The interaction of *Lactobacillus* strains with intestinal epithelial cell lines

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DECLARATION

Statement under O. Ph.D. 8/ (iii) of The Maharaja Sayajirao University of Baroda, Vadodara, India.

The work presented in this thesis has been carried out by me, under the guidance of **Prof. Tamishraha Bagchi**, Department of Microbiology and Biotechnology Centre, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from research studies undertaken by me.

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Date:

This is to certify that the above declaration is true.

……

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(Research Guide)

Place: Vadodara

Date:

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Dedicate to my dear parents and all loved ones .. ..

"Though my soul may set in darkness, it will rise in perfect light; I have loved the stars too fondly, to be fearful of the night."

- Sarah Williams

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"Gratitude unlocks the fullness of life. It turns what we have into enough, and more. It turns denial into acceptance, chaos to order, confusion to clarity. It can turn a meal into a feast, a house into a home, a stranger into a friend. Gratitude makes sense of our past, brings peace for today, and creates a vision for tomorrow."

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May the Almighty God richly bless all of you.

- Akhilesh S. Dhanani

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APS	Ammonium per sulphate
ATCC	American Type Culture Collection
bp	Base pair
BS^3	Bis[sulfosuccinimidyl] suberate
BSA	Bovine serum albumin
BSH	Bile salt hydrolases
cDNA	Complementary DNA
cfu	Colony forming unit
CnBP	Collagen-binding protein
DC	Dendritic cells
DDBJ	DNA Data Bank of Japan
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTPs	2'-deoxynucleotide-5'triphosphates
DTT	Dithiothreitol
DW	Distilled water
EDTA	Ethylene-diamine tetra acetic acid
EF-Tu	Elongation factor-thermo unstable
ELISA	Enzyme-linked immunosorbent assay
ENA	European Nucleotide Archive

EPS	Exopolysaccharides
FBS	Fetal bovine serum
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GIT	Gastrointestinal tract
HRP	Horse radish peroxidase
IBD	Inflammatory bowel diseases
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilo base pair
kDa	Kilo Dalton
LGG	*Lactobacillus rhamnosus* GG
LPS	Lipopolysaccharide
LPxTG	Leu-Pro-any-Thr-Gly
LRM	Low range DNA molecular weight ruler
LTA	Lipoteichoic acid
M	Molar
MAMPs	Microbes-associated molecular patterns
MapA	Mucus adhesion promoting protein
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex

M-MuLV	Moloney murine leukemia virus
mRNA	messenger RNA
MRS	de Man Rogosa and Sharpe
Mub	Mucus-binding protein
NAG	N-acetylglucosamine
NAM	N-acetlymuramic acid
NF-κB	Nuclear factor κB
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid
RNAsin	RNase inhibitor
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
Taq	*Thermus aquaticus*
TBE	Tris-Borate EDTA
TE	Tris EDTA

TEMED	NNN'N' Tetramethyl ethylenediamine
Temp	Temperature
Th	T-helper cells
TLRs	Toll-like receptors
Tm	Melting temperature
TNF	Tumor necrosis factor
Treg	Regulatory T cell
Tris	Tri(hydroxymethyl) amino methane hydrochloride
UV	Ultraviolet
WTA	Wall teichoic acid
X-gal	5-bromo-4-chloro-3-indoly-β-D-galactopyranosid

Note: Standard units (SI) of measurements and chemical formulae abbreviations are not included in the list above.

CHAPTER 1

REVIEW OF LITERATURE

"The most beautiful experience we can have is the mysterious - the fundamental emotion which stands at the cradle of true art and true science."

- Albert Einstein

Chapter 1

Review of literature

1.1. Introduction

Human beings and other higher life forms would have never arisen and could not have sustained till now without the microorganisms. The co-existence of microbes with higher forms of life and symbiotic relationship for several host related functions show the necessity of microbes. Over millions of years, the diversity and functions of microorganisms has continued to change so as to adapt the new habitats. Microorganisms, especially bacteria, exist in a wide variety of environmental niche and are found as complex communities rather than single cellular planktonic cells. The population present in each community is continually modulated in order to adapt and survive. The identification and culturing of all bacterial species in a given niche is not yet possible, but advancements in the field of biotechnology and culture independent techniques have enabled researchers to monitor the evolutionary divergence that can be used to identify and classify the bacteria.

Human epithelial surfaces are inhabited by a variety of bacteria throughout the life of the host. Bacterial presence in the respiratory tract, vaginal epithelium and gastrointestinal epithelium serves various functions. The gastrointestinal tract (GIT) is sterile at birth which is soon colonized by anaerobes and facultative anaerobes from oral inoculations of maternal milk. The intestinal microflora is subsequently established and after two years the faecal microflora is completely constituted (MacFarlane and McBain, 1999). The analysis of prokaryotic ribosomal gene analysis from human colon and feces suggests that approximately 80% of the population is among the uncultivable species and novel microorganisms. Most of the identified organisms are either *Firmicutes* or *Bacteroidetes* (Eckburg *et al.*, 2005). Although there is heterogeneity between subjects, the human intestinal microflora performs similar functions in each person. The benefits provided to the host include nutritional contributions, protection against infections, development and maturation of the immune system and mucosa (Nataro, 2005). The B-group vitamins necessary for

normal homeostasis and vitamin K required for proper blood coagulation are both produced by the resident intestinal microflora (Hill, 1997). Other products such as short chain fatty acids produced by intestinal microflora also provides additional energy source and supports the growth of intestinal epithelial cells (Simon and Gorbach, 1984). The high numbers of commensal bacteria in the intestine antagonize the activity of pathogens by various mechanisms such as competition for nutritional substances and common attachment sites, creating restrictive physiological barriers, besides production of antimicrobial substances (Fons *et al.*, 2000). Intestinal bacteria are also required for development of gut-associated lymphoid tissues (GALT), which perform a variety of host immune functions, such as mucosal immunity and oral tolerance. The gut commensal bacteria play an important role in the development of preimmune antibody (Ab) repertoire by promoting somatic diversification of Ig genes in B cells that have migrated to GALT (Rhee *et al.*, 2004). Finally, intestinal epithelial layer permeability and major part of epithelial cell function are shaped by modulating expression of associated genes upon the exposure of bacteria in the early stages of life and weaning (Hooper *et al.*, 2001). It is well established that the human immune system and GIT requires the presence of diverse and high numbers of microorganisms for proper maturation and function throughout the life of the host.

The commensal microflora populates and maintains homeostasis in the human GIT and any alteration in the number and diversity of microflora have a direct impact on the health of the host. For example, when antibiotic treatment decreases the levels of commensal, the pathogens get a chance to grow and can cause infection. The prospect that human health can be positively influenced by modulating and maintaining the commensal flora is an exciting possibility. The practice of replacing the commensal flora with beneficial microbes gave birth to the current field of probiotics. Probiotics are defined as 'Live microorganisms that when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). Probiotics have been found to be useful in maintaining the normal health and also prevention and treatment of diseases. Many of the probiotic bacteria are belong to Lactic acid bacteria (LAB), a family of microorganisms which ferments various substrates primarily into lactic acid. The majority of LAB are Gram positive, anaerobic or facultative anaerobic, non-sporulating and acid tolerant. *Lactobacillus* is an important genus among LAB family and many *Lactobacillus* strains are widely used as probiotic bacteria.

1.2. History of probiotics

The word probiotic comes from the Greek 'pro bios' which means 'for life'. The origin of fermented milk or dairy products dates back to the dawn of civilization. The use of these products in human diet is also mentioned in ancient books like Bible and the sacred books of Hinduism. The difference in milk preparation and climatic conditions lead to the development of many traditional soured milk or cultured dairy products such as kefir, koumiss, leben, clabber, amasi, lassi, yogurt and dahi. Many of these products were often used therapeutically before the existence of bacteria was recognized (Shortt, 1999).

At the beginning of the twentieth century, the main function of gut microbiota was completely unknown. Elie Metchnikoff, the Nobel Prize winner for the discovery of phagocytosis and director of Pasteur Institute, Paris, made a land-mark observation that the bacteria in fermented milk products have beneficial effects on human. In his book *The prolongation of life*, published in 1907, he postulated the link of human health and longevity to the ingestion of bacteria present in yogurt (Metchnikoff, 1907). With his interest in aging process, he stated the large bowel as a source of toxic substances (for example ammonia, amines) that damage the nervous and vascular system, when they are absorbed from the gut and circulated in blood. These toxic substances are produced from the digestion of proteins by “putrefactive” bacteria in large bowel and were thus responsible for “autointoxication” (Metchnikoff and Chalmers Mitchell, 1910). Metchnikoff proposed that the human can be benefited by encouraging the correct balance of microbial type and reducing the bacteria with putrefactive activity in large bowel. He attributed the enhanced health and longevity of Bulgarian peasant to their regular intake of yogurt containing *Lactobacillus* species.

Henry Tissier, a French paediatrician who was working independently observed that the children with diarrhea had a low number of peculiar, Y shaped bacteria in their stool compared to the healthy children. Later on it was characterized as Bifidobacteria. His observation also played a key role in establishing the concept that specific bacteria take part in maintaining health. In 1906, Tissier showed the benefits of modulating gut flora in infants with intestinal infection (Tissier, 1906). At this time, people were still sceptical about the use of bacterial therapy and also raised

question regarding the survival of yogurt bacteria in intestinal transit, reaching gut and conveying benefits to the host (Kulp and Rettger, 1924). In the early 1920s, *L. acidophilus* milk was demonstrated with therapeutic effects (Cheplin and Rettger, 1922). It is thereafter believed that since colonization and proliferation of bacteria in the gut is essential for their efficacy, any strain originating from the intestine would have better adaptability. During the world war in 1917, German Professor Alfred Nissle isolated a strain of *Escherichia coli* from the feces of a soldier who was not affected by the disease in the outbreak of shigellosis. There were no remedies to treat the diseases as antibiotics were also not discovered yet. He used the *E. coli* Nissle 1917 strain to treat patients with acute gastrointestinal infectious salmonellosis and shigellosis (Nissle, 1918). In Japan during early 1930s, Shirota focused his research on selecting bacterial strains originated from intestine which could survive passage through the gut and used such strain to prepare fermented milk for distribution in his clinic. The Yakult Honsha Company was established with his first product containing *L. acidophilus* Shirota, subsequently named as *L. casei* Shirota (Yakult Honsha Co. Ltd, 1998). In 1965, Lilly and Stillwell first time used the term 'Probiotics' in a different context to represent, substances secreted by one organisms which stimulates the growth of another. Parker (1974) defined probiotics as "organisms and substances which contribute to intestinal microbial balance". Fuller (1989) described probiotics as 'live microbial supplements which beneficially affects the host animal by improving its microbial balance. Recently, Salminen *et al.*, (1998) defined probiotics as 'foods containing live bacteria which are beneficial to health'.

Only at the end of the century, it became clear that intestinal microflora have several health beneficial functions, including metabolic, trophic and protective ones (Guarner and Malagelada, 2003). Metabolic functions are primarily characterized by production of vitamins, fermentation of non-digestible food residues, energy saving with the provision of short chain fatty acids, and absorption of ions. Trophic functions include the control of intestinal epithelial cell proliferation and differentiation, and development and homeostasis of the immune system. Finally, protective functions are related to antagonistic effect on pathogens to prevent infections (Del Piano *et al.*, 2006). *Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus* are mostly used as yogurt starter culture in dairy industry but it has limited health benefits and they are not natural inhabitant of the intestine. Therefore, for yogurt to be considered as

probiotic, the probiotic strains such as *L. acidophilus*, *L. casei* and *Bifidobacterium* are incorporated in preparation as dietary adjunct which has documented health benefits (Shah, 2007).

1.3. Criteria for probiotics

After a long history of safe use in fermented dairy products and increasing reports of positive impacts on human health, there is an increasing interest in and demand for functional foods containing probiotics,. In particular, strains belonging to *Bifidobacterium* and *Lactobacillus*, the predominant and subdominant groups of intestinal microbiota, respectively, are the most widely used probiotic bacteria and oftenly incorporated in many functional foods and dietary supplements. Other groups such as yeast *Saccharomyces boulardii* and Gram negative *E. coli* Nissle 1917 have also shown health effects. For probiotics to be successful, they must possess some characteristics as listed below.

Criteria	**Properties**
Safety	▪ Origin ▪ Non-pathogenic and Non-infectious ▪ Virulence factors- toxicity, metabolic activity and intrinsic properties mainly antibiotic resistance
Technological	▪ Genetically stable strains ▪ Desirable viability during processing and storage ▪ Phage resistance ▪ Ease of large scale production
Functional	▪ Tolerant to gastric acids and juice ▪ Resistant to bile acids ▪ Ability to colonize mucosal surfaces ▪ Validated and documented health beneficial effects
Desirable physiological properties	▪ Immunomodulation ▪ Secrete antimicrobial substance and inhibit colonization of pathogens

	▪ Lactose metabolism ▪ Reduce cholesterol level ▪ Antimutagenic and anticarcinogenic

The growing evidence-based clinical studies data clearly indicates the efficacy of probiotics in treatment of many diseases. The health promoting effect of lactobacilli have been widely explored and include stabilization of indigenous microbial population, protection against intestinal infection, alleviation of lactose intolerance, increased nutritional value of foods, reduction of serum cholesterol levels and non-specific enhancement of the immune systems (Hooper *et al.*, 1999; Perdigon *et al.*, 2002; Sullivan and Nord, 2005; Kim *et al.*, 2008). Although there is several evidence of health benefits linked to probiotics, the attributes among the probiotic strains cannot be generalized as the strains belong to same species could have varied health benefits. The probiotic attributes have been considered to be strain specific. The important steps involved in establishing a bacterial strain as a novel probiotic are given in Figure 1.1.

1.4. *Lactobacillus* strains

Lactobacillus was first isolated by Moro in 1990 and he named the strain as *Bacillus acidophilus* which is the generic name for *Lactobacillus acidophilus*. In general, lactobacilli are characterized as Gram positive, non-spore forming, non-flagellated rods or coccobacilli (Hammes and Vogel, 1995). They are either anaerobes or facultative anaerobes and strictly fermentative. Glucose is converted predominantly to lactic acid by homofermentative bacteria or equimolar amount of lactic acid, CO_2 and ethanol (and/or acetic acid) by heterofermentative counterpart (Gomes and Malcata, 1999).

Lactobacilli are present throughout in vagina and gastrointestinal tract and constitute an important part of the indigenous gut microbiota of human and higher animals. The environmental factors such as oxygen availability, pH, presence of specific substrates and bacterial interactions majorly affect their distribution in a given niche. They are rarely associated with any gastrointestinal or extraintestinal infections. The fermented dairy products are mainly dependant on use of lactobacilli at different stages of production. The strains used technologically are regarded as non-pathogenic and safe (Salminen *et al.*, 1996).

1.5. Source, isolation and identification of *Lactobacillus* strains

1.5.1. Source

The probiotic strains can be isolated from various sources like dairy related products, traditional fermented preparations, fruits, human and animal gut and/or breast milk. Spontaneous milk fermentation has a long history in Mongolia and use of beneficial bacteria in fermented milk product has been practiced for many generations. A study with 189 samples of traditional fermented milk products from over 13 regions of Mongolia showed the complex composition of lactic acid bacteria including *Lactobacillus* species (Yu *et al.*, 2011). Similarly in China, the analysis of 143 LAB isolated from Kurut-the traditional naturally fermented yak milk also showed the presence of diverse bacterial population comprising five genera and thirteen different species and subspecies. Moreover, *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* were the predominant bacteria in these samples (Sun *et al.*, 2010). The analysis of bacterial community in *tungrymbai*, a fermented soybean food, prepared using indigenous technology and consumed by the ethnic Khasi tribes of India indicated the presence of a strain belonging to *Lactobacillus* spp. along with other identified genera. The isolated *Lactobacillus* strain was found to be a probiotic candidate with desirable bile and acid tolerance, antimicrobial activity against both Gram-positive and Gram-negative bacteria. Additionally, the strain was also sensitive to most antibiotics tested which reduce the chance of antibiotic resistance gene transfer to pathogenic microbes (Thokchom and Joshi, 2012). These studies indicate the complex microbial community in traditionally fermented foods and thus provide an important source for selection of probiotic bacteria and starter culture for industrialization and production of traditional fermented products. The potential probiotic bacteria have been isolated from various other fermented products such as kefir grains, Masai milk and Koumiss (Lopitz-Otsoa *et al.*, 2006; Patrignani *et al.*, 2006; Ya *et al.*, 2008). And most of the microorganisms isolated from these fermented foods belong to *Lactobacillus* genus. Recently, isolation and characterization of bacteria from Nigerian fermented foods interestingly led to *Weisella* strain with potential probiotic properties (Ayeni *et al.*, 2011). But the use of such strains needs to be validated completely for possible virulence factors and non-infectious nature.

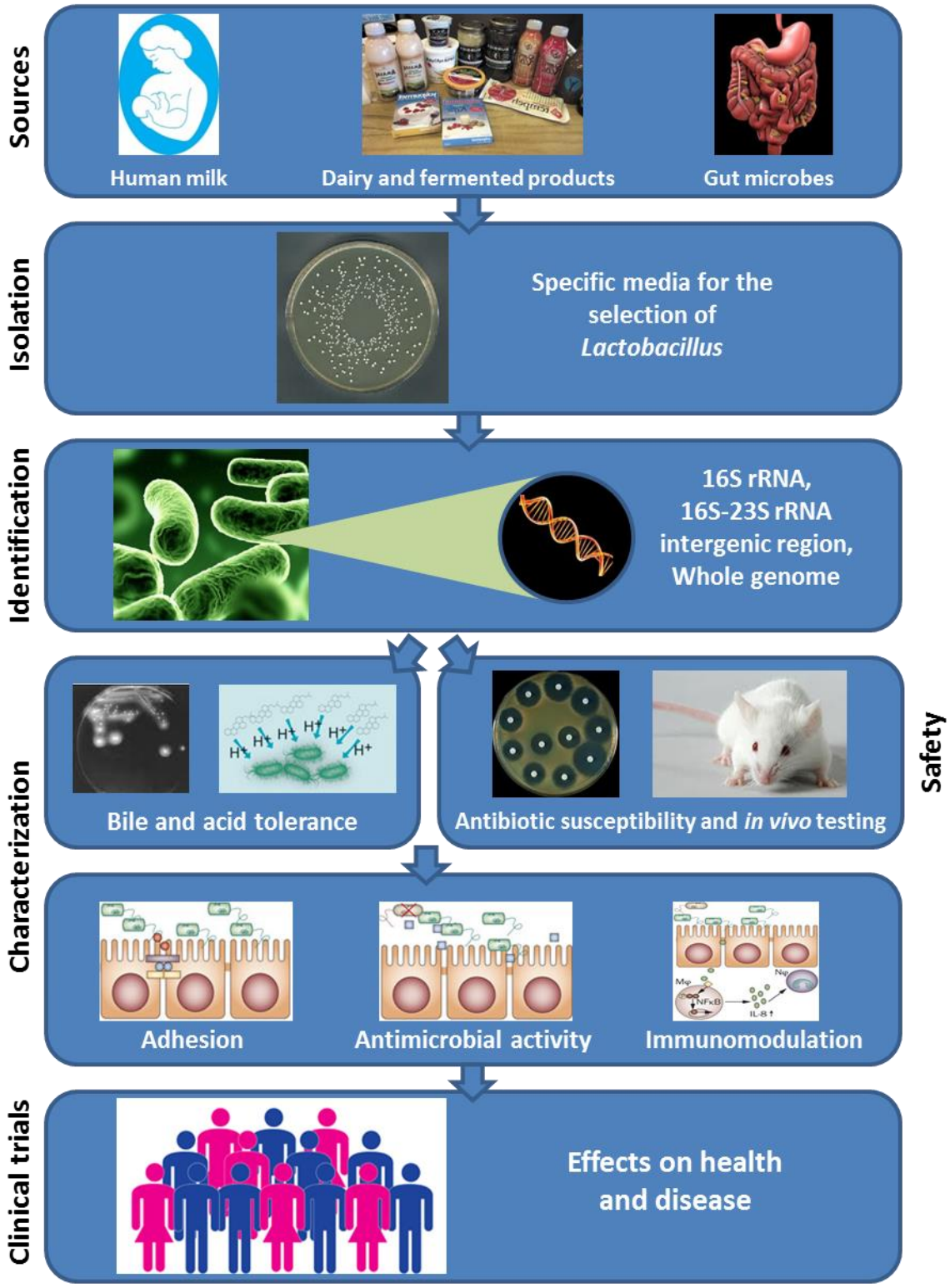


Figure 1.1. Flow chart indicating the various steps in order to isolate and characterize a novel probiotic strain.

The bacteria present in breast milk were long time considered to be skin contaminant although reports suggested the existence of genotypically different isolates in human

milk and skin (O'hara and Shanahan, 2006; Martin *et al.*, 2009). Later on, the aseptic collection also showed the presence of bifidobacteria and lactobacilli in breast milk, and isolates from breast milk and faeces of corresponding infants showed similarities (Martin *et al.*, 2003). Based on these evidences, it was recently become accepted that the human milk constitutes an important natural source for selection of probiotic LAB and bifidobacteria aimed to use in infant formulas (Arboleya et al., 2012). It is also reported that the breast-fed infants have fewer allergies and gastrointestinal infections than the formula-fed infants (Kalliomaki *et al.*, 2001). Thus, the use of strain isolated from breast milk in infant formula would be desirable as it increases the similarities between breast milk and infant formulas. Human breast milk comprises various bacteria belong to staphylococci, streptococci, micrococci, lactobacilli, enterococci, lactococci and bifidobacteria, and its regular intake favours dominance of bifidobacteria and lactobacilli in infant gut microbiota (Martin *et al.*, 2004; O'hara and Shanahan, 2006; Martin *et al.*, 2009; Solis *et al.*, 2010). In addition, four lactobacilli isolated from human breast milk displayed antimicrobial activity against pathogenic bacteria (Olivares *et al.*, 2006).

Apart from above mentioned sources, human GIT is considered to be a most promising source of isolation as more than 500 different bacterial species resides in human gut. Also there is an increased chance of obtaining probiotic bacteria with high bile and acid tolerance when it is isolated from human gut as they are already exposed to detrimental effects in human body before colonizing gut. In fact, many strains of lactobacilli currently used as probiotic are of human origin, such as *L. rhamnosus* GG, *L. plantarum* 299v, *L. gasseri* LA39, and *L. reuteri* (Kawai *et al.*, 2001; Doron *et al.*, 2005; Goossens *et al.*, 2005; Ryan *et al.*, 2008). *L. fermentum*, isolated from human colonic mucosal biopsy samples exhibited antimicrobial activities against enteric and food-borne pathogens (Varma *et al.*, 2010).

The isolation of probiotics is not just limited to the human GIT. Probiotic lactobacilli are isolated from several animal species including pigs, dogs, rats and even poultry (McCoy and Gilliland, 2007; Nazef *et al.*, 2008; Yun *et al.*, 2009; Jena *et al.*, 2013). Recently, *L. johnsonii* CRL 1647, isolated from *Apis mellifera* L. bee-gut, was found to exhibit a beneficial effect on honeybee colonies (Audisio and Benitez-Ahrendts, 2011). Further, probiotic strains have been characterized from various freshwater fish, such as wild European eel, perch and farmed African catfish (Bucio *et al.*, 2006).

Also *L. plantarum* was obtained from microbiota of marine fish and exhibited antimicrobial activity (Sahnouni *et al.*, 2012).

Probiotic bacteria are also found in non-dairy fermented substrates. Additionally, there is an increasing consumer demand for non-dairy-based probiotic products (Rivera-Espinoza and Gallardo-Navarro, 2010). A comparative *in vitro* study with bacterial strains, isolated from meat (*L. sakei*, *L. curvatus* and *Staphylococcus carnosus*) and fruits (*L. paracasei* and *L. plantarum*) showed functional and metabolic properties similar to those of human intestinal bacteria (Haller *et al.*, 2001). Recently, *L. pentosus* MP-10 obtained from brines of naturally fermented Alorena green table olives was reported to possess probiotic potential which includes inhibition of human pathogenic bacteria, acid and bile tolerance (Abriouel *et al.*, 2011). Moreover, *L. buchneri* P2 isolated from pickled juice, also demonstrated probiotic properties including cholesterol reduction (Zeng *et al.*, 2010).

