC 180
            ************************************************************

Lp9         TACCCACTTTACTACGATGCGATGAATGAAAAAGGATTGTGTATTGCGGGATTAAATTTT 240
Lp10        TACCCACTTTACTACGATGCGATGAATGAAAAAGGATTGTGTATTGCGGGATTAAATTTT 240
Lp201       TACCCACTTTACTACGATGCGATGAATGAAAAAGGATTGTGTATTGCGGGATTAAATTTT 240
A24002      TATCCACTTTACTACGATGCGATGAATGAAAAAGGCTTGTGTATTGCGGGATTAAATTTT 240
            ** ********************************** ************************

Lp9         GCAGGTTATGCTGATTATAAAAAATATGATGCTGATAAAGTTAATATCACACCATTTGAA 300
Lp10        GCAGGTTATGCTGATTATAAAAAATATGATGCTGATAAAGTTAATATCACACCATTTGAA 300
Lp201       GCAGGTTATGCTGATTATAAAAAATATGATGCTGATAAAGTTAATATCACACCATTTGAA 300
A24002      GCAGGTTATGCTGATTATAAAAAATATGATGCTGATAAAGTTAATATCACACCATTTGAA 300
            ************************************************************

Lp9         TTAATTCCTTGGTTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGAACATACAG 360
Lp10        TTAATTCCTTGGTTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGAACATACAA 360
Lp201       TTAATTCCTTGGCTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGAACATACAA 360
A24002      TTAATTCCTTGGTTATTGGGACAATTTTCAAGTGTTAGAGAAGTGAAAAAGAACATACAA 360
            ************ ************************************************

Lp9         AAACTAAACTTGGTTAATATTAATTTTAGTGAACAATTACCATTATCACCGCTACATTGG 420
Lp10        AAACTAAACTTGGTTAATATTAATTTTAGTGAACAATTACCATTATCACCGCTACATTGG 420
Lp201       AAACTAAACTTGGTTAATATTAATTTTAGTGAACAATTACCATTATCACCGCTACATTGG 420
A24002      AAACTAAACTTGGTTAATATTAATTTTAGTGAACAATTACCATTATCACCGCTACATTGG 420
            ************************************************************

Lp9         TTGGTTGCTGATAAACAGGAATCGATAGTTATTGAAAGTGTTAAAGAAGGACTAAAAATT 480
Lp10        TTGGTTGCTGATAAACAGGAATCGATAGTTATTGAAAGTGTTAAAGAAGGACTAAAAATT 480
Lp201       TTGGTTGCTGATAAACAGGAATCGATAGTTATTGAAAGTGTTAAAGAAGGACTAAAAATT 480
A24002      TTGGTTGCTGATAAACAGGAATCGATAGTTATTGAAAGTGTTAAAGAAGGACTAAAAATT 480
            ************************************************************

Lp9         TACGACAATCCAGTAGGTGTGTTAACAAACAATCCTAATTTTGACTACCAATTATTTAAT 540
Lp10        TACGACAATCCAGTAGGTGTGTTAACAAACAATCCTAATTTTGACTACCAATTATTTAAT 540
Lp201       TACGACAATCCAGTAGGTGTGTTAACAAACAATCCTAATTTTGACTACCAATTATTTAAT 540
A24002      TACGACAATCCAGTAGGTGTGTTAACAAACAATCCTAATTTTGACTACCAATTATTTAAT 540
            ************************************************************

Lp9         TTGAACAACTATCGTGCCTTATCAAATAGCACACCCCAAAATAGTTTTTCGGAAAAAGTG 600
Lp10        TTGAACAACTATCGTGCCTTATCAAATAGCACACCCCAAAATAGTTTTTCGGAAAAAGTG 600
Lp201       TTGAACAACTATCGTGCCTTATCAAATAGCACACCCCAAAATAGTTTTTCGGAAAAAGTG 600
A24002      TTGAACAACTATCGTGCCTTATCAAATAGCACACCTCAAAATAGTTTTTCGGAAAAAGTG 600
            ************************************ ************************