Ecological niches contain the diverse microbial populations and this complex interrelation cannot be mimicked in traditional culturing methods. The traditional cultivation based approach gives an incomplete picture of microbial diversity in many ecosystems. Techniques based on molecular approaches have become popular to identify bacterial diversity of different sources as they bypass the cultivation step. These methods have provided important information regarding microbial ecosystems, including the sources of probiotic bacteria. A key step for studying an ecosystem is the isolation of its members.

1.5.2. Isolation

The first step in isolation of probiotic bacteria is to preserve the sample in native condition before incubated in selective medium. Most probiotic bacteria are strict anaerobic or facultative anaerobic or microaerophilic so it is important to place the sample under adequate condition and process in order to isolate bacteria successfully. The sample has to be homogenized completely and diluted appropriately and cultured in selective media. Depending on the source of the sample, selective inhibitory agents can be incorporated in selective medium to avoid the growth of particular group of microbes predominating in sample. For example, we incorporated cycloheximide in selection medium for the isolation of lactobacilli from faecal samples in order to avoid the yeast overgrowth (Endo and Okada, 2007).

Several media have been developed for selective isolation and enumeration of lactobacilli and bifidobacteria (Rogosa *et al.*, 1951; Munoa and Pares, 1988; Dave and Shah, 1996; Hartemink and Rombouts, 1999). Rogosa *et al.* (1951) developed a selective medium for isolation and enumeration of oral and faecal lactobacilli which is still the widely used selective medium for isolation of lactobacilli. This medium is a Columbia agar base supplemented with propionic acid and has low pH which is tolerated by lactobacilli and bifidobacteria and inhibits the growth of other predominating organisms in human faeces, such as Bacteroides and Eubacterium species.

The cultured plate is incubated at 37°C for 48-72 h in microaerophilic or CO_2 rich environment for the growth of lactobacilli and anaerobic condition for bifidobacteria. Subsequently, the colonies appeared on selective medium is transferred to propagation medium called de Man Rogosa Sharpe (MRS). Each colony is further subjected to microscopic and biochemical analysis.

1.5.3. Identification

The identification of microbes from food and human sources is the first step in the selection of probiotic bacteria. For decades, the characterization of bacteria based on phenotypic properties has been used which depends on the type of sugar fermented and fermentation end product generated. The taxonomy relied on biochemical analysis for many years but many groups of bacteria cannot be differentiated at genus level with this approach and also lead to misidentification of bacteria. 16S rRNA gene sequencing is the single most powerful molecular technique presently used for bacterial species identification (Wilson, 1995). This conserved fragment is used for phylogenetic classification and identification is carried out based on relatedness with the sequences available in databases such as DDBJ (DNA Data Bank of Japan), ENA (European Nucleotide Archive), GenBank (Woese, 1987; Winker and Woese, 1991). Often, the 16S rRNA gene analysis is combined with other molecular methods to identify the bacterial diversity of gut and other complex ecosystems. The techniques generally coupled with 16S rRNA gene sequence analysis is gradient PAGE using temperature or chemical denaturation, hybridised using fluorescent oligonucleotide probes that target specific 16S rRNA gene (fluorescence in situ hybridisation) or

digested with restriction enzymes (Terminal restriction fragment length polymorphism) (Langendijk *et al.*, 1995; Muyzer and Smalla, 1998).

Although, 16S rRNA gene sequence analysis is widely accepted technique for phylogenetic analysis, 16S rRNA gene (1500 bp) represents only small portion of the whole genome of bacteria. Complementary information is needed in certain cases to discriminate the strains of a given species. Alternatively, the spacer sequence between 16S and 23S rRNA gene shows a great deal of sequence and length variation (Leblond-Bourget *et al.*, 1996) (Figure 1.2). This intergenic spacer region is about 200 bases in length if tRNA genes are absent (small spacer sequence) and hypervariable in case of lactobacilli (Berthier and Ehrlich, 1998; Tilsala-Timisjarvi and Alatossava, 1997). It is a simple way to identify the bacteria at species level. Tannock *et al.* (1999) showed the identification of lactobacilli at species level using intergenic region sequence analysis of isolates from human feces, rodent gastrointestinal samples and porcine gastrointestinal contents. Additionally, it can be used as a qualitative technique to confirm isolates as lactobacilli by simply comparing the electrophoretic mobility pattern of amplified products on agarose gel with the same of any standard lactobacilli strain.

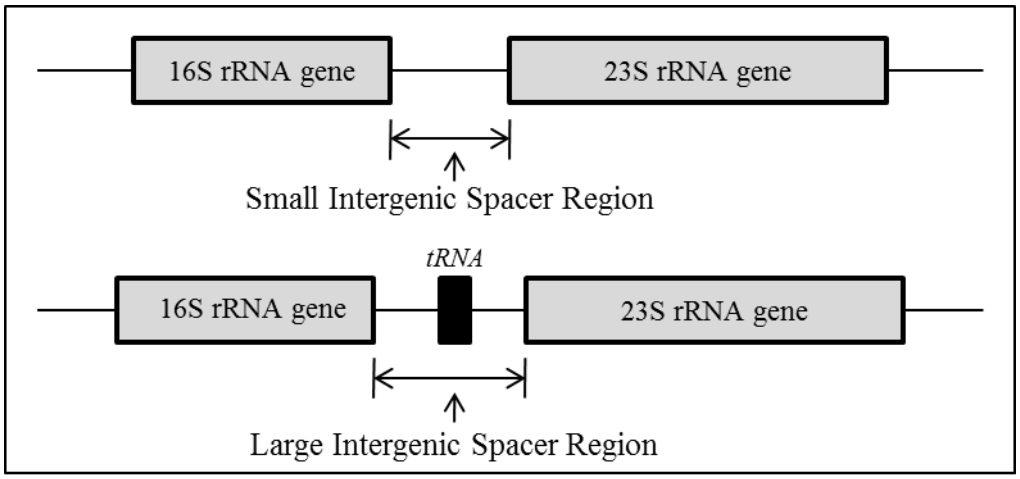


Figure 1.2. Schematic representation of 16S-23S rRNA gene intergenic region of bacteria.

Indeed, the sequencing of whole bacterial genome is the most useful tool to identify and characterize bacteria including probiotics. But genome sequencing is a laborious and relatively expensive technique hence it is not widely accepted. However, it is

being used to understand the underlying molecular mechanisms behind the properties of important probiotic and other dairy strains.

1.6. Characterization of lactobacilli as probiotics

1.6.1. Resistance to biliary salts

Bile salts are water soluble and synthesized in the liver. It is the metabolic end product of cholesterol and facilitates the enzymatic digestion of lipids in vertebrates. In humans, they are synthesized as the primary bile acids which include mainly cholic acids, deoxycholic acids and chenodeoxycholic acids. These acids are conjugated as an *N*-acyl amidate with glycine or taurine before being secreted into the small intestine via the bile duct (Savage *et al.,* 1995). The conjugated bile salts play an important role in solubilisation, digestion and absorption of dietary lipids and lipid-soluble vitamins in the small intestine. These secreted bile salts are reabsorbed at the distal ileum and transported to liver via bloodstream by the process called enterohepatic circulation (Carey and Duane, 1994). The glycoconjugated to tauroconjugated bile salts in human bile is usually present at the ratio of 3:1 (Hofmann, 1994). Bile salts come into contact with the bacterial population in the intestinal tract but mainly facultative and anaerobic microbes have the ability to transform the bile salts to secondary bile salts.

Among the few attributes sought for the selection of probiotic bacteria, bile tolerance is essentially important. During the transit from stomach to intestine, the bacteria come in contact with the bile salts which have detrimental effects. The intestinal bacteria including lactobacilli are reported to have several mechanisms to combat this adverse effect. It mainly includes the bile efflux and bile salt hydrolysis along with the change in composition of cell membrane profile.

Bile salt hydrolases

The bile salt hydrolases (Bsh; EC 3.5.1.24), also called choloylglycine hydrolase or conjugated bile acid hydrolase, are produced by the lactobacilli and other intestinal bacteria such as *Bifidobacterium*, *Enterococcus*, *Clostridium*, and *Bacteroides* spp (Bateup et al., 1995; Coleman and Hudson, 1995; Grill *et al.*, 1995; Kawamoto *et al.*, 1989; Franz *et al.*, 2001). Except *Bacteroides*, all other BSH positive strains reported

so far are Gram positive. The first step in bile salt metabolism by lactobacilli is the decojugation of bile salts by hydrolysis of the amide bond and release of glycine or taurine from the conjugated bile salts (Figure 1.3). The unconjugated bile acids can be further modified by intestinal bacteria to form secondary bile salts by oxidation and dehydroxylation or it can be excreted with the feces due to its lower water solubility (De Smet *et al.*, 1995).

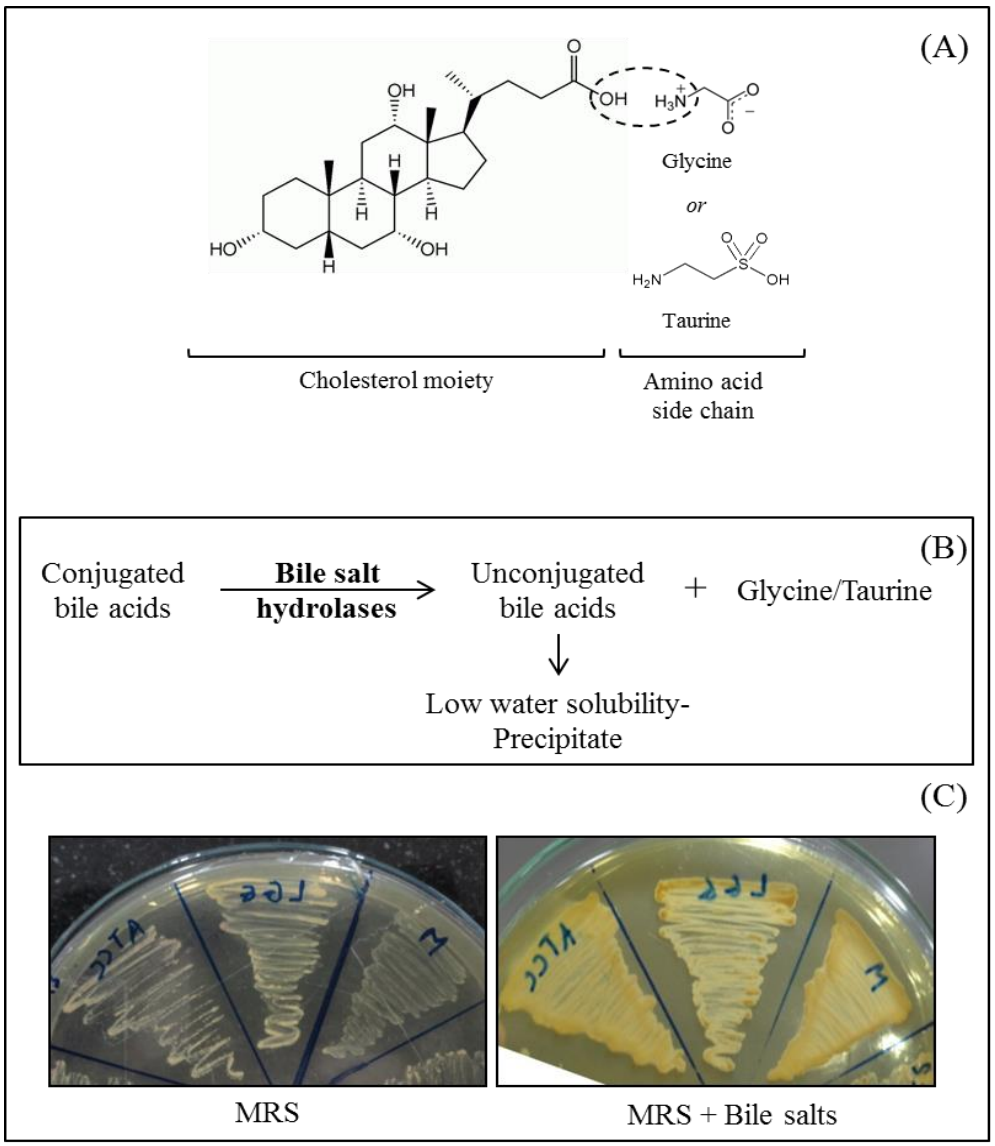


Figure 1.3. (A) Chemical structure of bile acids. Glycine or taurine is conjugated with cholesterol moiety to form toxic conjugated bile acids. (B) Bile salt hydrolase deconjugates these bile acids and releases amino acid side chain and the unconjugated bile acid gets precipitated. (C) Analysis of BSH activity on MRS agar plate containing bile salts with formation of visible cloudy precipitates near the growth of streaked culture.

Genetics of Bile salts hydrolases

The information gathered from available whole genome sequenced lactobacilli shows the presence of BSH in all the strains. Interestingly, some of the strains showed more than one BSH gene at different locations on the genome. *L. plantarum* WCFS1 possesses four BSH genes such as *bsh1*, *bsh2*, *bsh3* and *bsh4* which are not identical. BSHs have been purified and characterized from various gastrointestinal microflora and are generally intracellular, oxygen insensitive, and have slightly acidic pH optima between pH 5 and 6 (Kim and Lee, 2005). BSHs recognize the substrate at the amino acid moieties and kinetic data available in literatures suggests that most BSHs efficiently hydrolyse glycoconjugated bile salts than tauroconjugate bile salts (Taranto and Font de Valdez, 1999; Tanaka *et al.*, 2000). BSH-positive strains have nutritional advantages as amino acids liberated post BSH action could be used as carbon, nitrogen and energy sources. Glycine can be metabolized to ammonia and carbon dioxide, and taurine can be converted to ammonia, carbon dioxide and sulphate. In support of this hypothesis, it was observed that certain BSH-positive strains of *Clostridium* utilized the released free taurine as an electron acceptor and their growth rates improved in the presence of taurine and taurine-conjugated bile salts (Huijghebaert *et al.*, 1982; Van Eldere *et al.*, 1996).

The functional characterization of *bsh* with the generation of isogenic mutants in some studies provides the link between bile salt hydrolases and bile tolerance in lactobacilli. Lambert *et al.*, (2008) generated the bsh^{-} mutant for *bsh1*, *bsh2*, *bsh3* and *bsh4* of *L. plantarum* WCFS1 and observed that *bsh1* contributes the majority of activity in the strain. The *bsh1* mutant rendered cells susceptible to bile salts. The genetic analysis of *bshA* and *bshB* of *L. acidophilus* NCFM observed the different substrate specificities of BshA and BshB. BshA activity is dictated by the steroid moiety of the conjugated bile salt, while the specificity of BshB is determined by the presence of taurine in the bile salt structure (McAuliffe *et al.*, 2005). The precise mechanism behind relatedness between resistance to bile salts and activity of BSHs is not yet fully understood. However, it has been proposed that since the protonated form of bile salts may exhibit toxicity through intracellular acidification in a manner similar to organic acids, BSH activity may protect bacteria through the formation of the weaker unconjugated counterparts. This could help negate intracellular pH from drop by recapturing and exporting the co-transported proton (De Smet *et al.*, 1995).

The tauroconjugated bile salts have low toxic impact on bacteria compared to glycoconjugated bile salts, when studied *in vitro* at different pH value. It is observed that the glycoconjugated bile salts are extremely toxic at low pH (De Smet *et al.*, 1995). Given the fact that BSH preferentially hydrolyse glycoconjugated bile salts and have slightly acidic pH optima, BSH activity is of great importance at the point when bacteria enter the duodenum from stomach and are exposed to bile salts in an acidic environment.

Bron *et al.*, (2006) demonstrated an approximately 6-fold up regulation of *bsh1* with clone-based DNA microarray when *L. plantarum* WCFS1 was grown on MRS agar containing 0.1% porcine bile for 3 days compared to culture without presence of bile salts. Duary *et al.*, (2012) also found an approximately 6-fold increase of *bsh* in *L. plantarum* Lp91 at 2% bile salt concentration after 3 h.

There are however other reports that do not correlate BSH activity and tolerance to bile salts (Gopal *et al.*, 1996; Moser and Savage, 2001; Ahn *et al.*, 2003). There could be some other factors also playing an important role in tolerance to bile salts such as membrane characteristics and bile adaptation (Begley *et al.*, 2005). From the microorganism's point of view, it is certainly advantageous for bacteria to possess BSH to survive the toxic effects of bile acids in gastrointestinal tract.

BSHs and lowering cholesterol levels

The key factor responsible for the development of coronary heart disease is an elevated blood cholesterol level (Hypercholesterolemia). The available treatments are often suboptimal and expensive and also exhibit side effects (Schuster, 2004). The oral administration of probiotic bacteria in some human and animal studies showed the reduction in serum cholesterol level by 22% - 33% (Pereira and Gibson, 2002), or prevented increase of cholesterol level in mice fed with high fat containing diet (Taranto *et al.*, 2000). This cholesterol lowering ability is partially ascribed to BSHs of probiotics. Deconjugated bile salts are excreted with faeces due to its low solubility results in less efficiently reabsorbed by gut epithelial layer. To compensate the loss, the serum cholesterol is used for de novo synthesis of bile acids. The other mechanism is the assimilation of cholesterol to the bacterial cell wall (Gilliland *et al.*, 1985).

1.6.2. Acid tolerance

Acid tolerance and resistance to human gastric juice is considered as a desirable attribute for the selection of probiotic lactobacilli and eventual successful colonization in the gut. Sometimes the strain with high adhesive ability to epithelial cells is of no use if it cannot survive through the low gastric pH. Physiological studies with streptococci have shown that the cell membrane plays a major role in acid-base regulation by extrusion of protons through the membrane and exclusion of the environmental protons to enter the cell (Bender *et al.,* 1986). Amachi *et al.,* (1998) reported the action of cell membrane- bound H^+-ATPase for acid tolerance of *Lactococcus lactis*. It was demonstrated that it is important for bacteria to increase H^+-ATPase activity quickly and pump out H^+ in order to maintain intracellular pH. The acid sensitive bacteria are damaged when exposed to acidic condition since their H^+-ATPase activity cannot be increased (Matsumoto *et al.*, 2004).

The reported mechanisms in gram-positive bacteria to tolerate acid includes proton pumps, proteins engaged in repair and degradation of damaged cell components, alteration in the composition of cell envelop, and increased expression of regulator that promote minor or global responses (Cotter and Hill, 2003; Girgis *et al.,* 2003). Along with the multisubunit F_1F_0 ATPase, the amino acid decarboxylation-antiporter systems are the main proton pumps utilized by these microorganisms. The F_1F_0-ATPase system of *L. acidophilus* is well characterized and the *atp* operon contains eight genes (Kullen and Klaenhammer, 1999). Azcarate-Peril *et al.,* (2004) showed that the survival of *L. acidophilus* NCFM in acidic condition was mainly due to F_1F_0-ATPase and also showed overexpression of general stress proteins such as GroESL. Capozzi, *et al.,* (2011) also showed the overexpression of another general stress protein, small heat shock proteins (sHsps) in *L. acidophilus* NCFM upon exposure of bacterium to pH 4. The *atpD* of F_1F_0 ATPase complex in *L. plantarum* Lp91 was reported to be 4.7 fold up-regulated when bacterial cells were incubated at pH 2.5 (Duary *et al.*, 2010). Using microarray technology, Wall *et al.*, (2007) reported the differential expression of 72 genes at pH 2.7 in *L. reuteri* ATCC 55730. The genes induced were chaperon *clpL*, genes putatively involved in alteration of the cell membranes and cell wall such as esterase, genes encoding transcriptional regulators and some genes of unknown functions.

1.6.3. Production of antimicrobial compounds

Lactobacillus strains as probiotic bacteria are also being increasingly examined for their ability to inhibit the pathogenic bacteria. A predominance of lactobacilli in vaginal tract is known from many years and thought to protect from bacterial vaginosis by production of hydrogen peroxide (Eschenbach *et al.*, 1989) and maintaining low pH in vaginal ecosystem with the lactic acid production (Juarez Tomas *et al.*, 2003). The proportion of lactobacilli in this habitat is consistently higher than 70% (Eschenbach *et al.*, 1989; Redondo-Lopez *et al.*, 1990) and they play a fundamental role in protecting the cavity from pathological conditions such as the excessive proliferation of indigenous microorganism, *Gardnerella vaginalis*, whose dominance may lead to bacterial vaginosis (Sobel, 2000). Further, lactobacilli also inhibit the colonization of pathogens, such as *Candida spp.* and *Trichomonas vaginalis*, which may induce vaginitis or, less frequently, cervicitis and other regional and systemic problems (Johnston and Mabey, 2008; Nyirjesy, 2008).

Lactic acid is considered to be a key antimicrobial compound produced by lactobacilli (Servin, 2004). For instance, the strong anti- *S. enterica* serovar Typhimurium activity exhibited by *L. rhamnosus* GG was due to high accumulation of lactic acid (De Keersmaecker *et al.*, 2006). Although the exact mechanism is still not clearly understood, it was clearly observed that both *Salmonella* growth and expression of virulence factors was affected by lactic acid (Durant *et al.*, 2000). H_2O_2 production by lactobacilli is also suggested to be an important antimicrobial molecule, especially in the vagina of healthy woman (Servin, 2004). Pridmore et al. (2008) demonstrated role of H_2O_2 in the anti-*Salmonella* activity by *L. johnsonii* NCC533. The strain produced up to millimolar quantities of H_2O_2 when resting cells were incubated in the presence of oxygen. The genetic basis of H_2O_2 production is not yet understood.

Lactic acid bacteria are well known for their ability to produce structurally diverse ribosomally synthesized antimicrobial proteins or peptides, collectively known as bacteriocins. Since lactic acid bacteria are commonly used as starter culture in food fermentation, researchers have explored the use of bacteriocins producer as starter culture which can also inhibit the growth of food spoiling bacteria. Several different bacteriocins are characterized from different intestinal and other lactobacilli. Along with food protection, the use of such strain for human consumption is certainly

advantageous. Several investigators have shown the inhibition of gut pathogens by bacteriocin producing lactobacilli. Table 1.1 shows the functionally characterized and classified bacteriocins found in different *Lactobacillus* strains.

Classification of bacteriocins

Bacteriocins are commonly classified into four groups. Class I which is also termed as lantibiotics, are characterized by presence of unusual amino acids, such as lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine. Nisin was discovered in 1928 (Hurst, 1967), and widely used in processed cheese, meats, beverages etc. during production to extend shelf-life of food by suppressing the gram positive spoilage and pathogenic bacteria. Subtilin, a nisin analogue differing by 12 amino acid residues, was discovered in 1948 (Hansen, 1993). Typically, Class I peptides have from 19 to more than 50 amino acids. Further, Class I is subdivided into Class Ia and Class Ib. Class Ia bacteriocins, which include nisin, usually consist of cationic and hydrophobic peptides that form pores in target membranes and have a flexible structure compared to the more rigid Class Ib. Class Ib bacteriocins are globular peptides and have no net charge or a net negative charge (Altena *et al.*, 2000).

Class II bacteriocins are small heat-stable, non-modified peptides and can be further subgrouped. As per conventional classification, Class IIa includes Pediocin-like anti-*Listeria* peptides; Class IIb is a two peptide bacteriocin. Both the peptides are essentially required for activity. This class of bacteriocins are often found and characterized in lactobacilli. They are usually located in a single operon adjacent to each other and followed by a single immunity gene. Class IIc initially comprised the bacteriocins that are secreted by the general sec-system (Nes *et al.*, 1996). Later on, it was shown that Class IIa bacteriocins can also use this secretory system and consequently the class IIc was eradicated (Cintas *et al.*, 1997). Class III bacteriocins comprises the antimicrobial peptides with much less information. Class I and II bacteriocins are among the well-studied antimicrobial peptides used in food applications due to their target specificity and robustness.

Table 1.1. Functionally characterized bacteriocins in lactobacilli.

Microorganism	Source of organism	Name of bacteriocin/mol. wt.	Action against	Reference
L. reuteri LA6	Human infant feces	Reutericin 6/ 2.7 kDa	Food borne pathogenic bacteria	Kabuki *et al.*, (1997)
L. plantarum A-1	Tortilla - traditional Mexican corn bread	Plantaricin ASM1/ 5.1 kDa	*Lactobacillus*, *Leuconostoc*, and *Enterococcus*	Hata *et al.*, (2010)
L. salivarius CRL 1328	Human vagina	Salivaricin CRL 1328/4.5 kDa (Two peptide bacteriocin- Salα and Salβ)	Uropathogenic *Enterococcus faecalis* MP97	Vera Pingitore *et al.*, (2009)
L. brevis 925A	kimchi - a traditional Korean fermented dish made from Chinese cabbage	Brevicin 925A/6.9 kDa	*Listeria monocytogenes* and *Streptococcus mutans*	Wada *et al.*, (2009)
L. salivarius DPC6005	Porcine intestine	Salivaricin P (Two peptide bacteriocin - Sln1/4.1 kDa and Sln2/4.3 kDa)	*Enterobacteriaceae* family members	Barrett *et al.*, (2007)
L. curvatus LTH 1174	Fermented meat sausage	Curvacin A/ 4.3 kDa	*Listeria monocytogenes* and *Enterococcus faecalis*	Tichaczek *et al.*, (1993)
L. bulgaricus BB18	Authentic Bulgarian dairy products	Bulgaricin BB18/4.2 kDa	Wide inhibitory spectra including *Helicobacter pylori*	Simova *et al.*, (2009)

L. casei CRL705	Meat isolate	Lactocin 705/3.4 kDa (Two peptide bacteriocin - 705α and 705β)	Gram-positive bacteria, including food-borne pathogens	Castellano *et al.*, (2003)
L. salivarius BGHO1.	human oral isolate	Bacteriocin LS1/10 kDa	*Streptococcus mutans*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Micrococcus flavus*, and *Salmonella enteritidis*	Busarcevic *et al.*, (2008)
L. rhamnosus 160	Vaginal microflora of a healthy female	Lactocin 160/ 3.8 kDa	Active against the most prevalent species associated with bacterial vaginosis	Li *et al.*, (2005); Dover *et al.*, (2007)
L. acidophilus N2 (NCFM)	Human source	Lactacin B/6.5 kDa	*Lactobacilliaceae* family members	Barefoot and Klaenhammer, (1984)
L. plantarum C11	Human source	Plantaricin A, Plantaricin EF, Plantaricin JK and Plantaricin N	*Lactobacilliaceae* family members	Anderssen *et al.*, (1998)
L. salivarius UCC118	Human ileal-caecal region	Bacteriocin Abp118/6.2kDa	*Listeria monocytogenes*	Flynn *et al.*, (2002)
L. gasseri KT7	Human infant faeces	Gassericin KT7/5kDa	*Clostridium*, *Listeria* and *Enterococcus*	Zhu *et al.*, (2000)

1.6.4. Adhesion of *Lactobacillus* strains

Intestinal epithelial cells form a physical barrier that plays fundamental role in the cross talk between the host and the luminal contents. Although there are several reports of *in vitro* adhesion of lactobacilli, only few *in vivo*/*ex vivo* studies supports the adhesion of lactobacilli to intestinal epithelial cells. The underlying adhesion mechanisms of lactobacilli are still unknown. The interactive nature between microflora and intestinal mucosa requires an adequate technique to understand their relationship. Unfortunately, the ecosystem of human gut presents many obstacles to study the interaction between microflora and mucosa of host. To overcome these problems, several groups have developed and utilized *in vitro* cell culture models, and *in vivo* animal systems. Further, the advancement of cell culture methods such as co-culture of different cell types to generate complex conditions led to more reliable and realistic *in vitro* models to simulate *in vivo* condition. An accurate replica of human gastrointestinal tract cannot be developed but *in vitro* models provide insight into specific intestinal relationships of interest. Researchers have used different *in vitro* models to study the multi-factorial process of lactobacilli adhesion with epithelial cells and other components present in intestinal mucosa (von Kleist *et al.*, 1975; Lindgren *et al.*, 1992; Roos and Jonsson, 2002; Vesterlund *et al.*, 2005).

In vitro adhesion models

Intestinal epithelial cell lines

Most *in vitro* studies of the interaction of bacteria with intestinal mucosa involves culture of enterocytes or explanted section of intestinal mucosa. Enterocytes comprise around 70% of total cell population in human intestinal epithelial layer. Therefore, the use of cell lines which have originated from human epithelium and representing enterocytes cell types are of choice. Caco-2 cell line is widely accepted and routinely used for study related to human gut epithelium. Caco-2 cells are human colonic adenocarcinoma cells which express several morphological and enzymatic features of small intestinal enterocytes upon differentiation. These cells grow in a monolayer with a cylindrical polarized morphology expressing brush border microvilli and small intestinal hydrolase activity on the apical surface with tight junctions between cells (Sambuy *et al.*, 2005). For intestinal transport and bacterial invasion studies, Caco-2

cells are grown on permeable filter supports that allow access of ions and nutrients to both sides of the monolayer. For bacterial adhesion studies, Caco-2 cells are grown in monolayers on multi well plates specially treated for cell culture. Where as in intestinal transport and bacterial invasion studies, Caco-2 cells are grown on permeable filter supports that allows access of ions and nutrients to both sides of the monolayer but restrict the migration of cells from monolayers. When cultured, Caco-2 is a heterogeneous population of cells with morphological variations. Clonal cell lines have been isolated from the parental Caco-2 culture (ATCC HTB-37) to improve the homogeneity of the population and/or isolate a specific desired phenotype from a subpopulation. For example, the C2BBe cell line shows more homogeneous brush border expression comparable to *in vivo* human intestinal enterocytes (Peterson and Mooseker, 1992). Although Caco-2 cells are widely used for bacterial adhesion studies because of their morphological and functional similarity to human small intestinal enterocytes, the inherent heterogeneity of the Caco-2 cells is responsible for variant results obtained for the same *Lactobacillus* strain and makes the comparison of data between laboratories difficult.

Caco-2 cells do not accurately replicate the intestinal mucosa as they lack the mucus secretions. Another colonic carcinoma cell line, HT-29 cells are characterized with varied morphologies and mucin secretion. Upon exposure to either 5-fluorouracil (HT-29-FU) or methotrexate (HT-29-MTX), subpopulations were isolated that showed distinct morphology like goblet cells with higher mucus secretion. The mucins of the secretory HT-29-MTX goblet-shaped cells are similar to mucins of the human colon (Leteurtre *et al.*, 2004). The availability of mucin-secreting subclones helped researchers to study the adhesion of bacteria with intestinal cell as well as mucus. Further, the intestinal bacteria including lactobacilli induce the mucin secretion. The availability of immunoassays for specific mucin types provides the platform to study the same. For example, certain adhesive *Lactobacillus* species have the ability to induce the expression of MUC3 from intestinal epithelium. The MUC3 gene product is a secreted small intestinal mucin which has ability to inhibit the adhesion of enteric pathogen (Mack *et al.*, 2003). Mack *et al*., (1999) reported the induction of intestinal mucins MUC2 and MUC3 in HT-29 cells following exposure to probiotic agents *L. plantarum* 299v and *L. rhamnosus* GG, and both the mucins were able to inhibit the adherence of an attaching and effacing pathogenic *E. coli* to

HT-29 intestinal epithelial cells. Wherein nonintestinal HEp-2 cells expressed only minimal level MUC2 and no MUC3, and did not inhibit the pathogenic *E. coli* adhesion under same condition.

Apart from the above, other cell lines have also been used to study the adhesion of commensal and probiotic bacteria to the human intestinal mucosa. HeLa cells, isolated as malignant cervical cells, are mostly used other than intestinal cell lines. This is a very aggressive cell lines that can easily overwhelm other cell lines. Although HeLa cells do not necessarily express receptors and other phenotypes of the human intestinal epithelium due to their cervical origin, but their ease of maintenance makes them a popular choice for bacterial adhesion studies (Fourniat *et al.*, 1992; Mastromarino *et al.*, 2002). Intestinal 407 (Int-407) cells have been used occasionally in bacterial adhesion experiments (Kapczynski *et al.*, 2000; Avall-Jaaskelainen et al., 2003; Ingrassia *et al.*, 2005). Despite being originated from the small intestine of a human embryo and likely to show a greater similarity to the healthy human intestine, Int-407 cells were found to have a similar DNA fingerprinting profile to HeLa cells. HEp-2 cells, a human larynx epithelioma cell line, are also used for bacterial adhesion studies, but do not necessarily present an accurate model of the human intestinal mucosa (Mack *et al.*, 1999). Selection of cell line that mimics the *in vivo* environment as accurately as possible and proper maintenance of cell lines is of premier importance when surveying the adhesive mechanisms of pathogenic, commensal and potentially probiotic bacteria.

Mucin and extracellular matrix proteins

The epithelial cells of intestinal tract are covered with thick protective layer of mucus which is composed of glycolipids and a complex mixture of large and highly glycosylated proteins called mucin. Mucin is mainly synthesized by goblet cells but other cells such as enterocytes also produce in little amount. Apart from protective role, mucus layer serves as a primary site for adherence of bacteria. Due to the continuous renewal of mucus layer, the attachment of bacteria is facilitated for a short duration only. The interaction of lactobacilli with mucus has been reported *in vitro* by many researchers (Tuomola *et al.*, 2000; Jonsson *et al.*, 2001; Martin *et al.*, 2010). This interaction is also validated with *in vivo*/*ex vivo* microscopic analysis of biopsy samples (Macfarlane *et al.*, 2004; Macfarlane and Dillon, 2007).

Beneath the mucus layer, the host extracellular matrix (ECM) composed of various secreted proteins such as laminin, fibrin, heparin, fibronectin and collagen can also serve as adhesion sites in the intestinal mucosa. Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) is a term used for a group of bacterial surface adhesins that binds to ECM molecules (Patti *et al.*, 1994). Two of such adhesins, fibronectin-binding protein and collagen-binding protein, have been well characterized in lactobacilli (Toba *et al.*, 1995; Roos *et al.*, 1996; Buck *et al.*, 2005; Munoz-Provencio *et al.*, 2010). Fluorescent microscopy using anti-fibronectin antibody demonstrated that *L. acidophilus* and *L. agilis* localize in the area where fibronectin was detected on intestinal 407 cells. Additionally, the pre-treatment of lactobacilli with fibronectin resulted in decreased adhesion to intestinal 407 epithelial cell monolayers (Kapczynski *et al.*, 2000). An isogenic mutant of *L. acidophilus* NCFM for fibronectin binding protein (FbpA) was reported to have a 76% reduction in adhesion to Caco-2 cells compared to wild type control (Buck *et al.*, 2005). These and other some studies indicate that fibronectin serves as one of the eukaryotic receptors for adherence of lactobacilli to intestinal epithelium.

Lorca *et al.*, (2002) showed adhesion of *L. acidophilus* CRL 639 to both fibronectin and collagen. Although the molecular determinant of adhesion was not characterized, the strain was found to bind collagen more readily than fibronectin. Collageneous molecules are the major constitutes of the ECM and serve as most common target for pathogenic bacteria (Singh *et al.*, 2012). Thus, adhesion of lactobacilli to these ECM components represents an important criterion for selection of probiotic strains. In an effort to generate genetically engineered lactobacilli with an ability to adhere epithelial cell from non-adherent *Lactobacillus*, the gene (*cbsA*) encoding a collagen binding surface-layer protein from *L. cripatus* JCM5810 was expressed in *L. casei* (Martinez *et al.*, 2000). CbsA was successfully expressed at the surface of *L. casei* and showed collagen-binding properties in the transformed strain.

In vivo animal model system

In vitro models are commonly employed for adhesion related experiments due to their relatively low cost, ease of controlled target experimentation and manipulation. However, they lack the realism of human gastrointestinal environment which makes the use of animal models more attractive. Although the use of animal models has

much ethical restriction, they offer a more accurate representation and allied complexity of the human GIT. For example, animal models show the integration of mucosal and luminal surfaces along with the immune response which is the ideal replica of the human GIT (Boureau *et al.*, 2000). Conventional animal models present few ethical restrictions and high degree of similarities with human GIT, but the inherent complexity factors fail to depict the interaction and limit their use in bacterial adhesion studies. Therefore, germ free and gnotobiotic animals are most commonly used for bacterial adhesion studies as they provide ease to control experimental conditions, although they are not true representatives of the human GIT as conventional animals. The lack of complex microflora in gnotobiotic animals simplifies the interpretation of data obtained from bacterial adhesion and bacterial-host interaction studies. Further, the studies with germ-free animals give valuable information regarding the key role of single bacterial population on normal development, establishment and maintenance of the mucosa-associated immune system, and epithelial-cell functions (Boureau *et al.*, 2000).

Factors involved in lactobacilli adhesion

Some factors mediating the adhesion of lactobacilli to the different parts of gut epithelium i.e. the intestinal cells, mucus layer and/or ECM components have been characterized (Figure 1.4; Table 1.2). Lactobacilli adhesins can be classified according to their adhesion targets in intestinal mucosa (i.e. mucus and ECM components), their localization on the bacterial surface (i.e. surface layer proteins), and/or according to the way they are anchored in the bacterial surface (i.e. sortase-dependent proteins).

Mucus or mucin binding proteins

The functionally characterized extracellular mucin binding proteins of lactobacilli are mucus-binding protein (Mub) of *L. reuteri* 1063 (Roos and Jonsson, 2002), the lectin-like mannose specific adhesin (Msa) of *L. plantarum* WCFS1 (Pretzer *et al.*, 2005), and the Mub of *L. acidophilus* NCFM (Buck *et al.*, 2005). These three proteins share similar domain organization of typical cell surface protein of gram positive bacteria, characterized by N-terminal signal peptide, and a C-terminal anchoring motif (LPxTG) that is recognized by a family of enzymes called sortase for covalent attachment of transported protein to the peptidoglycan layer of the bacterial cell wall

(Navarre and Schneewind, 1994, 1999; Ton-That *et al.*, 2004). In addition to the typical N- and C- terminal similarities, these three proteins share a common feature of mucus binding domain (MUB) organization. This MUB domain is described as MucBP (MUCin-Binding Protein) in the Pfam database (PF06458), consist of 50 amino acid residues in length. Boekhorst *et al.* (2006a) performed an in silico search for mucin-binding proteins in available databases and could identify a total of 48 proteins containing atleast one MUB domain in 10 lactic acid bacteria. The identified MUB domains varied in size, ranging from 100 to more than 200 amino acid residues per domain, and appeared mostly but not exclusively in lactobacilli that belong to GIT.

Sortase-dependent proteins

This class of surface proteins contain the C-terminal motif LPxTG recognized by the enzyme sortase (SrtA), which cleaves between the T and G residues of transported proteins, and covalently links the carboxyl group of threonine to amino groups of peptidoglycan precursors in cell wall (Marraffini *et al.*, 2006). Therefore, these surface proteins are commonly known as sortase-dependent proteins. Bacterial genome encodes many sortase-dependent proteins, but the function of most proteins is unknown (Pallen *et al.*, 2001). So far five such sortase-dependent proteins have been identified in lactobacilli. Three of these proteins belong to mucin-binding proteins as already mentioned, *L. reuteri* 1063 Mub (Roos and Jonsson, 2002), *L. plantarum* WCFS1 Msa (Pretzer *et al.*, 2005) and *L. acidophilus* NCFM Mub (*Buck et al.*, 2005). The fourth characterized sortase-dependent protein is LspA of *L. salivarius* UCC118 which exhibited adhesion to human epithelial cells and mucus (Claesson *et al.*, 2006; van Pijkeren *et al.*, 2006). The domain composition of LspA revealed eight mucus-binding domains (MucBP; PF06458) and can be included in the group of the fully characterized lactobacilli mucus-binding proteins. Modulator of adhesion and biofilm, MabA of *L. rhamnosus* GG is the recently characterized LPxTG motif containing surface protein, involved in biofilm formation capacity on abiotic surfaces and adhesion to intestinal epithelial cells and tissues of the murine GIT (Velez *et al.*, 2010). Boekhorst *et al.* (2006b) identified 12 proteins as putative adhesins utilizing bioinformatics tool to predict secretome of *L. plantarum* WCFS1. Among these proteins, 10 contained an LPxTG sortase motif and predicted their role in the adherence to collagen, fibronectin, chitin or mucus.

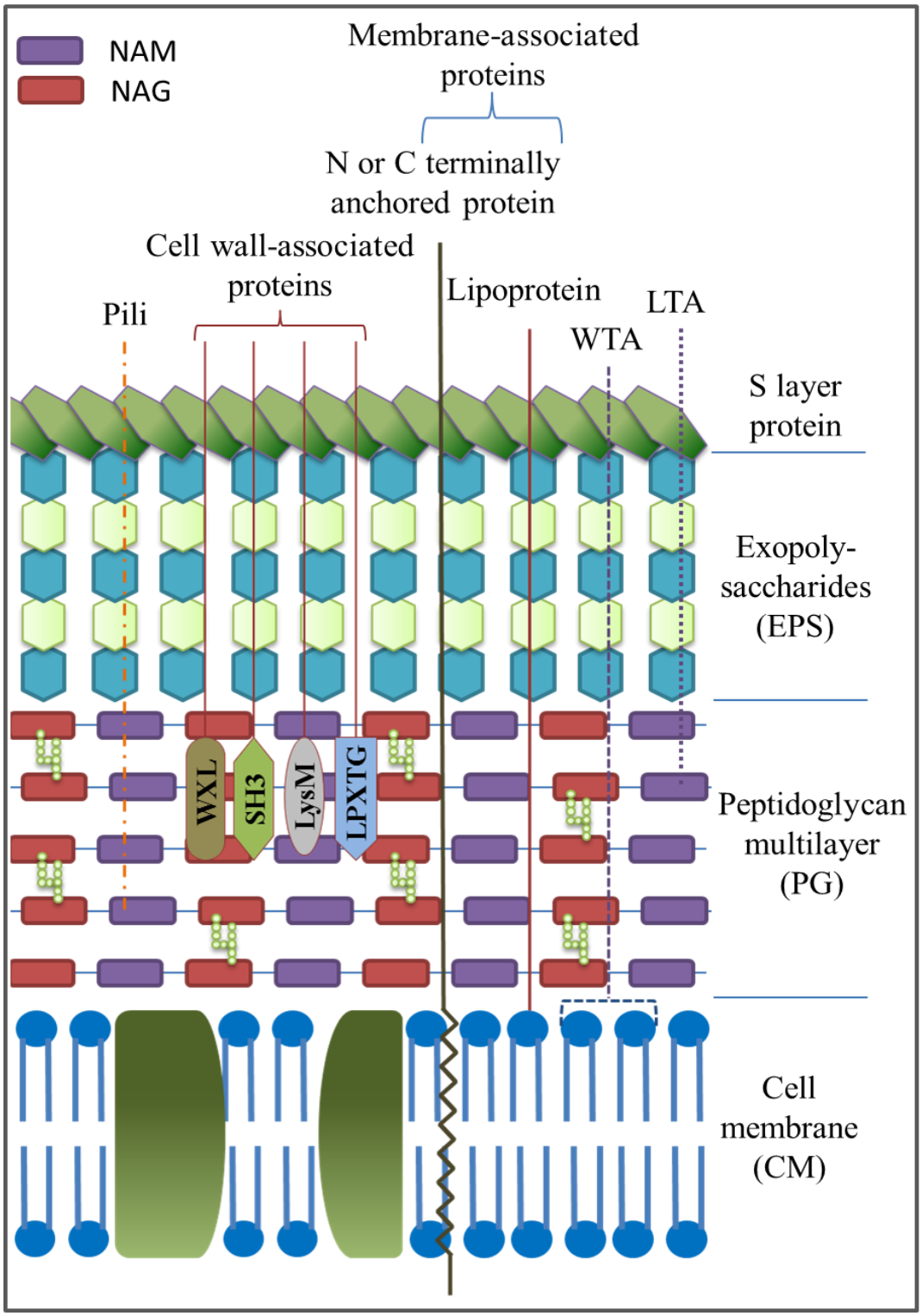


Figure 1.4. Schematic representation of cell wall, membrane and surface bound proteins of lactobacilli (Adapted from Sengupta *et al.*, 2013). The bilipidic cell membrane (CM) covered with multi-layered peptidoglycan (PG) shell decorated with embedded proteins, lipoteichoic acids (LTA), wall teichoic acid (WTA), pili and lipoproteins. Exopolysaccharides (EPS) form thick layer closely associated with PG and are surrounded by an outer envelope of S-layer proteins.

Surface layer proteins

Surface-layers (S-layers) are generally formed by a paracrystalline monomolecular assembly of proteins or glycoproteins on the surface of bacteria. At present, several lactobacilli S-layer proteins have been identified from lactobacilli, but the biological function of most of these proteins remains to be validated (Sara and Sleytr, 2000; Avall-Jaaskelainen and Palva, 2005), except for six *Lactobacillus* S-layer proteins i.e. S-layer protein of *L. acidophilus* spp. (Schneitz *et al.*, 1993), CbsA of *L. crispatus* JCM 5810 (Toba *et al.*, 1995; Sillanpaa *et al.*, 2000; Antikainen *et al.*, 2002), Slp of *L. helveticus* R0052 (Johnson-Henry *et al.*, 2007), SlpA of *L. brevis* ATCC 8287 (Vidgren *et al.*, 1992; Avall-Jaaskelainen *et al.*, 2002), SlpA of *L. acidophilus* M92 (Frece *et al.*, 2005) and SlpA of *L. acidophilus* NCFM (Buck *et al.*, 2005). These proteins were reported to mediate adhesion to the intestinal epithelial cells and ECM components. Additionally, some of these proteins were also involved in antagonistic activity of lactobacilli to prevent the adhesion of pathogenic bacteria to epithelial cells (Chen *et al.*, 2007; Johnson-Henry *et al.*, 2007).

Adhesins bind to extracellular matrices

One of the reported ECM adhesins is the collagen-binding protein (CnBP) of *L. reuteri* NCIB 11951 which helps the strain to adhere to solubilized type I collagen (Aleljung *et al.*, 1994; Roos *et al.*, 1996). The structural domain analysis of CnBP showed similarities to the solute-binding domain of bacterial ABC transporters in Pfam domain databases (PF00497). A similar domain was also found in MapA (mucus adhesion-promoting protein) of *L. reuteri* 104R and mucus- and mucin-binding protein (32-Mmubp) of *L. fermentum* BCS87 (Miyoshi *et al.*, 2006; Macias-Rodriguez *et al.*, 2009). Although, both proteins were involved in mucus adhesion, there was no mucin-binding domain detected according to Pfam. Other examples of collagen binding lactobacilli adhesin includes above discussed S-layer proteins such as CbsA of *L. crispatus* JCM 5810 (Toba *et al.*, 1995) and SlpA of *L. brevis* ATCC 8287 (de Leeuw *et al.*, 2006).

Another group of ECM adhesins are fibronectin binding proteins. These include fibronectin-binding protein (FbpA) of *L. acidophilus* NCFM (Buck *et al.*, 2005) and *L. casei* BL23 (Munoz-Provencio *et al.*, 2010) and previously discussed SlpA of *L. brevis* ATCC 8287 (Hynonen *et al.*, 2002).