Lp9         GATTTAGATAGTTATAGTAGAGGAATGGGCGGACTAGGATTACCTGGAGACTTGTCCTCA 660
Lp10        GATTTAGATAGTTATAGTAGAGGAATGGGCGGACTAGGATTACCTGGAGACTTGTCCTCA 660
Lp201       GATTTAGATAGTTATAGTAGAGGAATGGGCGGACTAGGATTACCTGGAGACTTGTCCTCA 660
A24002      GATTTAGATAGTTATAGTAGAGGAATGGGCGGACTAGGATTACCTGGAGACTTGTCCTCA 660
            ************************************************************
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Lp9             ATGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACTCGTTGCCGATGCAGACAGAG 720
Lp10            ATGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACTCGTTGCCGATGCAGACAGAG 720
Lp201           ATGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACTCGTTGCCGATGCAGACAGAG 720
A24002          ATGTCTAGATTTGTCAGAGCCGCTTTTACTAAATTAAACTCGTTGCCGATGCAGACAGAG 720
                ************************************************************

Lp9             AGTGGCAGTGTTAGTCAGTTTTTCCATATACTAGGGTCTGTAGAACAACAAAAAGGGCTA 780
Lp10            AGTGGCAGTGTTAGTCAGTTTTTCCATATACTAGGGTCTGTAGAACAACAAAAAGGGCTA 780
Lp201           AGTGGCAGTGTTAGTCAGTTTTTCCATATACTAGGGTCTGTAGAACAACAAAAAGGGCTA 780
A24002          AGTGGCAGTGTTAGTCAGTTTTTCCATATACTAGGGTCTGTAGAACAACAAAAAGGGCTA 780
                ************************************************************

Lp9             TGTGAAGTTACTGACGGAAAGTACGAATATACAATCTATTCTTCTTGTTGTGATATGGAC 840
Lp10            TGTGAAGTTACTGACGGAAAGTACGAATATACAATCTATTCTTCTTGTTGTGATATGGAC 840
Lp201           TGTGAAGTTACTGACGGAAAGTACGAATATACAATCTATTCTTCTTGTTGTGATATGGAC 840
A24002          TGTGAAGTTACTGACGGAAAGTACGAATATACAATCTATTCTTCTTGTTGTGATATGGAC 840
                ************************************************************

Lp9             AAGGGAGTTTATTACTATAGAACTTATGACAATAGTCAAATTAACAGTGTCAGTTTAAAC 900
Lp10            AAGGGAGTTTATTACTATAGAACTTATGACAATAGTCAAATTAACAGTGTCAGTTTAAAC 900
Lp201           AAGGGAGTTTATTACTATAGAACTTATGACAATAGTCAAATTAACAGTGTCAGTTTAAAC 900
A24002          AAGGGAGTTTATTACTATAGAACTTATGACAATAGTCAAATTAACAGTGTCAATTTAAAC 900
                ************************************************** *******

Lp9             CATGAGCACTTGGATACGACTGAATTAATTTCTTATCCATTACGATCAGAAGCACAATAC 960
Lp10            CATGAGCACTTGGATACGACTGAATTAATTTCTTATCCATTACGATCAGAAGCACAATAC 960
Lp201           CATGAGCACTTGGATACGACTGAATTAATTTCTTATCCATTACGATCAGAAGCACAATAC 960
A24002          CATGAGCACTTGGATACGACTGAATTAATTTCTTATCCATTACGATCAGAAGCACAATAC 960
                ************************************************************

Lp9             TATGCAGTTAACTAA 975
Lp10            TATGCAGTTAACTAA 975
Lp201           TATGCAGTTAACTAA 975
A24002          TATGCAGTTAACTAA 975
                ***************
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Fig. 25. CLUSTAL W (1.82) multiple amino-acid sequence alignment

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Lp9             MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGITADVES 60
Lp10            MCTAITYQSYNNYFFRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGITADVES 60
A24002          MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGITADVES 60
Lp201           MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGITADVES 60
                ************** ********************************************