Table 1.2. Functionally characterized adhesins in different *Lactobacillus* strains

***Lactobacillus* strain**	**Adhesin name/ amino acid length or mol. wt.**	**Target for binding**	**References**
L. acidophilus NCFM	Mub/4326 aa SlpA/1017 aa FbpA/563 aa	Mucus and Human epithelial cell lines Human epithelial cell lines Fibronectin and Human epithelial cell lines	Buck *et al.*, (2005)
L. plantarum LA 318	GAPDH/340 aa	Human colonic mucin	Kinoshita *et al.*, (2008)
L. brevis ATCC 8287	SlpA/465 aa	Human intestinal epithelial cell lines, laminin, collagen and fibronectin	Vidgren *et al.*, (1992); Avall-Jaaskelainen *et al.*, (2003); de Leeuw *et al.*, (2006); Hynonen *et al.*, (2002)
L. plantarum LM3	EnoA1/442 aa	Human fibronectin	Castaldo *et al.*, (2009)
L. crispatus JCM 5810	CbsA/440 aa	Collagen I, IV and laminin containing regions in human colon and ileum and bacterial LTA	Vidgren *et al.*, (1992); Toba *et al.*, (1995); Sillanpaa *et al.*, (2000); Antikainen *et al.*, (2002)
L. helveticus R0052	Slp/437 aa	Human intestinal cell lines	Johnson-Henry *et al.*, (2007)
L. johnsonii La1 NCC 533	EF-Tu/396 aa GroEL/543 aa	Human intestinal cell lines and mucus Human intestinal epithelial cells and mucus	Granato *et al.*, (2004) Bergonzelli *et al.*, (2006)
L. crispatus ZJ001	S-layer protein/42 kDa	Adhesion to HeLa cells	Chen *et al.*, (2007)

L. plantarum WCFS1	Msa/1010 aa	Mucus via Mannose binding	Kleerebezem *et al.*, (2003); Pretzer *et al.*, (2005)
L. reuteri 1063	Mub/3269 aa	Mucus components	Roos and Jonsson, (2002)
L. salivarius UCC118	LspA/1209 aa	Human epithelial cell lines and mucus	Claesson *et al.*, (2006); van Pijkeren *et al.*, 2006
L. reuteri 104R	MapA/263 aa	Caco-2 cells and mucus	Rojas *et al.*, (2002) ; Miyoshi *et al.*, (2006)
L. reuteri NCIB 11951	CnBP/263 aa	Collagen	Aleljung *et al.*, (1994); Roos *et al.*, (1996)
L. rhamnosus GG	MabA/2419 aa SpaB/241 aa SpaC/895 aa SpaF/983 aa	Human intestinal epithelial cell lines Intestinal mucosa Intestinal mucosa Human intestinal mucus	Velez *et al.*, (2010) Kankainen *et al.*, (2009) von Ossowski *et al.*, (2010) von Ossowski *et al.*, (2010)
L. casei BL23	FbpA/567 aa	Human fibronectin	Munoz-Provencio *et al.*, (2010)
L. acidophilus spp.	S-layer protein	Avian intestinal epithelial cells	Schneitz *et al.*, (1993)
L. acidophilus M92	SlpA/45 kDa	Murine ileal epithelial cells	Frece *et al.*, (2005)
L. reuteri 100-23	Lsp/185 kDa	Murine gut epithelium	Walter *et al.*, (2005)
L. fermentum BCS87	32-Mmubp/300 aa or 32 kDa	Porcine mucus and mucin	Macias-Rodriguez *et al.*, (2009)

Peculiar protein mediated adhesion

Moonlighting function of many cytoplasmic proteins have been observed. In lactobacilli, four such cytoplasmic proteins have been identified with role in adhesion apart from basic cytoplasmic functions. The mechanism of these anchorless cytoplasmic proteins is still unknown. Granato et al., (2004) first reported the role of elongation factor Tu (EF-Tu), a guanosine nucleotide-binding protein involved in protein synthesis in the cytoplasm as a surface located adhesin which mediated the adhesion of *L. johnsonii* La1 to the intestinal epithelial cell line and mucins. Pfam analysis of EF-Tu did not show any mucin-binding domain and domains typical to elongation factors were only found. Later on, the same strain was found to possess another such moonlight protein referred as heat shock protein GroEL, a chaperone of Hsp60 class (Bergonzelli *et al.*, 2006). GroEL was found on surface of *L. johnsonii* La1 with binding ability to Caco-2 cells and mucus.

L. plantarum LM3 was reported to have surface displayed alfa-enolase (EnoA1) which helps the bacterium to bind human fibronectin, an extracellular matrix protein (Castaldo *et al.*, 2009). Kinoshita *et al.*, (2008) characterized glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *L. plantarum* LA 318 as a principle molecule involved in binding to human colonic mucin.

Pili

Pili are multisubunit protein polymeric structure which was first and foremost functionally characterized only in *L. rhamnosus* GG (Kankainen *et al.*, 2009; Reunanen *et al.*, 2012), although they have been seen at the genome level in other lactobacilli (Forde *et al.*, 2011). After assembly, the pilin subunits are attached to cell wall by membrane bound transpeptidase also called housekeeping sortase (Scott and Zahner, 2006). The role of pili in bacteria adhesion, invasion, biofilm formation, aggregation and immunomodulation is well established (Danne and Dramsi, 2012; Lebeer *et al.*, 2012). The presence of pili was earlier observed only on gram positive pathogens, but *L. rhamnosus* GG was first reported to carry pili which also mediates adhesion to mucus of intestinal epithelium (Kankainen *et al.*, 2009).

Nonprotein-mediated adhesion

Certain cell surface associated factors such as lipoteichoic acid (LTA) and exopolysaccharides (EPS) have been seen to participate in the adhesion of lactobacilli to intestinal mucosa.

LTA is the main component of lactobacilli contributing to surface hydrophobicity. It is a strongly negatively charged polyol phosphate polymer which has membrane anchored glycolipids moiety and its poly(glycerophosphate) (Gro-P) chain extending into the cell wall (Neuhaus and Baddiley, 2003). LTA seems to participate in bacterial adherence in non-specific way. For instance, the cell surface associated LTA of *L. johnsonii* NCC533 was shown to mediate the adhesion to Caco-2 cells. Purified LTA from the cell surface inhibited the bacterial adhesion to Caco-2 cells by 60% in a concentration dependent manner (Granato *et al.*, 1999). Although, the hydrophobicity contributed by LTA largely depends on the D-alanine ester substitutions, the inactivation of *dltD* in L. rhamnosus GG suggested that D-alanylation of LTA is not necessary for short-term adherence to Caco-2 cells (Perea Velez *et al.*, 2007). But in case of *L. reuteri* 100-23, a *dltA* mutant resulted in impaired colonization of the mouse gastrointestinal tract (Walter *et al.*, 2007).

Apart from LTA, the exopolysaccharides (EPS) produced by lactobacilli have been reported to help bacterial adhesion to abiotic and biotic surfaces in intestinal mucosa. EPSs are long-chain polysaccharides made up of branched, repeating units of sugars or its derivatives loosely bound to the cell surface or secreted into the environment (Ruas-Madiedo and de los Reyes-Gavilan, 2005). EPSs have been shown to have indirect effect in adhesion of lactobacilli as they shed other cell surface adhesins. For example, *L. acidophilus* CRL 639 which produces no capsule in exponential growth phase, exhibited maximal binding compared to stationary phase capsular bacterium (Lorca *et al.*, 2002). Similarly, the knockout mutant of *L. johnsonii* NCC533 with deleted EPS biosynthesis cluster, slightly prolonged the gut persistence compared to the persistence of the wild type parental control strain (Denou *et al.*, 2008). Ruas-Madiedo *et al.* (2006) first reported both positive and negative effects of EPS on the adhesion properties of probiotic and enteropathogens to human intestinal mucus.

1.6.5. Immunomodulation

Lactobacilli can elicit innate and adaptive immune responses in the human via binding to pattern recognition receptors (PRRs) expressed on immune cells and other cells present in intestinal epithelium. The conserved microbial molecular structures, microbes-associated molecular patterns (MAMPs) recognized by PRR induces immune activation, antigen presentation and production of cytokines, chemokines and other innate effector molecules (Abreu, 2010; Kawai and Akira, 2010; Wells *et al.*, 2010). Same PRRs are also involved in recognition of pathogen-associated molecular patterns (PAMPs). PRRs can be divided into three families – i) Membrane bound PRRs includes Toll-like receptors (TLRs) and C-type lectin receptors (CLR) such as mannose and scavenger receptors, ii) Cytoplasmic PRRs includes retinoic acid inducible gene I (RIG-I)-like receptors and nucleotide oligomerization domain-like (NOD) receptors (NLR) and iii) Secreted PRRs includes Complement receptors, collectins, C-reactive protein, etc. There are around 10 TLRs that have been identified and characterized in humans (summarized by Kawai and Akira, 2010). Both TLR and NLR activate the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor κB (NF-κB) pathway signalling cascades (Janssens and Beyaert, 2002). In an inactivated state, NF-κB is located in the cytosol bound to the inhibitory protein IκBα but TLR and NLR signalling leads to degradation of IκBα. The free NF- κB then migrates to nucleus and induces the transcription of specific genes. PRR signalling pathways play an important role in activation of both innate and adaptive immune responses by influencing the skewing of naïve T cells, the regulation of regulatory T cells and also activation of antigen presenting cells (APCs) including dendritic cells (DC) and macrophages. DCs are specialized APCs which are found throughout the lamina propria of the intestine and gut-associated lymphoid tissues such as Peyer's patches and lymph nodes (Rescigno, 2010). Although most tissue resident DCs are immature and poorly immunogenic as they have low expression of MHC and co-stimulatory molecules, however in response to contact with MAMPs and other signals which induce PRR signalling and NF- κB pathway, this results in maturation and activation of DCs. Once activated, DCs express high level of MHC, Co-stimulatory molecules and cytokines which are required for antigen presentation and T cell activation, clonal expansion and differentiation (Kapsenberg, 2003). The level of cytokines produced by activated DCs depends on the nature of stimuli passed through

PRRs. This signalling is a key regulator for the induction of different T cell subsets. Different strains and species of lactobacilli can differentially modulate the immune response through the stimuli passed (Wells, 2011).

Intestinal epithelial cells also express a range of PRR to sense the presence of microbes. Upon activation, they produce a broad range of cytokines and chemokines including interleukins (ILs), tumour necrosis factor (TNF), growth factors and inducible small antimicrobial peptides called beta-defensins (BDs) (Cario and Podolsky, 2000). Enterocytes and goblet cells are the primary cell types engaged in cell signalling and pass signals to underlying cells upon microbial attachment to intestinal epithelium. In human, TLR2 and TLR4 were seen on enterocytes by immunohistochemical techniques (Cario and Podolsky, 2000; Abreu et al., 2002; Furrie *et al.*, 2005). Several mechanisms take part to prevent excessive immune responses to non-pathogenic bacteria in intestinal epithelium: a) regulating TLR expression, b) TLR localization, c) differential apical and basolateral TLR signalling, d) negative feedback regulation of NF-κB pathway and e) the attenuation of NF-κB activation by commensal bacteria. Combined effect of these adaptations helps to maintain homeostasis in gut.

Lactobacilli factors involved in immunomodulation

Cell wall components of lactobacilli can potentially bind to TLR2 along with TLR6 and initiate signalling. Bacterial lipoproteins were shown to recognize TLR2 over a decade ago (Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999). The surface located anionic molecules, the WTA and LTA may also generate signalling through their binding to scavenger receptors such as SRA which was seen earlier to bind purified LTA (36). The lipid chain of LTA in cytoplasmic membrane of gram positive bacteria also binds to TLR2. However, the wall teichoic acid (WLA) is covalently attached to the peptidoglycan and presumably cannot signal through TLR2/6 as they lack the lipid moiety. Both techoeic acids are anionic through the D-ala substitution by the enzymes of *dlt* operon. A Dlt-mutant generated for *L. plantarum* NCIMB8826 showed much less incorporation of D-alanine in its teichoic acid (Grangette *et al.*, 2005). This defect significantly affected the immunomodulatory properties of the strain with significantly reduced secretion of proinflammatory cytokines from peripheral blood mononuclear cells (PBMCs) and monocytes compared to wild type

strain (Grangette *et al.*, 2005). Interestingly, similar mutant of *L. rhamnosus* GG did not show altered immunomodulation in epithelial cells and PBMCs (Perea Velez *et al.*, 2007). The reasons are not clear but it may be related to species and strain dependent differences in LTA and WTA composition. For example the cell wall of *L. rhamnosus* and *L. casei* appears to contain only LTA in contrast to other lactobacilli which have WTA as well (Perea Velez *et al.*, 2007).

The human innate immune system has evolved specific mechanisms to recognize bacterial DNA. TLR9 binds bacterial Cytosine-phosphate-guanine (CpG motifs) DNA (Stacey *et al.*, 2003). The treatment of human epithelial monolayer with various bacterial DNA showed differential effects on secretion of IL-8 *in vitro* (Jijon et al., 2004). DNA of pathogenic species of *Salmonella* induced IL-8 secretion from human colonic cell line HT-29 but *Lactobacillus*, *Bifidobacterium* and *Streptococcus* present in the VSL3 probiotic mixture did not show the same. Further, the pre-treatment of HT-29 cells with DNA preparation from VSL3 resulted in an attenuation of IL-8 secretion in response to live *S. typhimurium* exposure or TNF-α. The effect was mediated via delayed NF-κB activation and stabilized levels of IκB (Jijon et al., 2004). Similar effects were also observed with purified DNA from *L. rhamnosus* GG for HT-29 and T84 polarized cell monolayers (Ghadimi *et al.*, 2010). Several studies reported that preparation of macromolecular peptidoglycan (PGNpolymer) can activate NF-κB through TLR2. However, studies indicated that the lipoprotein within the PGNpolymer but not PGN itself activates TLR2 (Volz *et al.*, 2010). Further, the peptidoglycan fragments are recognized by the NOD1 and NOD2 receptors of NLR family (Girardin *et al.*, 2003a; Girardin *et al.*, 2003b). In addition to teichoic acid and PGN, exopolysaccharides (EPSs) are also commonly found within the cell wall of lactobacilli which are either attached to cell wall or secreted into the milieu. The structural diversity of EPSs is very large due to the substitution of the different sugar monomers and branching (De Vuyst and Degeest, 1999). EPSs and other cell wall polysaccharides are readily recognized by C-type lectin receptors which are involved in the recognition and capture of antigens by antigen presenting cells such as dendritic cells and macrophages. The addition of heat-killed *L. casei* strain Shirota or its purified polysaccharide-peptidoglycan (PS-PG) complex showed decrease in production of IL-6 in LPS-treated mouse macrophage cell line RAW 264.7 (Matsumoto *et al.*, 2005).

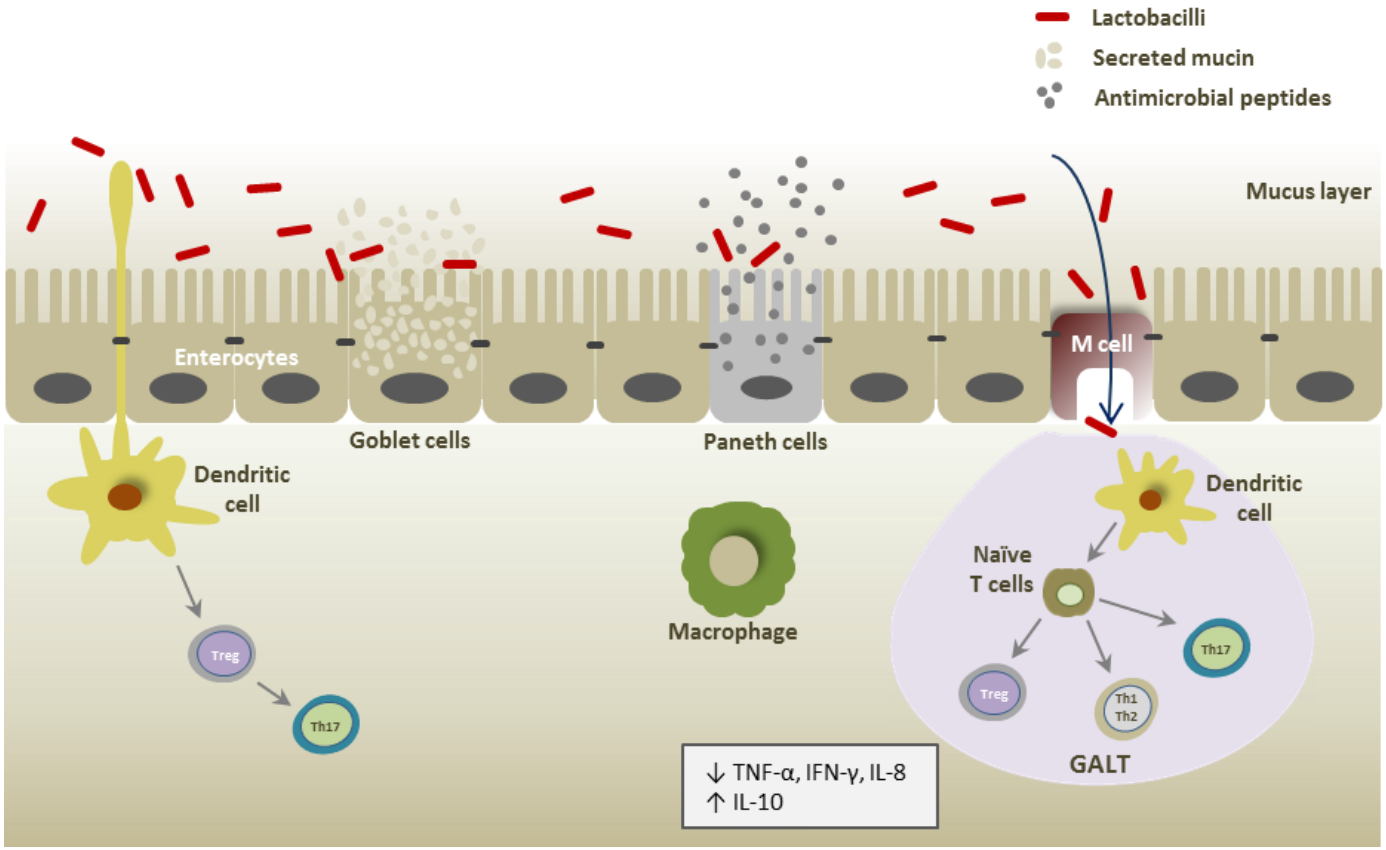


Figure 1.5. Schematic representation of lactobacilli mediated immunomodulation in intestinal mucosa. Probiotic lactobacilli induces 1) the mucin secretion from goblet cells, 2) secretion of antimicrobial peptides induced from Paneth cells, and 3) translocation via M cell to the localized lymphoid tissues and interact with immune cells which in turn alter the cytokines and chemokines expression and also activate the naïve T cells.

Lactobacilli mediated immunomodulation

Several lactobacilli have been shown to modulate the immune system under different *in vitro* co-culture systems, using different types of immune cells such as human monocytic cell line THP-1, human monocyte derived DCs, human PBMCs and mouse bone marrow derived DCs (Meijerink and Wells, 2010). IL-10 expression is more frequently seen in most reports because it is an anti-inflammatory cytokine that suppresses IL-12 production and subsequently IFN-γ production which eventually favours a T-helper 2 (Th2) or a regulatory T cell (Treg) response. Apart from that, IL-10 also down-regulates antigen presentation and inhibits the activation of pro-inflammatory cytokines and chemokines. Till date, there is still lack of evidence correlating *in vitro* data to *in vivo* data but some studies indicated possible correlation. For example three studies indicated that the ability of different lactobacilli to induce high ratio of IL-10/IL-12 or IL-10/TNF-α production from immune cells correlates with their capacity to protect mice and rats from TNBS induced colitis (Peran *et al.*, 2005; Foligne *et al.*, 2007; Zoumpopoulou *et al.*, 2009). Several studies have also shown that differential immunomodulation by lactobacilli depends on both the species and the strain (Miettinen *et al.*, 1996; Miettinen *et al.*, 1998; Christensen *et al.*, 2002; Foligne *et al.*, 2007; Meijerink *et al.*, 2010; van Hemert *et al.*, 2010; Vissers *et al.*, 2010). Although the different subsets of DCs with distinct immunological phenotypes exist within the mucosal tissues, *in vitro* models serve as an important tool to evaluate the immunomodulatory properties of bacteria. Peña and Versalovic (2003) have shown that secretory molecules of *L. rhamnosus* GG decreases TNF-α production in LPS-activated murine macrophages cell line RAW 264.7.

Along with change in cytokines and chemokine expression, lactobacilli can induce mucin production from epithelial cells and goblet cells which in turn can help to eliminate pathogen adhesion to gut epithelium. Mack *et al.*, (2003) demonstrated increased extracellular MUC3 gene expression in intestinal epithelial cell line HT-29 upon adherence by lactobacilli. The probiotic mixture VSL#3 also showed an increase in basal luminal mucin content by 60% post seven days dose with preparation in Wister rats (Caballero-Franco *et al.*, 2007). Probiotic lactobacilli also induce the expression of antimicrobial peptides including β-defensins (BD) from epithelial cells. Schlee *et al.*, (2008) have shown that lactobacilli and probiotic mixture VSL#3 strengthen intestinal barrier function through the up-regulation of

hBD-2 via induction of proinflammatory pathways including NF-κB and AP-1 as well as MAPKs.

1.7. Beneficial role of probiotics in Human health and Disease

The discovery of antibiotics in the 1950's facilitated their prevalent use as therapeutic agents to treat infections and as growth stimulants for animals. However, growing concerns over the years due to the emergence of multi-drug resistant pathogens and the associated side effects of antibiotic use, led consumers and manufacturers to seek alternate measures. Probiotics provide an effective and attractive alternative to overcome these problems (Fuller, 1989). The mechanisms by which probiotics function, especially in eliminating pathogens are diverse, thus reducing the chances of the emergence of pathogens resistant to secreted antimicrobial molecules from probiotics (Fioramonti *et al.*, 2003). In addition, the re-establishment of the normal gut microflora is required post antibiotic therapy. The use of probiotics also eliminates this necessity (Fuller, 1989, Rolfe, 2000). Further, the use of probiotic therapy would be cheaper compared to antibiotics (Fuller, 1989). Currently, there is a large volume of scientific literatures suggest that the use of probiotics benefits the human and animals in a number of ways. The new health benefits are also being reported incessantly.

Intestinal infections

In developing countries, intestinal infectious diseases caused by the pathogenic microorganisms such as *Escherichia coli, Vibrio cholerae, Shigella* spp., *Campylobacter* spp., *Clostridium difficile* and rotavirus are the main cause of death (Nomoto, 2005). Even in developed countries like the USA, 16.5 million children less than 5 years of age have between 21 and 37 million episodes of diarrhea annually (Glass *et al.*, 1991). Further, the excess use of antibiotics also resulted in an increase of nosocomial infections caused by multi-drug resistant pathogens. Rotaviral diarrhoea, characterized by vomiting and watery stool, is more prevalent in children aged between 6 to 24 months. Current treatment includes fluid replacement to counteract dehydration and nutritional deficiency. The efficacy of different probiotics to treat rotaviral diarrhoea was investigated in various double-blind placebo-controlled trials (Isolauri *et al.*, 1991; Shornikova *et al.*, 1997). *L. rhamnosus* GG in

the form of fermented milk or freeze-dried powder effectively shortened the course of acute diarrhea in children aged 4-45 months compared to the placebo group (Isolauri *et al.*, 1991). Similar results were obtained when *L. reuteri* was consumed by patients aged between 6-36 months (Shornikova *et al.*, 1997). *Clostridium difficile,* a Gram-positive bacterium, produces toxin that causes colitis. Antibiotic treatment with metronidazole and vancomycin is effective, but there were incidences of recurrence of the disease in some cases (Surawicz, 2003). The recurrence and pathophysiology of the disease is still not clearly known but the production of *Clostridium* spores are considered to be contributing factor to the recurrence of the disease (McFarland *et al.*, 2002). Re-treatment with antibiotics is usually prescribed. The probiotic yeast, *Saccharomyces boulardii* showed positive effects on managing the infection by *C. difficile* in mice and human (Surawicz, 2003). The probiotic yeast prevented the adherence of *C. difficile* to Vero cells in a dose dependent manner (Tasteyre *et al.*, 2002) and also stimulated the specific IgA intestinal anti-toxin A levels by 4.4-fold in mice (Qamar *et al.*, 2001). Similarly, *L. delbrueckii* ssp. *bulgaricus* B-30892 also showed elimination of *C. difficile*-mediated cytotoxicity, using Caco-2 cells as a model (Banerjee *et al.*, 2009). In clinical studies, *L. rhamnosus* GG cured patients from *C. difficile* associated diarrhoea when used as adjunct therapy (Gorbach *et al.*, 1987; Biller *et al.*, 1995).

Shigellosis, also known as bacillary dysentery caused by *Shigella dysenteriae* 1, is a highly contagious infection characterized by diarrhoea, fever, vomiting, blood, pus, or mucus in stools (Lindberg and Pal, 1993). Increasing incidence of epidemic outbreaks and the fatality rate of young children pose a major concern in developing countries. Animal studies provided the valuable information to understand the pathogenesis of the disease and also to evaluate the effect of probiotics. The pre-treatment with *L. rhamnosus* and *L. acidophilus* exhibited a protective role with reduced inflammation and protected from *S. dysenteriae* 1 infection in rats (Moorthy *et al.*, 2007).

Campylobacter jejuni is a Gram-negative pathogenic bacterium which causes gut infection resulting into gastroenteritis, characterized by abdominal pain, diarrhoea and fever. It is frequently isolated from animal faeces. Amongst human, food contaminated with *C. jejuni* is one of the most common causes of diarrhoea. *Bifidobacterium breve* was evaluated for the treatment of Campylobacter enteritis in 133 patients aged between 6 months to 15 years. Although the strain did not shorten

the duration of diarrhoea, *B. breve* administration was successful in eradicating *C. jejuni* from faeces and restoring the normal intestinal flora (Tojo *et al.*, 1987). The lactobacilli mixtures prepared by mixing four lactobacilli cultures, including *L. acidophilus, L. fermenentum*, *L. crispatus*, and *L. brevis* completely eradicated *C. jejuni* in different sections of a simulated chicken digestive system (Chang and Chen, 2000). The antagonistic effect of lactobacilli on *E. coli* is frequently used as indicator of antimicrobial activity for selection of potential probiotic strains (Cole and Fuller, 1984). The administration of fermented milk with *L. salivarius* decreased the number of coliforms including *E. coli* in the gut of new born rats (Cole and Fuller, 1984). Similar results were obtained with strains of *L. acidophilus* and *L. lactis* in new born pigs (Kohler and Bohl, 1964, Muralidhara *et al.*, 1977).

Prolonged treatment with antibiotics such as clindamycin, cephalosporin and penicillin imbalance the endogenous gut microflora and facilitates abnormal proliferation of opportunistic enteropathogens (Nomoto, 2005). This imbalance in normal gut microflora causes diarrhoea commonly referred to as antibiotic associated diarrhoea and occurs in about 20% of treated patients (Nomoto, 2005). Probiotic therapy was effectively utilized to treat this condition with the administration of *L. rhamnosus* GG, *B. longum* and *E. faecium* SF68 in patients on antibiotic treatment. A significant decrease in the incidence of antibiotic associated diarrhoea was observed in the double-blind placebo controlled trials with probiotic cultures (Colombel *et al.*, 1987; Buydens and Debeuckelaere, 1996; Pant *et al.*, 1996).

Clostridium difficile can cause severe diarrhoea. *C. difficile* bacteria are usually present at very low number in the intestine and antibiotic treatment makes patient susceptible to the associated infection (Nomoto, 2005). Diarrhoea may result in pseudomembranous colitis and recurrence of the disease may occur. When *S. boulardii* was concomitantly consumed with standard antibiotic therapy using vancomycin, the double blind placebo controlled clinical studies showed that the recurrence of the disease was considerably reduced compared to the placebo group (McFarland *et al.*, 1995). The mechanism of action is not completely understood but it was seen that probiotic produces proteolytic enzymes that digests toxin A or B of the pathogen and prevents adsorption of the toxin to receptors on the intestinal mucosa (McFarland *et al.*, 1995). Traveller's diarrhoea is a more frequent disease among the people travelling from developed countries to developing countries and

generally acquired by the ingestion of contaminated food or water. Most of the reported cases (80-85%) have shown the infection with bacterial pathogens such as enterotoxigenic *E. coli* (ETEC) and *Campylobacter jejuni* (Hill, 2000, Yates, 2005). Antibiotic therapy has limited use due to the diversity in the cause and etiology of the disease. Probiotics offer a safe and effective method to prevent Traveller's diarrhoea. A supplement containing Probiotic strains - *S. boulardii* and a mixture of *L. acidophilus* and *B. bifidum* prevented onset of disease in the 12 controlled clinical trials (McFarland, 2007).

Helicobacter pylori infection

Helicobacter pylori causes gastritis and peptic ulcers and is also one of the risk factors of gastric cancer (Hohenberger and Gretschel, 2003). In most cases, patients infected with *H. pylori* are asymptomatic (Malfertheiner *et al.*, 2002). Lactobacilli and their metabolic products have shown ability to eliminate or inhibit the pathogen in various *in vitro* studies. Some of these strains include *L. acidophilus* CRL 639 and its autolysin product (Lorca *et al.*, 2001), *L. johnsonii* LA1 (Michetti *et al.*, 1999) and *L. salivarius* WB1004 (Aiba *et al.*, 1998). Among most researchers, the common factor for eradication of *H. pylori* was found to be high lactate production by lactobacilli (Midolo *et al.*, 1995; Aiba *et al.*, 1998). Other researches have shown inhibitory effects by the production of antibacterial substances including bacteriocins along with exclusion effects from lactobacilli (Lesbros-Pantoflickova *et al.*, 2007; Chen *et al.*, 2012). Mukai *et al.*, (2002) showed the inhibition of *H. pylori* with strains of *L. reuteri* and reported the role of homologous surface glycolipid binding proteins in these strains competing for common receptor sites on the host. *L. salivarius* WB1004 effectively inhibited the adhesion of *H. pylori* to human and mouse gastric epithelial cells and also protected gnotobiotic BALB/c mice from *H. pylori* infection (Kabir *et al.*, 1997). In human trials, treatment with inactivated *L. acidophilus* was effective in increasing eradication rates of a standard anti-*H. pylori* therapy to 88% in clinical studies with 120 *H. pylori* positive patients (Canducci *et al.*, 2000). Sykora *et al.*, (2005) reported that live probiotic *L. casei* DN-114 001 containing fermented milk confers an enhanced therapeutic benefit on *H. pylori* eradication in children with gastritis on triple therapy.

Inflammatory bowel diseases

The gastrointestinal barrier is made up of physical components such as epithelial layer with mucus and functional components include gut associated immune cells (Fioramonti *et al.*, 2003). This barrier plays a pivotal role in restricting colonization of pathogens, in the systemic translocation of foreign antigens and in eliciting antigen specific immune response (Sanderson and Walker, 1993). Under certain conditions, this fine array of network is disturbed and leads to an inflammation (Isolauri *et al.*, 2002). Several lactobacilli have shown various mechanisms to counteract the inflammation and these include stabilization of normal microbiota during infection, degradation of enteral antigens and reduction in production of inflammatory mediators (Isolauri *et al.*, 2002). In most infectious conditions, the gut microbial diversity is reduced and the positive host-microbial interaction is compromised which leads to inflammation. Probiotics help to re-establish these interactions via restoring the gut microbiota (Isolauri *et al.*, 2002). Probiotics are being explored as a therapeutic agent for the treatment of inflammatory bowel diseases (IBD) such as irritable bowel syndrome (IBS), Crohn's disease and ulcerative colitis (Isolauri *et al.*, 2002). The aetiology of these diseases is not completely understood, but studies indicate that the genetic predisposition and gut microbiota are thought to play an important role. Indeed selected probiotics showed positive effects on reducing the number of relapses and prolonging the period of remission. Interestingly, not only lactobacilli, *L. salivarius* UCC118 and *L.rhamnosus* GG, but also *S. cerevisiae* (*boulardii*) and a strain of *E. coli* (Nissle) were effective in alleviating the symptoms of IBD (Mattila-Sandholm *et al.*, 1999; Gupta *et al.*, 2000; Guslandi *et al.*, 2000; Jonkers *et al.*, 2012).

Anti-carcinogenic activity

Anti-carcinogenic properties among probiotic bacteria were first reported in *L. bulgaricus* (Bogdonov *et al.*, 1962). Enteric bacteria produces certain enzymes such as β-glucuronidase, β-glucosidase, azoreductase and nitroreductasewhich are involved in the production of carcinogens from innocuous complexes. Probiotic bacteria exhibit the anti-carcinogenic properties by suppressing bacteria that produce these enzymes and also degrade the produced carcinogens in the gastrointestinal tract. The reduction in release of these enzymes was observed in individual that consumed

fermented milk prepared from *L. rhamnosus* GG and *Bifidobacterium* spp. in two independent reports (Goldin *et al.*, 1992; Bouhnik *et al.*, 1996). N-nitrosamine is a carcinogen which is produced as a result of enzyme nitroreductase. Intestinal bacteria including *Lactobacillus* spp. readily degrade N-nitrosamine (Rowland and Grasso, 1975) and also observed that there was decrease in production of enzyme in human subjects post regular consumption of fermented milk product prepared from *L. acidophilus* (Goldin and Gorbach, 1984). In an animal study with rats, the genotoxic carcinogen N-methyl-N'-nitro-N-nitrosoguanidin (MNNG) and 1, 2 dimethylhydrazine (DMH) administered orally led to DNA damage of gastrointestinal cells within 24 h. When *L. casei* was orally given 8 h before MNNG, the rats could be protected from the detrimental effects of carcinogens. Further, the heat treatment of bacteria resulted in loss of anti-carcinogenic activity (Pool-Zobel *et al.*, 1993) which indicates the role of heat-labile molecules in anti-carcinogenic activity.

Lactose intolerance

The consumption of probiotics can have beneficial effects on improving the digestion ability of humans and animals. In humans, the use of probiotics has been explored to compensate lactase in lactose intolerant individuals (Fioramonti *et al.*, 2003). These individuals have a congenital deficiency for the enzyme β-galactosidase in the brush boarders of the small intestine (Rowland, 1992). As a result of impaired expression of enzyme, undigested lactose reaches to the colon and it is utilized by colonic bacteria which results in abdominal discomfort and diarrhoea (Marcon, 1997). LAB including lactobacilli produces enzyme lactase upon culturing. The yogurt prepared from these bacteria can help to reduce the symptoms in lactose intolerant individuals by facilitating lactose digestion (Marcon, 1997). It has been hypothesized that ingestion of *Lactobacillus* strain with high β-galactosidase activity and intestinal adherence would lead to prolonged intestinal colonization of lactobacilli and possibly the conversion from a lactose-intolerant to a lactose-tolerant state. Ojetti *et al.*, (2010) used *L. reuteri* strain to treat lactose intolerant patient in clinical studies by oral supplementation and suggested that probiotic therapy may represent an interesting treatment option since its use is simple and its effect may last in the time after stopping administration. The effectiveness of probiotic on improved utilization of lactose in lactose intolerant patients is usually monitored by hydrogen breath analysis

method; a measure of hydrogen excretion in the breath is correlated with colonic fermentation and lactose maldigestion (Levitt and Donaldson, 1970).

Under the present study, the isolation of lactobacilli was aimed from different sources in search of identifying novel probiotic with significant probiotic attributes for human consumption. In order to establish as probiotic, the isolates must possess probiotic properties such as acid and bile salts tolerance, antimicrobial activity towards pathogens, adhesion ability to intestinal epithelial cells. Additionally, the cell surface properties such as surface hydrophobicity and electron donor and acceptor ability, autoaggregation and coaggregation ability with enteropathogens can be correlated to the adhesion potential and antagonistic ability of lactobacilli. To mimic in vivo conditions, the different adhesion assays were designed with lactobacilli and enteropathogens. The most probiotic effects are achieved by immunomodulation ability of lactobacilli. Thus, the study of lactobacilli isolates to modulate the array of cytokines and chemokines was undertaken. Most lactobacilli established as probiotic are on the basis of superior phenotypic properties of bacteria. But the molecular mechanisms behind the probiotic attributes and effects remained largely unknown. To address this limitation, the role of selected adhesins in the adhesion potential of different lactobacilli and possible adhesin mediated inhibition of enteropathogens was commenced. So, the objectives of the present work are:

1. Isolation, identification and biochemical characterization of *Lactobacillus* strains from different sources.

2. Adhesion ability of *Lactobacillus* strains to intestinal epithelial cell lines and competitive adhesion inhibition of enteropathogens.

3. Immunomodulatory potential of *Lactobacillus* strains co-incubated with intestinal epithelial cell lines.

4. Study the role of selected adhesins in the different *Lactobacillus* strains and the interaction study with intestinal epithelial cell lines.

CHAPTER 2

ISOLATION, IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF *LACTOBACILLUS* STRAINS FROM DIFFERENT SOURCES

"If we find ourselves with a desire that nothing in this world can satisfy, the most probable explanation is that we were made for another world."

- C.S. Lewis

Chapter 2

Isolation, identification and biochemical characterization of *Lactobacillus* strains from different sources

2.1. Introduction

The human body contains diverse groups of commensal microbiota including both aerobes and anaerobes. The largest population amongst that resides in the gastrointestinal tract which is colonized by more than 400 bacterial species in the adult (Falk *et al.,* 1998; Sghir *et al.,* 2000). The commensal bacteria regulate intestinal epithelial development and function and any interruption of these interactions may result in disease condition (Isolauri *et al.,* 2001; Guarner and Malagelada, 2003). The beneficial effects of the gut microbiota are attributed to probiotics which are defined as 'Live microorganisms that when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). Currently, several strains of *Lactobacillus* and *Bifidobacterium* are being widely used as a probiotics for human health and nutrition.

Lactobacillus species are Gram positive, non-pathogenic and desirable members of intestinal tract. The health promoting effect of lactobacilli have been widely explored and include stabilization of indigenous microbial population, protection against intestinal infection, alleviation of lactose intolerance, increased nutritional value of foods, reduction of serum cholesterol levels and non-specific enhancement of the immune systems (Hooper *et al.,* 1999; Perdigon *et al.,* 2002; Suvarna and Boby, 2005; Kim *et al.,* 2008). Several lactobacilli which act as probiotic bacteria are currently being explored as novel biological therapeutic agents (Sullivan and Nord, 2005; Reid *et al.,* 2006). Since not all lactobacilli possess ability of health benefits to host, it becomes necessary to screen and characterize numerous strains in order to obtain ideal probiotic strain. The colonization of the gastrointestinal tract is desirable

for any probiotic strain which depends on several factors including the ability of the bacteria to tolerate acidic pH of the stomach, tolerance to bile salts and on the adhesion of bacteria to intestinal cells and mucus (van Belkum and Nieuwenhuis, 2007). Upon the colonization, probiotic bacteria do exhibit antimicrobial activity and inhibit the colonization of pathogenic bacteria in gastrointestinal tract with competing for common attachment sites and secreting antimicrobial substances like bacteriocins and superoxide radicals (Jin *et al.*, 1996).

Physiochemical properties, especially cell surface hydrophobicity do have a role in aggregation and adhesion ability of bacteria (Handley *et al.*, 1987). Autoaggregation ability of probiotic strain is reported to help in adhesion to intestinal epithelium (Kos *et al.*, 2003) and may subsequently help colonization by forming a biofilm like structure. Autoaggregation feature has also been correlated with adhesion ability by several investigators (Handley *et al.*, 1987; Kos *et al.*, 2003; Del Re *et al.*, 2000). Studies on mechanisms of autoaggregation have demonstrated the role of both soluble protein molecules present in spent culture medium and also surface located protein or lipoproteins in cell aggregation (Kmet and Lucchini, 1997; Roos *et al.*, 1999). Additionally, probiotic strains have been shown to aggregate with enteropathogens and interfere with their adhesion to the gut epithelium (Ekmekci *et al.*, 2009). The secretion of antimicrobial compounds and ability to aggregate enteropathogens is also expected attributes from probiotic strain which would help to minimize the colonization of enteropathogens in the GIT tract. In a broad way, the isolation from human, capability to tolerate acidic pH and bile, antimicrobial activity and high adhesion ability are principle desirable properties in potential probiotic strain (Dunne *et al.*, 2001).

The probiotic properties are varied from strain to strain and not any single probiotic strain has all superior abilities like tolerance to bile and acid, antimicrobial activity against pathogens, ability to colonize in intestinal tract and/or immunomodulatory ability. Thus, it becomes necessary to isolate numerous *Lactobacillus* strains to identify novel probiotic strain with better abilities than the currently established probiotic strains. The present study was intended to isolate *Lactobacillus* strains from different sources, characterize them for probiotic properties and compare the individual abilities with the established probiotic strain – *L. rhamnosus* GG.

2.2. Materials and methods

2.2.1. Standard *Lactobacillus* strains

Standard strain *L. plantarum* ATCC 8014 and the established probiotic strain *L. rhamnosus* GG (LGG) were obtained as kind gift from Food and Drugs Laboratory (FDL), Vadodara, India and Dr. Shira Doron, MD, Department of Medicine, Tufts–New England Medical Center, USA respectively.

2.2.2. Isolation and identification of *Lactobacillus* strains

Isolation was carried out from eight sources including one curd of buffalo milk, faeces from five lactating human children aged 4-6 months and one human adult aged 27 years and one cow dung sample. About 1 g of faecal, curd or cow dung sample was suspended in 10 ml sterile normal saline, vigorously mixed and allowed to settle. A 100 µl aliquot of the suspension was then spread on Rogosa SL agar (a selective medium for *Lactobacillus* isolation; Himedia, Mumbai, India) plates containing 100 µg/ml cycloheximide (Sisco research laboratories, Mumbai, India) for each sample (Rogosa *et al.* 1951). The plates were incubated at 37^{o}C till sufficient growth was observed. About 6-8 isolated colonies were then picked from each Rogosa SL agar plate and transferred to de Man, Rogosa and Sharpe (MRS; Himedia) agar plates and subjected to further microscopic and biochemical tests. The primary characterization was carried out on the basis of Gram and endospore staining as well as with catalase test. The isolates which showed Gram positive nature, endospore and catalase negative phenotype were further analysed with molecular technique to identify them on species level. Molecular identification of isolates was carried out by amplification of 16S-23S rRNA gene intergenic region as reported by Tannock *et al.* (1999).

The 16S-23S rRNA gene intergenic regions of the isolates were amplified using primer 16-1A (5′-GAATCGCTAGTAATCG-3′) and 23-1B (5′-GGGTTCCCCCATTCGGA-3′) by colony PCR.

Reaction system for 16S-23S rRNA gene intergenic region amplification

Reaction Components	**Volume (µl)**
R.O water	16.9
10X Buffer for Taq DNA Polymerase	2.5
dNTP mix (2.5 mM each)	2.0
Forward primer (100 pmol/µl)	0.8
Reverse primer (100 pmol/µl)	0.8
Taq DNA polymerase (2.5 U/µl) (Sigma-Aldrich)	1.0
Colony suspension	2
Total volume	**25**

Conditions for 16S-23S rRNA gene intergenic region amplification

Steps		**Temperature (˚C)**	**Time**	**No. of cycles**
1	Pre-cycle denaturation	94	5 min	1
2	Denaturation	94	45 sec	30
3	Primer annealing	55	30 sec	
4	Primer extension	72	1 min	
5	Post-cycle elongation	72	6 min	1

Agarose gel electrophoresis

The PCR amplified products were analyzed by electrophoresis on 0.8% agarose gel in 0.5X TBE followed by staining with ethidium bromide and viewing under UV light.

Composition of Tris Borate EDTA (5X; for 1 L solution)

Tris-Cl	54 g
Boric acid	27.5 g
EDTA (0.5 M)	20 ml
Distilled water (DW)	Make up volume to 1 L

2.2.3. Bile and acid tolerance

Minimal inhibitory concentration of bile was determined for individual lactobacilli strains by inoculating 1 $\times 10^6$ bacterial cells in MRS broth containing 1-5% (w/v) bile salts (Himedia) for 24 h. Based on this subsequently, bacterial cells grown overnight in normal MRS broth, were washed with PBS and 20 µl each of selected lactobacilli (1×10^8 cfu/ml) were transferred to 980 µl MRS broth containing 3% bile salts and incubated at 37°C for 2 h. To examine survival rate of different lactobacilli under acidic condition at 37°C, 20 µl each of selected lactobacilli (1×10^8 cfu/ml) were transferred to 980 µl of acidic buffer (Casey *et al.* 2004). For both bile and acid tolerance, the aliquots were taken at 0 min and 2 h and cultures were plated after appropriate dilution on MRS agar plate and the enumeration was done following 48 h incubation at 37°C.

Composition of acidic buffer (For 1L solution)

D-glucose	3.5 g
NaCl	2.05 g
KH_2PO_4	0.6 g
$CaCl_2$	0.11 g
KCl	0.37 g

pH was adjusted to 2.0 and 2.5 using HCl

2.2.4. Antimicrobial activity

Antimicrobial activity of lactobacilli was determined by agar spot test as described by Schillinger and Lueke (1989) with minor modifications. Briefly, 5 µl each of *Lactobacillus* strains (1×10^8 colony forming unit/ml) was spotted on the surface of MRS agar plate and incubated for 24 h at 37°C for the spots to develop. A 150 µl aliquot of each of the indicator bacteria (listed below) grown overnight in Luria broth under shaking condition at 37°C, were vigorously mixed with 15 ml of Luria soft agar (0.6% agar, w/v) and poured over the MRS agar plates containing developed colonies of lactobacilli. The plates were then incubated for 24 h at 37°C and the zones of inhibition were measured and expressed as described by Baccigalupi *et al*. (2005). Test strains used are *Shigella dysentery*, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 25668, *E. coli* serotype O26:H11 (EPEC),

Salmonella enterica serovar Typhi MTCC 733 obtained from culture collection facility at our department.

2.2.5. Analysis of cell surface hydrophobicity

Cell surface properties were determined by the bacterial adhesion to solvent test as described by Bellon-Fontaine *et al.* (1996) with minor modification. The adhesion to xylene (apolar solvent), chloroform (polar acidic solvent) and ethyl acetate (polar basic solvent) demonstrates hydrophobic cell surface characteristic, electron donor and electron acceptor properties of bacteria respectively. The overnight grown lactobacilli were washed twice and resuspended in Dulbecco's phosphate buffered saline (PBS), pH 7.0 to achieve 0.5 OD at 600 nm. The cell suspension (5 ml) was mixed with 1 ml of solvent and vortexed for 15 s and allowed to stand 1 h for the separation of the two phases at 37 °C; the aqueous phase was measured at 600 nm using a spectrophotometer. The affinities to solvents with different physiochemical properties (hydrophobicity and electron donor-acceptor properties) were expressed using the following formula.

$$\text{BATS (\%)} = (1 - A_t / A_0) \times 100$$

Where A_t represents the absorbance at time = 1 h and A_0 the absorbance at time = 0 h.

2.2.6. Aggregation assays

Autoaggregation assay was performed according to Del Re *et al.* (2000) with certain modifications. The overnight grown culture was resuspended in PBS to give viable cell count of approximately 10^8 cfu/ml. Cell suspension (4 ml) was mixed by vortexing for 15 s and autoaggregation was determined after 4 h of incubation at 37°C by monitoring the absorbance at 600 nm. To determine the effect of heating on autoaggregation, the cultures were heated at 85°C for 30 min before the autoaggregation assay was carried out as described above. Additionally, the effect of any secretory compounds on autoaggregation was determined by adding 10% of cell free culture supernatant to the autoaggregation reaction mixture. To prepare cell free culture supernatant, the overnight grown culture of lactobacilli were harvested by centrifugation and culture supernatant was collected in fresh tube.

$$\text{Autoaggregation (\%)}: 1 - (A_t / A_0) \times 100$$

Where A_t represents the absorbance at time = 4 h and A_0 the absorbance at time = 0 h.

The method for preparing the cell suspensions for coaggregation assay was essentially similar to that used for the autoaggregation assay. Equal volumes (2 ml) of each cell suspension (lactobacilli and enteropathogen) were mixed together by vortexing for 15 s. Control tubes were set up at the same time, containing 4 ml of each of the individual bacterial suspension. The absorbance at 600 nm of the suspensions was measured after 4 h of incubation at 37 °C. To determine effect of heating on coaggregation, individual lactobacilli cultures were heated as described above and mixed with enteropathogen.

$$\text{Coaggregation (\%)} = [((Ax + Ay) / 2) – A(x + y)] / [(Ax + Ay) / 2] \times 100$$

Where x and y represent each of the two strains in the control tubes, and (x + y) represents the mixture.