Lp9             YPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQFSSVREVKKNIQ 120
Lp10            YPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQFSSVREVKKNIQ 120
A24002          YPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQFSSVREVKKNIQ 120
Lp201           YPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQFSSVREVKKNIQ 120
                ************************************************************

Lp9             KLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYDNPVGVLTNNPNFDYQLFN 180
Lp10            KLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYDNPVGVLTNNPNFDYQLFN 180
A24002          KLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYDNPVGVLTNNPNFDYQLFN 180
Lp201           KLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYDNPVGVLTNNPNFDYQLFN 180
                ************************************************************

Lp9             LNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPGDLSSMSRFVRAAFTKLNSLPMQTE 240
Lp10            LNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPGDLSSMSRFVRAAFTKLNSLPMQTE 240
A24002          LNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPGDLSSMSRFVRAAFTKLNSLPMQTE 240
Lp201           LNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPGDLSSMSRFVRAAFTKLNSLPMQTE 240
                ************************************************************

Lp9             SGSVSQFFHILGSVEQQKGLCEVTDGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVSLN 300
Lp10            SGSVSQFFHILGSVEQQKGLCEVTDGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVSLN 300
A24002          SGSVSQFFHILGSVEQQKGLCEVTDGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVNLN 300
Lp201           SGSVSQFFHILGSVEQQKGLCEVTDGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVSLN 300
                ************************************************************

Lp9             HEHLDTTELISYPLRSEAQYYAVN 324
Lp10            HEHLDTTELISYPLRSEAQYYAVN 324
A24002          HEHLDTTELISYPLRSEAQYYAVN 324
Lp201           HEHLDTTELISYPLRSEAQYYAVN 324
                ************************
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indicating very high level of sequence homology at protein level. Elkins *et al* (2001) also observed high level of similarity between cbsT1 and cbsT2 at nucleotide and predicted amino-acid sequence level in *Lb. johnsonii* 100-100. Similarly, based on their sequence alignment studies, these workers further concluded that bsh operon identical in genomic architecture in this strain was highly conserved in *Lb. acidophilus* KS13 which further supported our own results on these lines.

5.0 SUMMARY AND CONCLUSIONS

The salient findings of this investigation are given below.

1. A total of 25 human faecal samples including infant faecal samples, 5 human milk samples, 5 buffalo milk samples and two probiotic preparations were used for the isolation of Lactobacilli.

2. A total of 100 isolates based on Gram and spore staining and catalase negative reaction were used in the study.

3. Out of a total of 100 isolates, 35 along with three standard probiotic cultures namely *Lb. johnsonii La1, Lb. acidophilus LA7 and Lb. acidophilus P* showed the positive signal in the form of a distinct 250 bp band when subjected to genus specific PCR assay using LbLMA1/R16-1.

4. On the basis of sugar fermentation profiles of few selected isolates, 10 of the isolates namely Lb1, Lb3, Lb4,Lb5, Lb6,Lb8, Lb10, Lb14, Lb16 and Lb18 were tentatively identified as *Lb. plantarum*, one as *Lb. casei* (Lb2), two as *Lb. fermentum* (Lb7, Lb13) and one as *Lb. acidophilus* (Lb12).

5. All the 35 PCR positive Lactobacillus isolates that emerged from this study were subjected to a battery of standard tests recommended for determining their probiotic attributes as per WHO guidelines.

6. Some of the isolates namely Lb10, Lb12 and Lb26 were able to survive even at pH 1.0 for 1-3 hrs as indicated by relatively high OD values of 0.3009, 0.3316 and 0.2963 respectively.

7. The maximum bile tolerance at 1% level (OD values 0.5287 and 0.5130) was recorded with Lb27 and Lb26 respectively after 3 hrs of treatment.

8. The most bile sensitive isolate in this study was found to be Lb7 which could produce an OD_{600} value of only 0.0541.

9. Maximum hydrophobicity (68.4%) towards hexadecane was recorded with Lb19 followed by Lb27 (63.0%).

10. Almost all the cultures were sensitive to both erythromycin and tetracycline whereas streptomycin and gentamycin were weakly to moderately effective against these cultures.