2.2.7. Statistics

Values are given as mean along with the standard deviation (SD) of triplicate independent experiments. Significant ANOVAs were followed by Dunnett test to compare with the respective controls ($p<0.05$). All analysis was conducted using SigmaStat 3.5 software.

2.3. Results

2.3.1. Isolation of *Lactobacillus* strains from different sources

Out of 147 isolates screened, twenty-eight isolates which were Gram positive rods, found negative for the presence of endospore and for the production of catalase, and which could grow on Rogosa SL and MRS agar plates were selected for subsequent molecular identification on the basis of 16S-23S rRNA gene intergenic region amplification. The agarose gel mobility of the amplification products generated from the 16S-23S rRNA gene intergenic region of eighteen isolates matched with that of standard strains LGG and/or *L. plantarum* ATCC 8014. The smaller intergenic region of ten of these eighteen isolates was sequenced. In addition, the smaller intergenic region of three of ten others whose amplified product size was distinct from that of standard strains was sequenced and following sequence alignment with the NCBI database only the former ten were found to belong to *Lactobacillus* genus. The 16S-

23S rRNA gene intergenic region sequences of these strains have been held in GenBank and their corresponding accession number are collected in Table 2.1. The three isolates whose 16S-23S profile was distinct from the standard *Lactobacillus* strains were identified as belong to other genera like *Weissella* and *Enterococcus.* These isolates were not used for further study.

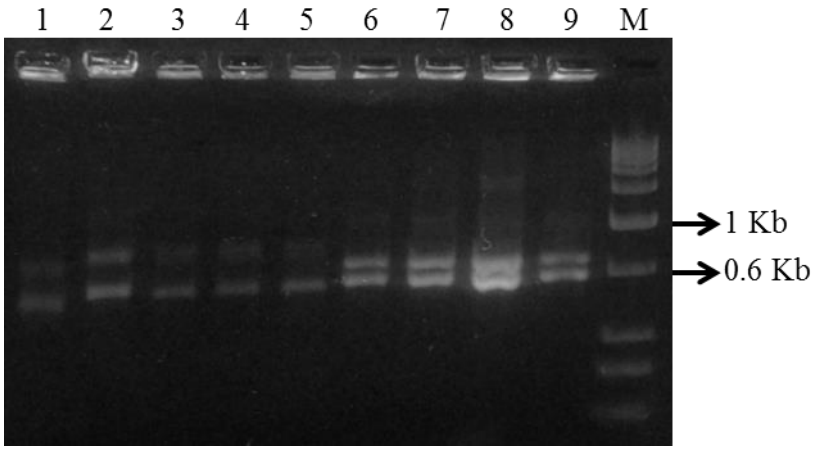


Figure 2.1. 0.8% Agarose gel stained with ethidium bromide for 16S-23S rRNA gene intergenic region PCR amplification.

Lane 1: LGG (Positive control)

Lane 2 - 9: Putative isolates (1-8)

Lane M: Low range DNA marker

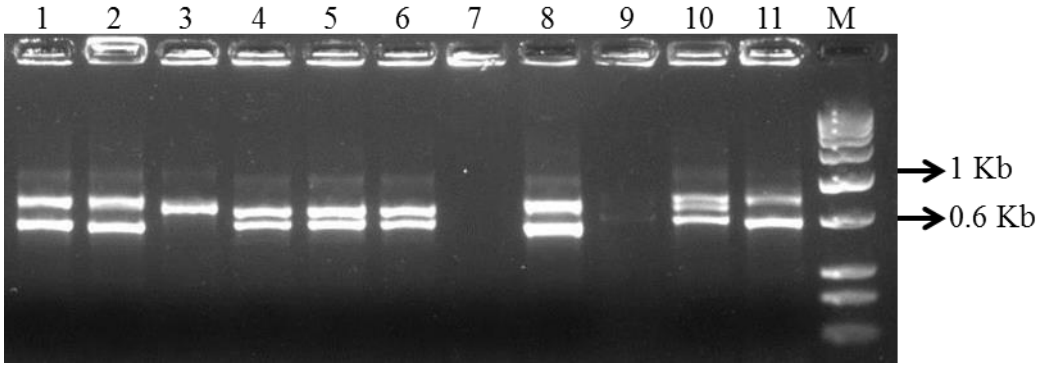


Figure 2.2. 0.8% Agarose gel stained with ethidium bromide for 16S-23S rRNA gene intergenic region PCR amplification.

Lane 1: LGG (Positive control)

Lane 2 - 11: Putative isolates (9-18)

Lane M: Low range DNA marker

The 16S-23S rRNA gene intergenic region amplification profile of isolates was compared with the same of standard *Lactobacillus* strain – LGG on agarose gel. The putative isolates – 1, 2, 3, 4 and 9, 15, 18 in corresponding wells of Figure 2.1 (Lane 2-5) and Figure 2.2 (Lane 2, 8, 11) showed the profile same as standard, respectively.

Table 2.1. 16S-23S rRNA gene intergenic sequence analysis and GenBank submission of selected isolates.

Isolates	Source	GenBank accession no. of 16S-23S sequence	16S-23S sequence based species identification	% similarity of 16S-23S sequence to that of reference strain in GenBank
ASt1	Faeces of adult human	FJ899642	*L. fermentum*	99%
M	Curd of buffalo milk	FJ899641	*L. delbrueckii*	98%
CS5.2	Faeces of human child	FJ899643	*L. casei*	99%
CS23	Faeces of human child	FJ899639	*L. plantarum*	99%
CS24.2	Faeces of human child	FJ870560	*L. plantarum*	98%
CS25	Faeces of human child	FJ899640	*L. rhamnosus*	99%
SCA	Faeces of human child	JX118842	*L. rhamnosus*	99%
SCB	Faeces of human child	JX118841	*L. rhamnosus*	99%
CD12D	Cow dung	JX118840	*L. fermentum*	94%

2.3.2. Bile tolerance

From different concentration of bile salts taken, 3% bile salts in MRS broth was minimal inhibitory concentration of bile for most *Lactobacillus* strains (data not shown), hence the survival of lactobacilli post 2 h incubation in MRS broth containing 3% bile salts was analyzed and the results were subjected to statistical analysis ($p<0.05$). As given in Table 2.2, isolate *L. plantarum* CS23 showed an excellent survival of 14.04% in contrast to standard strains LGG and *L. plantarum* ATCC 8014 which showed survival of 5.34% and 3.88% respectively. Among the other isolates, *L. fermentum* ASt1 showed the high tolerance with 8.27% compared to

both the standard strains. *L. plantarum* CS24.2 and *L. rhamnosus* CS25 showed the survival of 5.40% and 5.91%, respectively which was statistically higher than that of *L. plantarum* ATCC 8014. The cow dung isolate, *L. fermentum* CD12D showed no detectable viable count after 2 h incubation in bile salts containing media.

Table 2.2. Survival rate of *Lactobacillus* strains in the presence of 3% bile salts.

***Lactobacillus* strains**	**Pre incubation**		**Bile tolerance (% survival)**	
	Mean (% survival)	**SD**	**Mean (% survival)**	**SD**
LGG	6.57 (100)	0.13	5.30 (5.34)	0.07
ATCC 8014	6.43 (100)	0.18	5.04 (3.88)	0.05
ASt1	5.93 (100)	0.05	4.85 (8.27)†*	0.02
M	6.43 (100)	0.10	5.11 (4.83)	0.10
CS5.2	6.20 (100)	0.16	4.58 (2.27)	0.05
CS23	6.18 (100)	0.12	5.34 (14.04)†*	0.04
CS24.2	6.21 (100)	0.11	4.96 (5.40)*	0.02
CS25	6.19 (100)	0.13	4.98 (5.91)*	0.03
SCA	6.51 (100)	0.09	4.54 (1.08)	0.06
SCB	6.65 (100)	0.08	4.56 (0.82)	0.08
CD12D	6.59 (100)	0.07	ND	

Mean values (log_{10} cfu/ml) and standard deviations of *Lactobacillus* strains challenged to the presence of 3% bile. The percentage of survival is presented between brackets and results were obtained from three independent experiments. The strains were compared with two different controls (LGG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett test for multiple comparisons versus control group. † mean value of isolates was significantly higher than that of LGG ($P<0.05$). * mean value of isolates was significantly higher than that of *L .plantarum* ATCC 8014 ($P<0.05$).

2.3.3. Acid tolerance

The survival rates of lactobacilli in acidic buffer (pH 2.5 and 2.0) were examined by the difference in viable cell counts following 0 min and 2 h incubation, as shown in Table 2.3. The isolate *L. rhamnosus* CS25 (6.90% at pH 2.5, 5.12% at pH 2.0) showed the highest survival in acidic pH, immediately followed by *L. plantarum* CS23, *L. delbrueckii* M, *L. plantarum* CS24.2 and *L. fermentum* ASt1 (5.70%, 5.40%, 5.40% and 4.03% at pH 2.5, 4.90%, 4.31%, 4.73% and 2.33% at pH 2.0, respectively). LGG and *L. plantarum* ATCC 8014 showed poor tolerance to acid at both the pH.

Table 2.3. Survival rate of *Lactobacillus* strains under acidic condition.

***Lactobacillus* strains**	**Pre incubation**		**Acid tolerance (% survival)**			
			pH 2.5		**pH 2.0**	
	Mean (% survival)	**SD**	**Mean (% survival)**	**SD**	**Mean (% survival)**	**SD**
LGG	6.57 (100)	0.13	4.57 (1.0)	0.11	4.54 (0.91)	0.04
ATCC 8014	6.43 (100)	0.18	4.34 (0.80)	0.14	4.07 (0.42)	0.09
ASt1	5.93 (100)	0.05	4.53 (4.03)†*	0.07	4.29 (2.33)†*	0.06
M	6.43 (100)	0.10	5.18 (5.40)†*	0.09	5.07 (4.31)†*	0.06
CS5.2	6.20 (100)	0.16	4.58 (2.27)†*	0.05	4.56 (2.20)†*	0.07
CS23	6.18 (100)	0.12	4.95 (5.70)†*	0.01	4.88 (4.90)†*	0.01
CS24.2	6.21 (100)	0.11	4.96 (5.40)†*	0.02	4.90 (4.73)†*	0.04
CS25	6.19 (100)	0.13	5.05 (6.90)†*	0.02	4.92 (5.12)†*	0.01
SCA	6.51 (100)	0.09	5.18 (4.65)†*	0.02	5.08 (3.96)†*	0.04
SCB	6.65 (100)	0.08	3.73 (0.12)	0.05	3.29 (0.04)	0.11
CD12D	6.59 (100)	0.07	ND		ND	

Mean values (log_{10} cfu/ml) and standard deviations of *Lactobacillus* strains challenged to acidic conditions. The percentage of survival is presented between brackets and results were obtained from three independent experiments. The strains were compared with two different controls (LGG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett test for multiple comparisons versus control group. † mean value of isolates

was significantly higher than that of LGG ($P<0.05$). * mean value of isolates was significantly higher than that of *L .plantarum* ATCC 8014 ($P<0.05$).

2.3.4. Antimicrobial activity

As shown in Table 2.4, the antagonistic activities of the lactobacilli were examined against Gram negative *S. dysentery*, *P. aeruginosa*, *E. coli* O26:H11, *S* Typhi as well as Gram positive *S. aureus*.

The isolates *L. plantarum* CS24.2, *L. rhamnosus* CS25 and standard strain *L. plantarum* ATCC 8014 had the highest inhibitory activity against both Gram positive and Gram negative bacteria, followed by *L. delbrueckii* M and *L. plantarum* CS23. All the strains tested have shown a strong inhibition towards *E. coli* O26:H11 except *L. fermentum* CD12D.

Table 2.4. Spectrum of antimicrobial activity exhibited by various lactobacilli *

***Lactobacillus* strains**	***Shigella dysentry***	***Staphylococcus aureus* ATCC 6538**	***Pseudomonas aeruginosa* ATCC 5668**	***Salmonella* Typhi MTCC 733**	***Escherichia coli* O26:H11**
LGG	++	+	+	+++	+++
ATCC 8014	+++	++	++	+++	+++
ASt1	+++	+	+/–	+++	+++
M	++	++	++	+++	+++
CS5.2	++	+/–	+	+++	+++
CS23	+++	++	+/–	+++	+++
CS24.2	+++	++	+++	+++	+++
CS25	+++	++	++	+++	+++
SCA	++	++	++	++	++
SCB	++	++	+++	++	+++
CD12D	+/–	+/–	+/–	+/–	+/–

* Symbols refer to the size of the growth inhibition halo: +/–, 1mm; +, 2mm; ++, from 2 to 5 mm; +++, more than 5 mm.

2.3.5. Bacterial adhesion to solvents

The affinities of lactobacilli strains to solvents are shown in Figure 2.3. The isolate *L. rhamnosus* CS25 showed the highest hydrophobicity with 57.9%, followed by *L. rhamnosus* SCB (48.1%), LGG (40.9%), *L. fermentum* ASt1 (33.7%) and *L. plantarum* CS24.2 (32.8%). Most of the strains were observed to have good affinity for chloroform, an electron acceptor, except *L. plantarum* ATCC 8014 (34.4%) and *L. plantarum* CS23 (30.4%). The highest affinities were found with *L. rhamnosus* CS25 (87.2%), followed by *L. delbrueckii* M (78.7%), *L. rhamnosus* SCA (72.5%), LGG (71.6%) and *L. fermentum* ASt1 (71.2%). The affinity for ethyl acetate, an electron donor, on the other hand was quite varied among strains and ranged between as low as 17% to as high as 64%. The isolates *L. delbrueckii* M, *L. casei* CS5.2 and *L. rhamnosus* CS25 exhibited affinities 59.2%, 60.7% and 63.8% while the other strains showed very low affinity for ethyl acetate. The isolate *L. rhamnosus* CS25 showed highest affinity for chloroform and ethyl acetate while *L. plantarum* CS23 exhibited lowest affinity for the same.

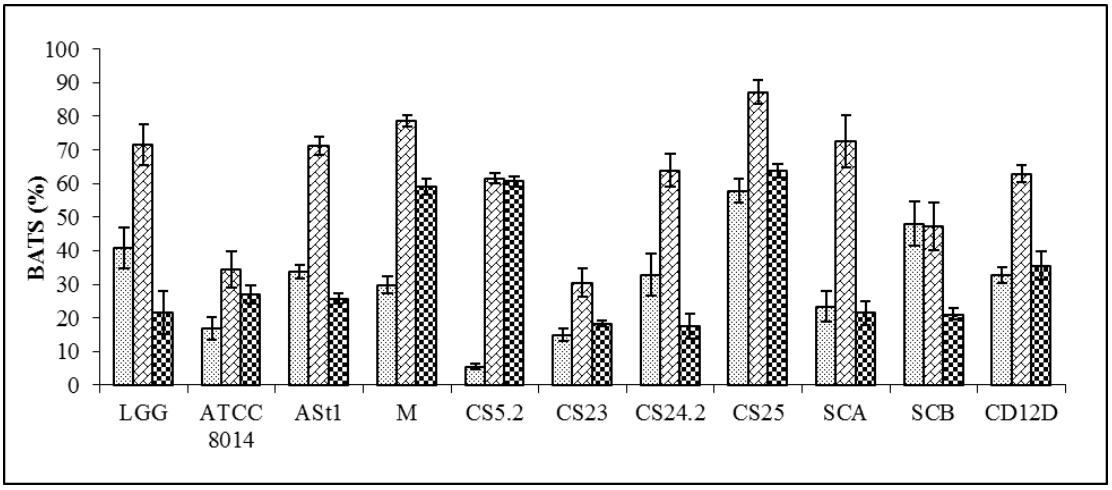


Figure 2.3. Determination of cell surface hydrophobicity (▨), electron donor (▱) and electron acceptor (▮) characteristics of *Lactobacillus* strains by bacterial adhesion to solvent test (BATS).

2.3.6. Aggregation assays

The autoaggregation and coaggregation ability of *Lactobacillus* strains are given in Table 2.5. The autoaggregation ability of *L. plantarum* ATCC 8014 (39%), *L. casei* CS5.2 (46%) and *L. plantarum* CS23 (46%) was comparatively very high, followed by LGG (32.3%), *L. fermentum* ASt1 (29.3%) and *L. plantarum* CS24.2 (28.7%). The ability of *L. rhamnosus* SCA (8.5%), *L. rhamnosus* SCA (11.3%) and *L. fermentum* CD12D (5.9%) to autoaggregate was poor. Heat treatment significantly reduced autoaggregation ability of *Lactobacillus* strains except *L. plantarum* ATCC 8014, *L. casei* CS5.2, *L. rhamnosus* CS25, *L. rhamnosus* SCA and *L. fermentum* CD12D. In order to check role of secreted factors, culture supernatant containing system showed significant increase in autoaggregation property in case of *L. fermentum* ASt1 (41%), *L. plantarum* CS24.2 (40.7%) and *L. rhamnosus* CS25 (21.3%). All the *Lactobacillus* strains were able to coaggregate with both the enteropathogens but the degree of coaggregation was strain specific. *L. plantarum* ATCC 8014, *L. rhamnosus* CS25 and *L. delbrueckii* M aggregated well with *E. coli* (17.7%, 16.0% and 11.7% respectively) as well as *S.* Typhi (13.3%, 9.3% and 9.7% respectively). *L. plantarum* CS23 showed highest coaggregation with *S.* Typhi (17.3%) among other strains tested but coaggregated poorly with *E. coli* (6.3%). The heat labile coaggregation ability was observed in accordance with the autoaggregation ability except in case of *L. fermentum* ASt1 with *E. coli* and *L. plantarum* ATCC 8015 and *L. plantarum* CS24.2 with *S.* Typhi.

Table 2.5. Autoaggregation and coaggregation ability of *Lactobacillus* strains.

	Autoaggregation, mean ± SD (%)			Coaggregation, mean ± SD (%)			
				E. coli O26:H11		*S.* Typhi MTCC 733	
	Control	Heat	10% CFS	Control	Heat	Control	Heat
L. rhamnosus GG	32.3 ± 8.0†	12.0 ± 2.0^{a}	38.0 ± 6.2	6.7 ± 1.5¶	2.3 ± 0.6^{a}	10.7 ± 2.1†	6.0 ± 1.0^{a}
L. plantarum ATCC 8014	39.0 ± 5.6*	38.3 ± 6.4	40.0 ± 1.0	17.7 ± 2.1*	17.3 ± 3.5	13.3 ± 2.1†	4.3 ± 2.1^{a}
L. fermentum ASt1	29.3 ± 2.1†	8.0 ± 2.0^{a}	41.0 ± 2.0^{a}	12.3 ± 1.5†	12.0 ± 1.0	7.3 ± 1.5¶	2.3 ± 1.5^{a}
L. delbrueckii M	10.0 ± 2.6¶	5.0 ± 1.0^{a}	10.7 ± 3.8	11.7 ± 1.5†	2.3 ± 1.5^{a}	9.7 ± 2.1†	7.3 ± 3.1^{a}
L. casei CS5.2	46.0 ± 5.6*	43.0 ± 2.0	44.3 ± 4.7	11.3 ± 1.5†	8.7 ± 2.1	8.3 ± 0.6†	7.7 ± 1.5
L. plantarum CS23	46.0 ± 2.0*	12.7 ± 2.5^{a}	44.7 ± 5.5	6.3 ± 2.1¶	3.3 ± 0.6^{a}	17.3 ± 1.5*	10.0 ± 1.0^{a}
L. plantarum CS24.2	28.7 ± 7.0†	15.7 ± 2.5^{a}	40.7 ± 4.7^{a}	5.7 ± 1.2¶	2.7 ± 1.5^{a}	4.7 ± 0.6¶	4.7 ± 1.2
L. rhamnosus CS25	13.0 ± 4.4¶	10.7 ± 2.1	21.3 ± 3.2^{a}	16.0 ± 1.0*	13.3 ± 1.5	9.3 ± 1.5†	8.7 ± 1.5
L. rhamnosus SCA	8.5 ± 1.3¶	6.7 ± 2.3	7.8 ± 1.5	6.9 ± 1.3¶	7.2 ± 0.9	7.2 ± 1.2¶	7.5 ± 2.2
L. rhamnosus SCB	11.3 ± 2.8¶	4.8 ± 1.3^{a}	10.9 ± 3.5	5.9 ± 0.3¶	3.2 ± 1.2 ^{a}	6.9 ± 0.8¶	2.9 ± 1.6^{a}
L. fermentum CD12D	5.9 ± 3.5	4.3 ± 1.6	5.2 ± 1.9	2.8 ± 1.2	1.9 ± 1.2	2.1 ± 0.9	2.6 ± 0.5

Mean values ± standard deviation; CFS, Cell Free Supernatant.

Results were obtained from three independent experiments. Significant ANOVA was followed by Duncan's multiple comparison test to compare aggregation ability to each other. $^{*, \dagger, \P}$ indicates no significant difference ($p<0.05$) among the group with same symbol Dunnett's test was carried out to compare with respective control group. ^{a} Mean value of treated system with either heat treatment at 85 °C for 30 min or addition of 10% cell free supernatant was significantly different from that of control group ($p<0.05$).

2.4. Discussion

A potential probiotic organism is preferred to be of human origin so that it can lead us to a candidate probiotic eventually targeted for human consumption (Tannock, 1997; Teitelbaum and Walker, 2002). Commercially available probiotic lactobacilli such as LGG, *L. plantarum* 299v, *L. gasseri* LA39 have all been of human origin, as they have been isolated from human faeces (Kawai *et al.*, 2001; Doron *et al.*, 2005; Goossens *et al.*, 2005). With this background, we carried out isolation of *Lactobacillus* strains from human faecal and food samples. The isolates have been assayed *in vitro* for significant probiotic properties such as their tolerance to bile and acidic environment, the degree of antagonism they exhibit against selected pathogenic organisms, cell surface properties which includes cell surface hydrophobicity and aggregation phenotypes.

Before the lactobacilli reach the hindgut region where they are generally known to colonize in human hosts, the organism needs to survive through acidic pH and bile. So the tolerance of an organism to these factors is equally important in selecting a potential probiotic. Lactobacilli do possess bile salt hydrolases (BSH; De Smet *et al.*, 1995). BSH deconjugate the bile salts into lesser toxic form which help them to survive in presence of toxic conjugated bile salts. Among the isolates, *L. plantarum* CS23, *L. fermentum* ASt1, *L. rhamnosus* CS25 and *L. plantarum* CS24.2 showed higher tolerance to bile salts compared to other strains in study. Acid tolerance is reported to be mediated by the change in cell wall structure, a proton-translocating F_1F_0-ATPase, several sodium-proton antiporters, amino acid decarboxylases that use an intracellular hydrogen ion for the decarboxylation of an imported amino acid and the genes involved in malolactic fermentation, during which extracellular malate is imported and decarboxylated (van de Guchte *et al.*, 2002; Cotter and Hill, 2003; Azcarate-Peril *et al.*, 2004). In studies by other authors, the survival of *Lactobacillus* isolates in pH 2.5 yielded similar results; however the survival has not been more than 0.2% (Kim *et al.*, 2007; Perelmuter *et al.*, 2008).

Baccigalupi *et al.* (2005) had observed a range of antimicrobial responses by lactobacilli to various indicator bacteria and also indicated a strong possibility that these activities were not caused due to acidic environment. In the present study, though *L. plantarum* CS23 and *L. plantarum* ATCC 8014 and similarly LGG and *L.*

rhamnosus CS25 are from the same species respectively, the difference in their antimicrobial activity suggests that antimicrobial attributes must be strain specific. Once colonized, the probiotics get an opportunity to exhibit its antimicrobial and immunomodulatory properties in favour of the host organism. The antimicrobial activity of *Lactobacillus* is generally due to production of lactic acid, hydrogen peroxide and/or other antibacterial molecules such as bacteriocin (Eschenbach *et al.*, 1989; Perdigon *et al.*, 2000). It should be interesting to study the mode of antimicrobial activity in different strains.

Xylene, chloroform and ethyl acetate were used to analyse the hydrophobic, electron donor and electron acceptor properties of bacterial surface, respectively. Cell surface hydrophobicity is an important surface characteristic of probiotic bacteria for colonization in GIT because of the hydrophobic nature of the intestinal mucus layer (Colloca *et al.*, 2000). The high hydrophobicity may help in non-specific interaction with components of the mucus layer. The presence of proteinaceous molecules at the bacterial surface contributes to the high hydrophobicity, whereas hydrophilic surface is attributed to the presence of polysaccharides (Greene and Klaenhammer, 1994; Rojas and Conway, 1996). Most of the strains tested had moderate to high hydrophobic surface property. *L. rhamnosus* CS25 (57.9%) showed the highest surface hydrophobicity. *Lactobacillus* strains are reported to be carrying diverse hydrophobicity ranged between 4% - 86% (Colloca *et al.*, 2000). LGG showed 40.9% hydrophobicity which was in agreement with earlier report from another author (Xu *et al.*, 2009). A negative correlation between the affinity of lactobacilli to chloroform and ethyl acetate was observed as reported by Pelletier *et al.* (1997) except *L. casei* CS5.2. All the strains showed strong affinity to chloroform which suggests the acidic or good electron donor bacterial surface property.

Aggregation ability is a very important characteristic as the coaggregation ability of probiotic strains can prevent colonization of invading enteric pathogens. It also leads to the neutralization of carcinogens by coaggregation with bacteria that secrete enzymes which are involved in synthesis or activation of carcinogens (Spencer and Chesson, 1994; Pool-Zobel *et al*., 1996). Bacterial aggregates are formed on the basis of interaction between cell surface molecules such as lipoteichoic acid, carbohydrates and proteins as well as soluble factors. *L. plantarum* ATCC 8014 exhibited strong autoaggregation and coaggregation ability with enteropathogens. Similarly, LGG and

L. plantarum CS23 showed good autoaggregation ability and also coaggregated well with *S.* Typhi. Interestingly, *L. delbrueckii* M and *L. rhamnosus* CS25 showed very low autoaggregation ability but comparatively good coaggregation with both the enteropathogens. This might be due to very high surface charge properties of these strains as observed in our study which enabled them to coaggregate well with test pathogens which may have different surface charge properties. Heat treatment remarkably inhibited the autoaggregation ability of lactobacilli studied except *L. plantarum* ATCC 8014, *L. rhamnosus* CS25 and *L. casei* CS5.2 ($p<0.05$). Similar effects of heat treatment were also observed on coaggregation ability of these strains with enteropathogens except *L. fermentum* ASt1 with *E. coli* and *L. plantarum* CS24.2 with *S.* Typhi. This suggests the role of heat labile proteinaceous surface molecules in aggregation ability of these strains. There was an inhibition of coaggregation ability of *L. plantarum* ATCC 8014 with *S.* Typhi by heat treatment but no effect on autoaggregation and coaggregation with *E. coli* which can be correlated with its limiting surface proteins role represented by low hydrophobicity of this strain. The inability of heat treatment to affect aggregation ability of other strains might be because of heat stable highly glycosylated proteinaceous molecules and/or non proteinaceous molecules in aggregation phenotype. Additionally, in order to assess the role of secreted molecules in autoaggregation ability, bacterial cells with its own spent medium showed significantly increased autoaggregation ability in case of *L. fermentum* ASt1, *L. plantarum* CS24.2 and *L. rhamnosus* CS25 ($p<0.05$). In general, the present data shows the different mechanism and factors playing a role in aggregation ability of different *Lactobacillus* strains and suggests the strain specific cell surface characteristics.

The primary characterization of *Lactobacillus* strains isolated from different sources showed varied attributes. The isolates were further analysed for adhesion abilities to various *in vitro* and strain specific antagonistic activities towards enteropathogens in subsequent chapters.

CHAPTER 3

ADHESION ABILITY OF *LACTOBACILLUS* STRAINS TO INTESTINAL EPITHELIAL CELL LINES AND COMPETITIVE ADHESION INHIBITION OF ENTEROPATHOGENS

"A universe with a God would look quite different from a universe without one. A physics, a biology where there is a God is bound to look different. So the most basic claims of religion are scientific. Religion is a scientific theory."

- Richard Dawkins

Chapter 3

Adhesion ability of *Lactobacillus* strains to intestinal epithelial cell lines and competitive adhesion inhibition of enteropathogens.

3.1. Introduction

The adherence of bacteria to the surface of intestinal mucosa is a key process for colonization and persistence in gastrointestinal tract (GIT) and prevents elimination by peristaltic force. Therefore, adhesion is an important property for both pathogenic bacteria as well as bacteria belonging to normal gut microbiota (Coconnier *et al.*, 1993; Servin and Coconnier 2003). Once established in GIT, probiotic strains can perform health promoting functions such as modulating the host immune system and exerting antagonistic activity against enteropathogens (Perdigon *et al.* 2002; Kim *et al.* 2008). Bacterial adhesion is reported to be a non-specific physical interaction between two surfaces which is followed by specific interaction between surface adhesion molecules of bacteria and complementary receptors present on the mucus layer and the underlying gut epithelial cells (Rojas and Conway 1996; Perez *et al.* 1998). The intestinal epithelial cells are covered with thick mucus layer that physically protect these cells from direct exposure to luminal bacteria and food antigens. The mucus layer is made up of a glycoprotein called mucin and provides the first site for bacterial adherence (Lievin-Le Moal and Servin 2006). Mucin is majorly synthesized by goblet cells and helps in colonization of bacteria by providing a site for attachment. Several *in vitro* models have been established to study bacterial adhesion and competitive inhibition ability of probiotic strains (Coconnier *et al.* 1992; Kim *et al.* 2007). Adherence of different probiotic bacteria has been studied using various eukaryotic cell lines of human origin such as Caco-2 and HT-29 (Coconnier *et al.*, 1992; Tuomola and Salminen, 1998; Laparra and Sanz, 2009). The human intestinal epithelial cell line Caco-2 has been extensively used to study adhesion

ability of lactobacilli. This is primarily because the Caco-2 cells express morphological and functional differentiation of mature enterocytes including polarization and functional brush border *in vitro* (Sambuy *et al.*, 2005). The bacteria are first exposed to mucin before it reaches the intestinal epithelial layer. Thus, the adhesion of *Lactobacillus* strains to immobilized mucin is also important for screening and selection of ideal probiotic strain.

Gut pathogens do possess enzymes that degrade mucin and cross the protective barrier which lead to infection (Smith *et al.* 1994; Lievin-Le Moal and Servin 2006). Several diseases of GIT are caused by *Salmonella enterica* serotype Typhimurium and *Escherichia coli* (Doyle and Schoeni, 1984; Rabsch *et al.*, 2002). To cause infection, adhesion to epithelium is essential; preventing pathogenic bacterial adhesion to the epithelial cells is an effective strategy to reduce pathogen associated illness. The epithelium of GIT can be protected from colonization of pathogen by a number of mechanisms including antibiotic treatment. Probiotic therapy is seen as potential attractive alternative to antibiotic treatment to overcome problems such as emergence of multidrug-resistant bacteria and imbalance of resident gut microbiota (Fooks and Gibson, 2002; Servin, 2004). *Lactobacillus* strains can inhibit the adhesion of pathogens by direct competition for mucus and other attachment surfaces in GIT. It is believed while inhibition of pathogens is through soluble effectors; competitive exclusion of pathogenic bacteria is through direct competition for common attachment sites on gut epithelium (Coconnier *et al.*, 1997; Lehto and Salminen, 1997; Jankowska *et al.*, 2008). The competitive inhibition studies with enteropathogens *in vitro* would give primary information about the efficacy of selected probiotic strains to antagonize the mucin adhesion of that enteropathogen. Several *Lactobacillus* strains are being explored as probiotic bacteria to restore and maintain normal gut microbiota as well as treatment of gastrointestinal diseases. Out of these, LGG is the best studied and widely accepted standard probiotic strain (Yan and Polk, 2002). This strain which has originated from intestinal tract of a healthy human is being studied for treatment of acute diarrhea and prevention of inflammatory bowel diseases (Doron *et al.*, 2005; Bousvaros *et al.*, 2005).

In present chapter, *Lactobacillus* strains isolated were further characterized for their adhesion ability with different *in vitro* models such as Caco-2 cells, HT-29 cells and mucin and compared with established probiotic strain LGG. The competitive

adhesion assays were also designed to mimic different *in vivo* conditions with lactobacilli and enteropathogens such as *E. coli* O26:H11and *S.* Typhi MTCC 733. Further, the secreted molecules from lactobacilli were also analyzed for inhibition of enteropathogens inhibition to intestinal epithelial cell lines.

3.2. Materials and Methods

3.2.1. Cell culture

The human colonic adenocarcinoma cell line Caco-2 and HT-29 were obtained from National Centre for Cell Science (NCCS), Pune, India, which were routinely cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) media, at 37°C temperature in a humidified atmosphere containing 5% CO_2 / 95% air atmosphere. The media were supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), 10 mM non-essential amino acids, 1 mM sodium pyruvate and 50 µg/ml gentamycin. The media lacked gentamycin whenever antibiotic free medium was used.

3.2.2. Adhesion assays to intestinal epithelial cell lines

For adhesion assay, the Caco-2 cells were seeded at a density of 10^4 cells/well in 24-well standard tissue culture plates (Corning Incorporated, NY, USA) and maintained for 2 weeks following confluence. Before the adhesion assay, Caco-2 monolayers were pre incubated with antibiotic free medium for 4 hours. The pH of the media used for adhesion assay was adjusted to 6.5 with 1 M HCl before use. Lactobacilli cells were harvested by centrifugation (10,000 *g*, 2 min, and 4°C) and washed twice with Dulbecco's phosphate buffered saline (PBS), pH 7.0 (Sigma-Aldrich) and cell density was adjusted to desired level by measuring absorbance at 600nm. The exact number of viable lactobacilli used in the assays was determined for each experiment by plate counting on MRS agar. Wells with Caco-2 monolayers were inoculated with 1×10^8 viable cells of each bacterial cell suspension and incubated at 37°C for 90 min in 5% CO_2 / 95% air atmosphere. Un-adhered bacterial cells were then withdrawn from the wells and the Caco-2 monolayers were washed twice with 1 ml PBS each. The Caco-2 cells were lysed by treatment with 0.5 ml 0.05% (v/v) Triton X-100 in PBS for 20 min at 37°C. The Caco-2 lysate including bound lactobacilli were plated after

appropriate dilution on MRS agar plate and the enumeration was done following 48 h incubation at 37°C. It was also determined that a 30 min treatment of 0.05% (v/v) Triton X-100 in PBS at 37°C did not affect the viability of lactobacilli (data not shown). At the end of each experiment, three randomly preselected unused wells were trypsinized and numbers of Caco-2 cells were counted on hemocytometer. The average value of Caco-2 cell count was used for expressing the adhered bacteria per Caco-2 cell.

For adhesion assay to HT-29 cells, the same procedure was followed except that the HT-29 cells were seeded at a density of 1×10^5 cells/ml in 24-wells tissue culture plates and maintained for a week to achieve confluent growth.

3.2.3. *In vitro* competitive adhesion assays

For competitive adhesion assays, post confluent HT-29 cells were pre incubated with antibiotic free medium for 4 h. Lactobacilli and pathogenic strains were harvested as described above and the cell density was adjusted by measuring absorbance at 600nm to get 1×10^8 cfu/well in 50 µl cell suspension for each culture.

To mimic the different *in vivo* conditions, the different adhesion assays were designed as competitive inhibition, adhesion inhibition and displacement of enteropathogenic *E. coli* O26:H11 by lactobacilli. For competitive inhibition assay, lactobacilli and pathogen were provided with an equal chance for binding at the same ratio. An adhesion inhibition assay was performed to investigate the role of lactobacilli growth and its ability to protect intestinal cells from being colonized by pathogen. In displacement assay, the ability of lactobacilli to displace colonized pathogen from intestinal epithelium was evaluated. The pathogen was allowed to adhere first to HT-29 cells before lactobacilli adhesion. In competitive inhibition, lactobacilli and *E. coli* were added at the same time to the HT-29 cells in same number (1×10^8 cfu/well), and co-incubated for 90 min. In adhesion inhibition and displacement assay, lactobacilli and *E. coli* were allowed to adhere to HT-29 cells for 90 min, respectively. Un-adhered bacterial cells were then removed by washing wells thrice with 1 ml PBS each time. The *E. coli* and lactobacilli were then added to respective wells and incubated for an additional 90 min. At the end of each assay, HT-29 cells

were lysed by treatment with 0.5 ml of 0.05% (v/v) Triton X-100 in PBS for 20 min at 37°C. The HT-29 lysate including bound bacterial cells were plated after appropriate dilution on Luria and MRS agar plate for *E. coli* and lactobacilli, respectively. The enumeration was done after 18-24 h incubation at 37°C. HT-29 cells co incubated with *E. coli* alone were taken as control and the number of bacteria adhering to HT-29 cells was considered as 100%. The treatment with 0.05% (v/v) Triton X-100 in PBS for 30 min at 37°C did not affect the viability of *E. coli* (data not shown).

To check the role of any secretary molecules in adhesion inhibition of *E. coli*, the cell free supernatant (CFS) of lactobacilli were analyzed for antimicrobial activity. CFS was obtained from overnight grown culture of lactobacilli by separating cells by centrifugation (10,000 g, 15 min, and 4°C). One microlitre of filter sterilized CFS was mixed with equal volume of 1×10^8 cfu in PBS and incubated at 37°C for 1 h. After the incubation, cells were washed twice with PBS before being resuspended in 1 ml of DMEM and co incubated with HT-29 cells for 90 min. The enumeration was done as described above. The incubation of *E. coli* with MRS did not affect the viability of bacteria in 90 min (data not shown). All assays were performed between 40 to 60 passages of HT-29 cells and repeated three times in duplicates.

3.2.4. Mucin adhesion assay

Lactobacillus strains were assayed for adhesion to immobilized mucin in 96-well microtiter plates (Nunc, Nunclone Delta SI, Denmark) under sterile condition. Plates were coated with 300 µl of porcine mucin (0.5 mg/ml; Sigma-Aldrich) in sterile Dulbecco's phosphate buffered saline (PBS), pH 7.0 (Sigma-Aldrich) at 4 °C overnight. Wells were washed twice with sterile PBS to remove unbound mucin. Two hundred microliters of each strain (1×10^8 cfu/ml) were added to respective wells and allowed to adhere for 90 min at 37°C. Un-adhered bacterial cells were then withdrawn and wells were washed five times with 300 µl sterile PBS each. Adhered cells were released by treatment with 300 µl 0.05% (v/v) Triton X-100 in sterile PBS for 20 min at 37°C. The released bacterial cells were plated after appropriate dilution on MRS agar and enumeration was done following 48 h incubation at 37 °C.

3.2.5. Competitive adhesion assay to mucin

Lactobacillus strains were also assayed for competitive adhesion to mucin in the presence of enteropathogens – *E. coli* and *S.* Typhi. Bacterial cells were processed as described above and cell density was adjusted to get 2×10^8 cfu/ml in sterile PBS. For competitive adhesion assay, one hundred microliters each of lactobacilli and enteropathogens were added to the mucin coated wells at the same time and co-incubated for 90 min. The adhered bacteria were released by treatment with Triton X-100 and enteropathogens were enumerated by plating on Luria agar plates. Adhesion of respective enteropathogens alone was taken as control and the number of bacteria adhered to mucin was considered as 100% in order to express percentage inhibition.

3.2.6. Statistics

Values are given as mean along with the standard deviation (SD) of triplicate independent experiments. Significant ANOVAs were followed by Dunnett test in the case of adhesion assays and compared with the respective controls ($p<0.05$). All analysis was conducted using SigmaStat 3.5 software.

3.3. Results

3.3.1. Adhesion of lactobacilli to intestinal epithelial cell lines

Two different cell lines (Caco-2 and HT-29) of human intestinal epithelium origin were employed to evaluate the adhesion potential of lactobacilli. The established probiotic strain, LGG was used to compare the adhesion ability and dairy strain, *L. plantarum* ATCC 8014 was also used for the same.

Among the isolates, none showed adherence ability to Caco-2 cells as good LGG. But isolates, *L. plantarum* CS23, *L. plantarum* CS24.2 and *L. rhamnosus* SCA were able to adhere significantly higher compared to *L. plantarum* ATCC 8014 (8 cells/Caco-2) with 17, 19 and 18 cells per Caco-2 cells, respectively. Adhesion ability of *L. fermentum* ASt1 (3 cells/Caco-2) and *L. fermentum* CD12D (3 cells/Caco-2) was very poor among the isolates (Figure 3.1).

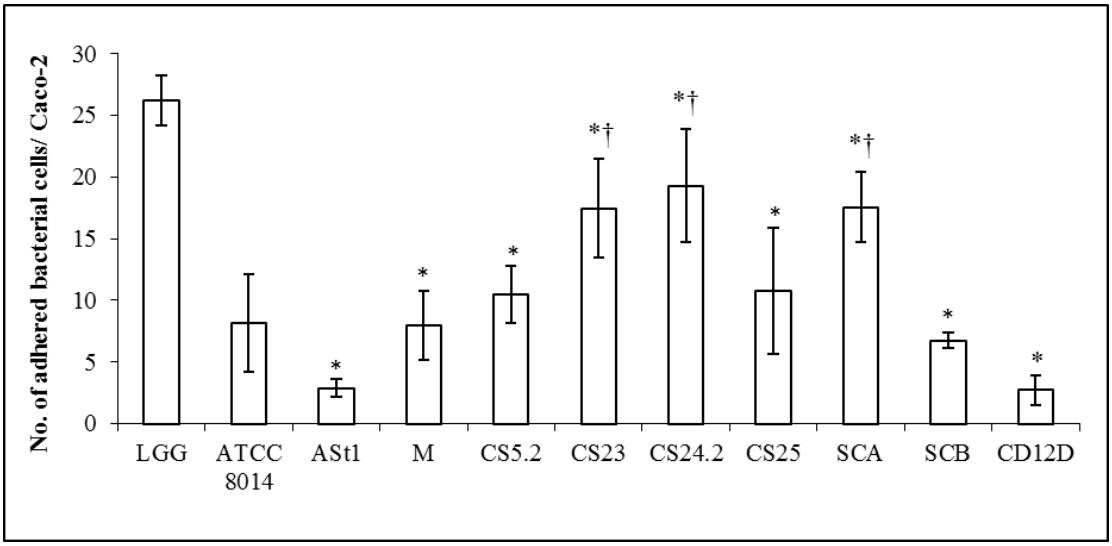


Figure 3.1. Adhesion of *Lactobacillus* isolates to Caco-2 epithelial cell line compared with standard strains LGG and *L. plantarum* ATCC 8014. Numbers of adhering bacteria are the means and error bar represents the standard deviation from three independent experiments. The strains were compared with two different controls (LGG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett test for multiple comparisons versus control group. * mean value of isolates was significantly different than that of LGG ($P<0.05$). † mean value of isolates was significantly different than that of *L .plantarum* ATCC 8014 ($P<0.05$).

Similarly, adhesion potential of lactobacilli was also analysed with HT-29 cells. Overall, the adhesion ability of all lactobacilli was high with HT-29 cells compared to the same with Caco-2 cells (Figure 3.2). Adhesion ability of *L. delbrueckii* M (27 cells/HT-29), *L. casei* CS5.2 (30 cells/HT-29), *L. plantarum* CS23 (29 cells/HT-29) and *L. plantarum* CS24.2 (33 cells/HT-29) was statistically same to that of LGG (30 cells/HT-29). All the isolates, except *L. fermentum* ASt1 (8 cells/HT-29) and *L. fermentum* CD12D (2 cells/HT-29) showed significantly high adhesion ability to HT-29 cells when compared with *L. plantarum* ATCC 8014 (18 cells/HT-29).

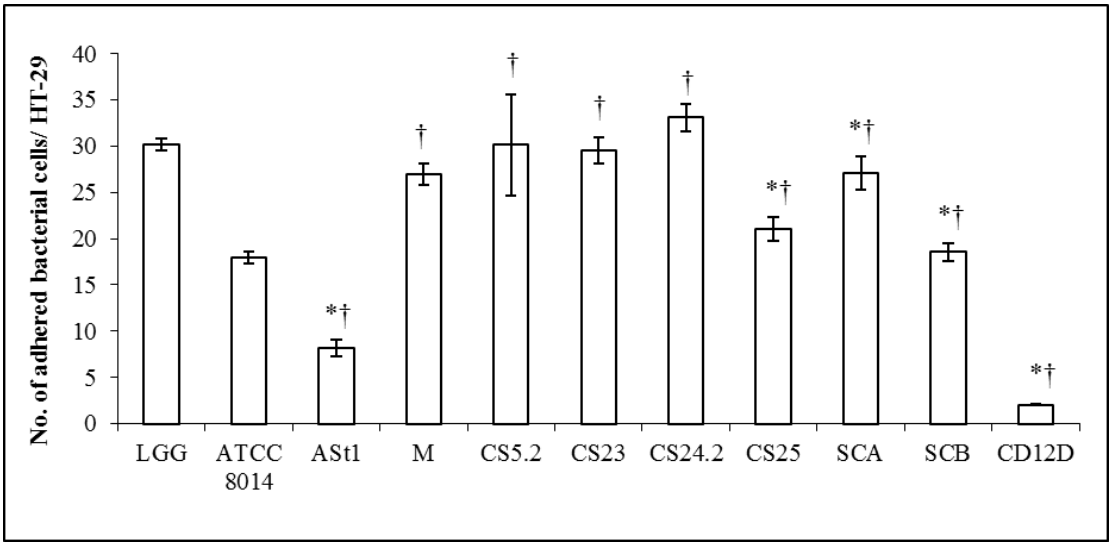


Figure 3.2. Adhesion of *Lactobacillus* isolates to HT-29 epithelial cell line compared with standard strains LGG and *L. plantarum* ATCC 8014. Numbers of adhering bacteria are the means and error bar represents the standard deviation from three independent experiments. The strains were compared with two different controls (LGG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett test for multiple comparisons versus control group. * mean value of isolates was significantly different than that of LGG ($P<0.05$). † mean value of isolates was significantly different than that of *L .plantarum* ATCC 8014 ($P<0.05$).

3.3.2. Adhesion of lactobacilli to immobilized mucin

All the strains were able to adhere immobilized mucin in microtiter plates. However, the degree of adhesion varied among the strains studied, including the strains belonging to the same species of lactobacilli (Figure 3.3). LGG showed highest adhesion to mucin among the strains analysed. The isolates - *L. delbrueckii* M and *L. plantarum* CS24.2 showed statistically significant adhesion which was comparable with LGG ($p<0.05$). The adhesion ability of *L. plantarum* ATCC 8014, *L. plantarum* CS23, *L. rhamnosus* CS25 and *L. rhamnosus* SCA was comparatively moderate and statistically similar to each other ($p<0.05$). The isolates *L. casei* CS5.2 and *L. rhamnosus* SCB were less adherent to mucin compared to other adhering strains. The adult stool isolate *L. fermentum* ASt1 and cow dung isolate *L. fermentum* CS12D showed the poorest binding capacity. The same results were obtained when

considering the percentage of the number of bacteria bound to the number of bacteria added to each well (data not shown).