11. Majority of the isolates except Lb2, Lb3 and Lb5 were resistant to vancomycin.

12. Almost all the selected Lactobacillus isolates examined in this study except one (Lb29) exhibited a moderate antibacterial activity against *L. monocytogenes*.

13. Only four of the cultures namely Lb26, Lb27, Lb29 and LaP were able to show weak inhibitory activity against *E. coli* 0157: H7.

14. The maximum antibacterial activity against *L. monocytogenes* was recorded with Lb10 as revealed by big inhibition zone (28 mm) in the cut well assay.

15. A total of seven pairs of primers namely LaBSH1F/951R; LjBSHAF/AR; LjBSHBF/BR; LgBSHF/R; LaBSHF/R; LpBSHF/R and Lp1F/975R based on the available lactobacillus bsh gene sequences were designed and used in this study for screening of bsh positive Lactobacillus isolates.

16. Initially, all the bsh primers were tested against three known probiotic lactobacillus cultures namely *Lb. johnsonii La1, Lb. acidophilus LA7 and Lb. acidophilus P*.

17. No amplification was observed when primer pairs LaBSH1F/951R; LgBSHF/R; LaBSHF/R and LpBSHF/R were used in their respective PCR assays with any of the template DNA from the three probiotic cultures.

18. The primer pairs LjBSHAF/AR and LjBSHBF/BR gave amplified products of 171 bp and 168 bp with *Lb. johnsonii* La1 only, thereby, suggesting that these primer pairs were specific for *Lb. johnsonii.*

19. LjBSHAF/AR, LjBSHBF/BR, LgBSHF/R and LaBSHF/R did not result into amplification of the expected PCR products with any of the non-targeted cultures such as *E. coli* 0157:H7, *Enterobacter aerogenes*. *Salmonella*, *Shigella, Klebsiella pneuminae*, *Enterococcus faecalis*, *E. faecium, L. monocytogenes*, Bifidobacteria etc.

20. The bsh PCR exploring the primer pair LpBSHF/R however gave cross reactivity with some non-targeted cultures such as *Bifidobacterium bifidum*, *E. coli* 0157:H7 and *L. monocytogenes* etc.

21. Multiplexing of LjBSHAF/AR and LjBSHBF/BR with that of genus specific primers LbLMA1/pH-1 resulted into concurrent amplification of 171, 250 bp and 168, 250 bp products respectively with template DNA from *Lb. johnsonii* La1 only.

22. Out of four additional primers namely BbBSH1F/951R; BbBSHF/R; BIBSHF/R and BbHSHAF/AR designed for Bifidobacteria, primer pairs BLBSHF/ R and BbBSHAF/AR also cross reacted with probiotic lactobacilli.

23. Ten of the 35 isolates were found to be bsh positive when screened with all the sets of primers.

24. Majority of the bsh positive isolates belonged to *Lb. plantarum* of human origin.

25. The entire PCR amplified 975 bp product representing the ORF of *Lb. plantarum 201* and two of the bsh positive lactobacillus isolates Lb9 and Lb10 could be cloned in *E. coli.*

26. The nucleotide sequence of bsh positive clones was determined and analysed for nucleotide and amino-acid identity.

27. High degree of homology (99%) was observed between the test cultures and the sequence from the database both at nucleotide and amino-acid level.

CONCLUSION

From the foregoing results, it can be concluded that bsh based PCR assays developed in this study could be very useful not only in Lactobacillus identification at species or strain level but also can provide a relatively simple, quick and realistic solution for mass *in vitro* screening of bsh positive lactobacilli of human origin. The short listed cultures based on this screening strategy could result into a repository of our own indigenous promising Lactobacillus cultures with strong probiotic attributes after establishing their specific health promoting functions by *in vivo* studies. Hence, the application of these molecular tools after appropriate validation could be extremely handy in pursuit of our search for novel and unique indigenous probiotic lactobacilli with additional health promoting functional attributes.

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