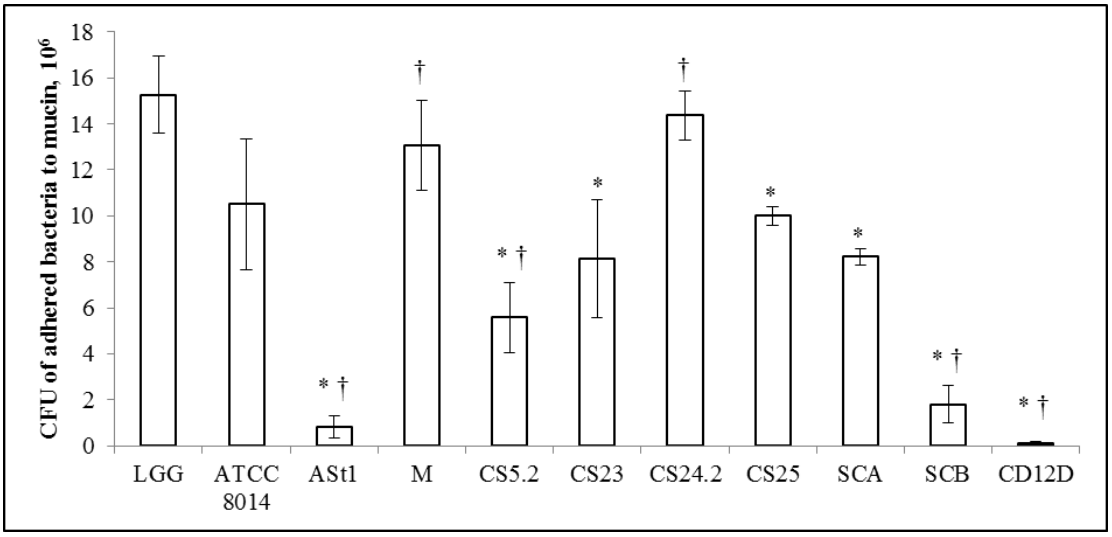


Figure 3.3. Adhesion of *Lactobacillus* strains to immobilized mucin in microtiter plate. Numbers of adhering bacteria are the means and error bar represents the standard deviation from three independent experiments. The strains were compared with two different controls (LGG and *L. plantarum* ATCC 8014) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett test for multiple comparisons versus control group. * mean value of isolates was significantly different than that of LGG ($P<0.05$). † mean value of isolates was significantly different than that of *L. plantarum* ATCC 8014 ($P<0.05$).

3.3.3. Competitive adhesion assays to HT-29 cells

To mimic various *in vivo* conditions, adhesion assays were designed to include competitive inhibition, adhesion inhibition and displacement assays between lactobacilli and enteropathogenic *E. coli* O26:H11. The numbers of enteropathogen bound to HT-29 cells under different competitive adhesion assays are given in Figure 3.4.

Under competitive inhibition, lactobacilli and *E. coli* were allowed to adhere to HT-29 cells at the same time in equal ratio. All the strain showed inhibition of *E. coli* to HT-29 cells. LGG, *L. delbrueckii* M and *L. plantarum* CS24.2 significantly reduced the adhesion of *E. coli* by 47.5%, 61.8% and 41.2%, respectively ($p<0.05$). To study

the ability of adhered lactobacilli to inhibit adhesion of *E. coli*, the adhesion inhibition assay was carried out. The adhesion inhibition ability of lactobacilli was slightly poor compared to competitive inhibition. The pre-colonization of LGG, *L. delbrueckii* M, *L. casei* CS5.2 and *L. plantarum* CS24.2 to HT-29 cells inhibited the *E. coli* adhesion by 36.5%, 67%, 55.1% and 33.9%, respectively. *L. plantarum* ATCC 8014 did not show any significant effect on *E. coli* adhesion to HT-29 cells. To analyze the ability of lactobacilli to displace the adhered enteropathogen from intestinal epithelial cells, displacement assay was carried out with initially allowing adhesion of *E. coli* prior to addition of lactobacilli to HT-29 cells. *L. plantarum* ATCC 8014, *L. delbrueckii* M, *L. casei* CS5.2, *L. plantarum* CS23, *L. plantarum* CS24.2 and *L. rhamnosus* CS25 significantly displaced the *E. coli* by 73.9%, 89.5%, 87.1%, 90.9%, 62.9% and 71.5%, respectively ($p<0.05$).

Overall, all the strains were able to interfere with *E. coli* adhesion to HT-29 cells under three adhesion assays. The effects were strain specific and varied under different assays.

Further, we carried out enumeration of adhered lactobacilli in all three adhesion assays for selected lactobacilli. LGG and *L. plantarum* CS24.2 were able to adhere to HT-29 cells under all the three competitive conditions. Figure 3.5 shows the number of adhered lactobacilli and *E. coli* in different adhesion assays.

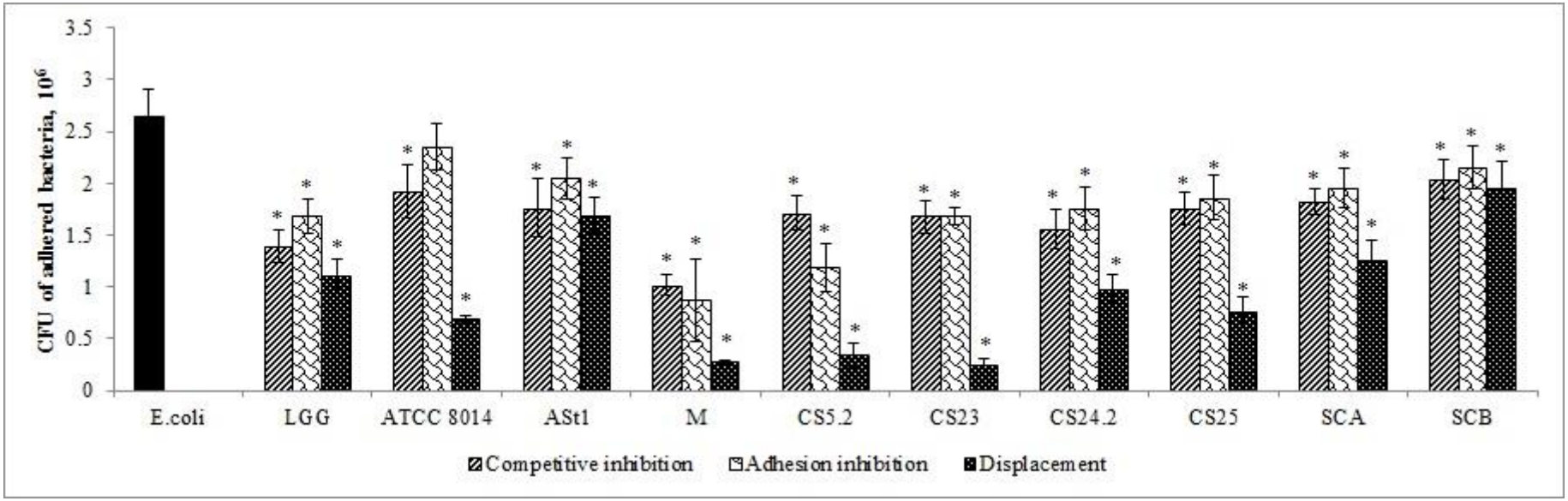


Figure 3.4. Adhesion of *Escherichia coli* O26:H11 to HT-29 cells following competition with, inhibition from and displacement by lactobacilli. Three different adhesion assays were carried out i.e. competitive inhibition, adhesion inhibition and displacement. Adhesion of *E. coli* in the absence of lactobacilli is considered as control. Each bar represents mean value and standard deviation as error bar. Significant ANOVAs were followed by Dennett test for multiple comparisons versus control group.

* mean value of adhesion was significantly lower than that of control ($P<0.05$).

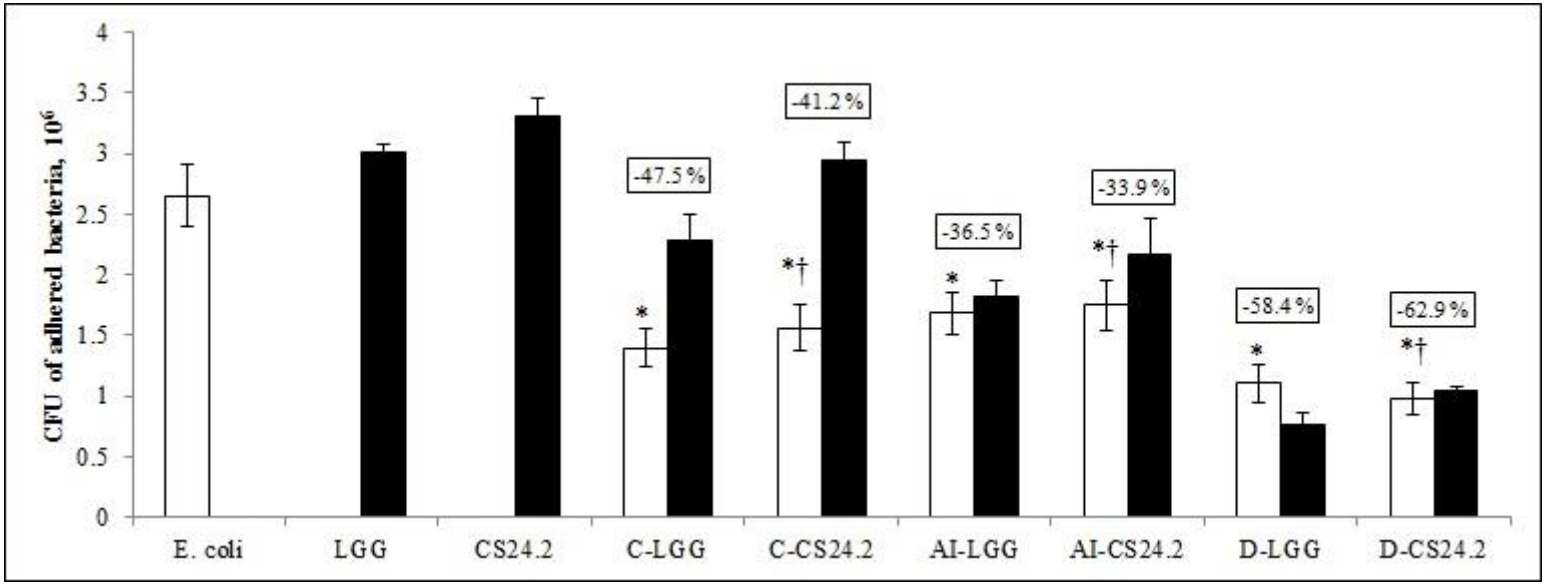


Figure 3.5. Adhesion of *Escherichia coli* O26:H11 to HT-29 cells in the presence of lactobacilli under different competitive conditions. The adhesion assays were competition inhibition (C), adhesion inhibition (AI) and displacement (D). Each bar represents mean value and standard deviation as error bar of three independent experiments. Black bars represent LGG or *L. plantarum* CS24.2; white bars represent the adhesion of *E. coli*. HT-29 cells co incubated with *E. coli* alone were taken as control. The square box above each bar shows the percentage reduction in adhesion of *E. coli* as compared to control. Significant ANOVAs were followed by Dunnett test for multiple comparisons *v.* control group. * mean value of adhesion was significantly lower than that of control ($P<0.05$). † Within LGG or *L. plantarum* CS24.2, mean value of adhesion of *E. coli* was statistically similar under same adhesion assay ($p<0.05$).

3.3.4. Competitive inhibition of enteropathogens to mucin

The competitive inhibition of enteropathogens by lactobacilli is shown in Figure 3.6. The isolates- *L. fermentum* ASt1 and *L. casei* CS5.2 were unable to interfere with *E. coli* adhesion to mucin while all other strains showed statistically significant competitive inhibition ($p<0.05$). LGG, *L. plantarum* ATCC 8014 and *L. delbrueckii* M exhibited high competition ability compared to other strains and inhibited the adhesion of *E. coli* by 59%, 79.1% and 62.2%, respectively. All the strains competitively inhibited the adhesion of *S.* Typhi and the degree of inhibition were overall similar and ranged between 27.3% - 58.9%.

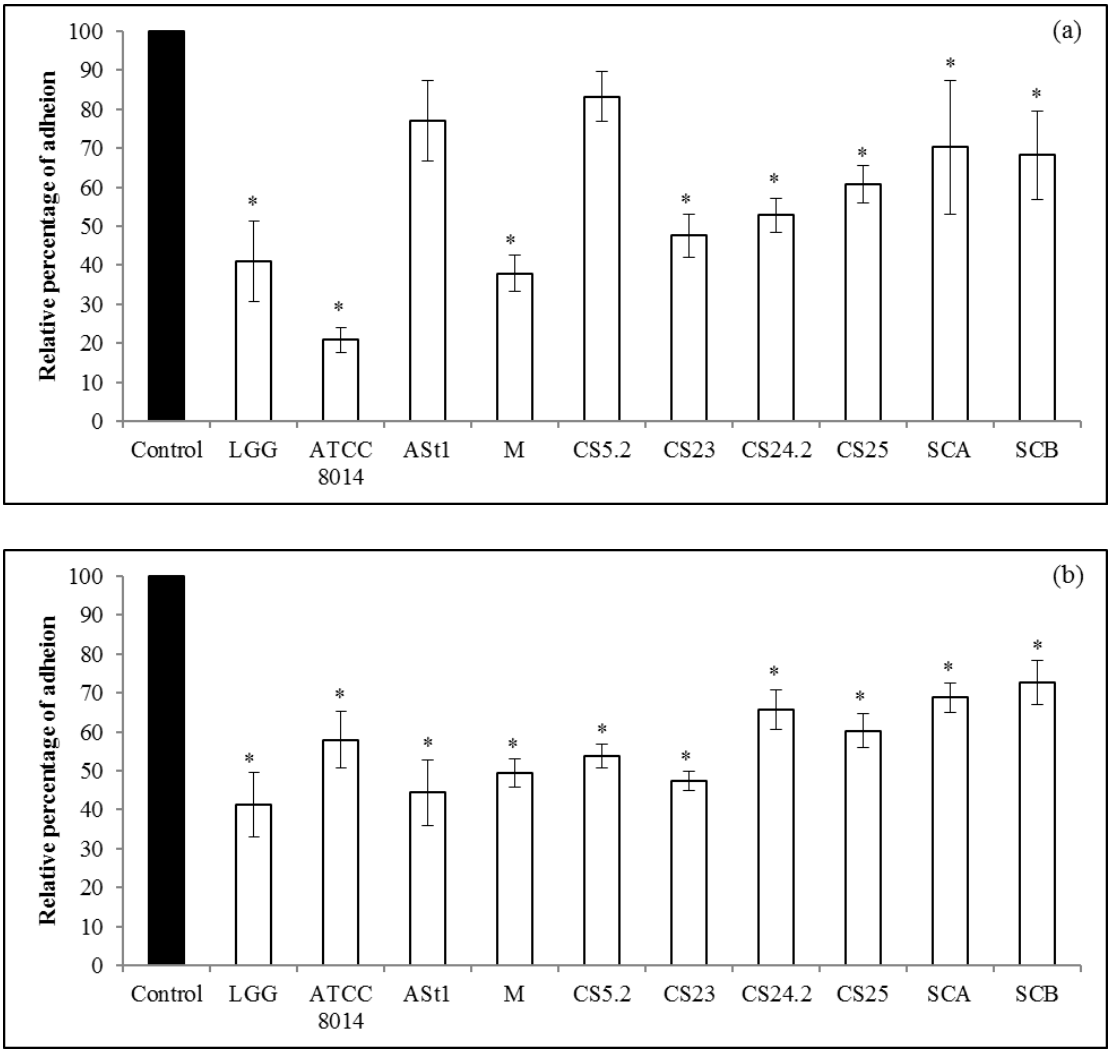


Figure 3.6. Competitive inhibition of (**a**) *Escherichia coli* O26:H11 and (**b**) *Salmonella enterica* serovar Typhi MTCC 733 to mucin by *Lactobacillus* strains in microtiter plate assay. Adhesion of *E. coli* and *S.* Typhi in the absence of lactobacilli is denoted as control. Each bar shows the mean value and error bar as standard

deviation of three independent experiments. Significant ANOVA was followed by Dunnett test to compare with respective control group. * mean value was significantly lower than that of control ($p<0.05$).

3.3.5. Role of cell-free supernatant of lactobacilli in inhibitory activity

To check the role of any secretory molecules of lactobacilli in the adhesion inhibition of *E. coli* O26:H11, the pathogen was pre-incubated with cell-free supernatant of lactobacilli before adhesion to HT-29 cells (Figure 3.7). All the strains showed significant reduction in adhesion of *E. coli* to HT-29 cells. *L. delbrueckii* M and *L. plantarum* CS24.2 showed highest inhibition with decrease in adhesion of *E. coli* by 62.5% and 59.1%, respectively.

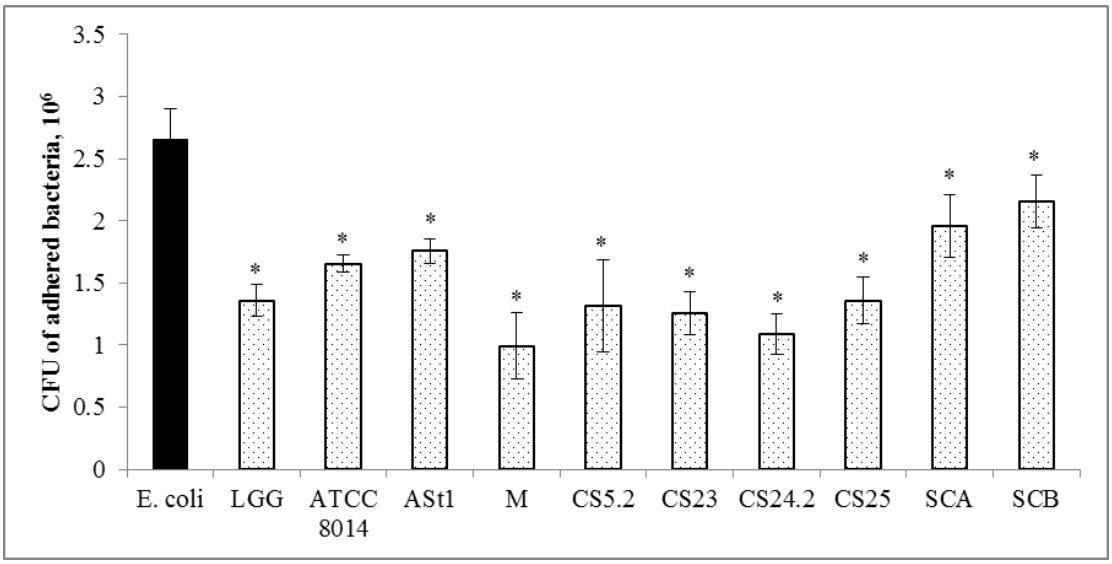


Figure 3.7. Adhesion of *E. coli* O26:H11 to HT-29 cells in the presence of cell-free supernatant of different lactobacilli. Each bar represents mean value and standard deviation as error bar of three independent experiments. HT-29 cells co incubated with *E. coli* alone were taken as control. Significant ANOVAs were followed by Dunnett test for multiple comparisons *v.* control group. * mean value of adhesion was significantly lower than that of control ($P<0.05$).

3.4. Discussion

The importance of lactic acid bacteria in the ecological niche of the gastrointestinal tract is well established. An imbalance in the normal flora is directly linked with a disease condition, wherein lactobacilli and Bifidobacteria are particularly used as

important indicators of health (Resnick and Levin, 1981; Walter, 2008). Many strains of lactobacilli are being used as a starter culture in dairy and fermentation industries and also used as a supplement in food products for improving human health (Tannock *et al.* 1999; Doron *et al.* 2005). *Lactobacillus* strains have also been widely explored as therapeutic agents, as many of them possess health promoting and corrective properties (Isolauri *et al.*, 2001; Kim *et al.*, 2008). To colonize and persist in the GIT, adhesion to mucosal surface becomes a key criterion for any probiotic bacteria. The bacterial adherence is in turn a complex process and involves several mechanisms which depend on cell surface properties and extracellular protein profile of bacteria (Gibbons, 1996). Adhesion to intestinal epithelium is a desirable criterion for *Lactobacillus* colonization and persistence in gastrointestinal tract before it can confer its probiotic effects to the host (Guarner and Malagelada, 2003; Winkler *et al.*, 2007). The mucus layer of intestinal tract provides a primary site for bacterial attachment and subsequent colonization. The screening of probiotic bacteria on the basis of its ability to bind mucin and intestinal epithelial cells *in vitro* are considered important criteria in order to meet ideal probiotic for *in vivo* application. Intestinal epithelial cell lines (Caco-2 and HT-29) are widely used and accepted for study related to probiotic and pathogens adhesion to intestinal epithelium (Sambuy *et al.*, 2005; Laparra and Sanz, 2009). The probiotic effects are reported to be strain specific and varied among the different strains of same species (Luyer *et al.*, 2005). Thus, the rational selection of probiotic lactobacilli is important to target the specific disease condition. LGG is widely studied and considered as one of the best probiotic strain. It is found to be an effective therapeutic agent in several gut associated infections and disorders (Doron *et al.*, 2005).

Among the strains tested the adhesion ability of *L. plantarum* CS24.2 was highest and also comparable with LGG in case of adhesion to HT-29 cells and mucin. *L. delbrueckii* M also showed statistically comparable adhesion ability with LGG ($p<0.05$) for HT-29 cells and mucin. *L. plantarum* CS23 and *L. rhamnosus* SCA showed comparative good adhesion ability to Caco-2 and HT-29 cells and it was high compared to that of *L. plantarum* ATCC 8014. Interestingly, *L. delbrueckii* M showed moderate adhesion to Caco-2 cells compared to LGG, whereas the adhesion ability of this strain to HT-29 cells and mucin was similar to LGG. Hence, it is important to study bacterial adhesion to different *in vitro* models in order to gain knowledge on the

overall adhesion ability of probiotic strains to different components of intestinal mucus layer and underlying receptors on epithelial cells. This information can then be extrapolated to select probiotic strains for *in vivo* application. Laparra and Sanz (2009) suggested that the difference in adhesion ability depends on the strain and the type of *in vitro* model used for evaluation and it can be explained with the presence of molecules involved in the interaction.

Adhesion is necessary for pathogens to cause enteric infection and this host-microbial interaction can be effectively inhibited by probiotic bacteria (Spencer and Chesson, 1994; Boris *et al.*, 1998). In order to study the role played by lactobacilli to protect against pathogen colonization, the competition of lactobacilli and adhesive enteropathogenic *E. coli* O26:H11 for adhesion to HT-29 cells was studied. When incubated along with pathogen, all lactobacilli showed good competitive inhibition and pathogen adhesion to HT-29 cells decreased by around 23-61%. Lee *et al.*, (2003) under similar assay condition reported around 20-50% inhibition of strains of *E. coli* and *S.* Typhimurium adhesion to Caco-2 cells by LGG. It is suggested that the degree of competition is strain dependent and can be determined by the affinity of adhesion molecules present on surface of respective bacteria for the receptor binding sites that they are competing for (Lee and Puong, 2002). In our study, we observed the high number of adhered lactobacilli compared to the same numbers of *E. coli* which supports the competition for common attachment sites.

In adhesion inhibition studies, the capacity of lactobacilli to prevent pathogens adhesion was analyzed. Except *L. plantarum* ATCC 8014, all lactobacilli were found to exclude pathogen adhesion by around 19-67% when HT-29 cells were pre-incubated with lactobacilli. Whether the exclusion of pathogens is due to competition for common adhesion receptors or steric hindrance of adhered lactobacilli, needs to be understood. Collado *et al.*, (2007) have shown 30.3% and 27.9% adhesion inhibition of *E. coli* and *S.* Typhimurium by LGG. Additionally, Lee and Poung (2002) have shown that the degree of adhesion inhibition depends on relative position of the hydrophobic surface and adhesion receptors.

In the present study, most lactobacilli exerted strong displacement ability towards *E. coli* with adhesion reduction by 26-91%. Candela *et al.* (2008) showed 40-90% displacement of *E. coli* H10407 and *S.* Typhimurium from Caco-2 monolayers by

strains of *L. acidophilus*. The displacement activity exerted by probiotic bacteria depends on the production of antimicrobial compounds or anti-adhesion factors rather than mere competition for common adhesion receptors (Lievin *et al.*, 2000). Our study also supports this observation. The ability of LGG and *L. plantarum* CS24.2 to adhere was poor when HT-29 cells were pre-colonized with *E. coli*. The cell free supernatant of both lactobacilli had ability to inhibit adhesion of *E. coli* to HT-29 cells. Thus, this ability can be best utilized for the treatment of gastrointestinal disorders involving pre-colonized adhesive pathogenic bacteria.

All the *Lactobacillus* strains significantly ($p<0.05$) reduced the adhesion of both the enteropathogens to mucin under competitive adhesion assay. The inability of *L. fermentum* ASt1 and *L. casei* CS5.2 to reduce *E. coli* adhesion can be related to its low binding ability to mucin. However, it was not the case with *S.* Typhi so it shows the role of different mechanisms in the ability of probiotic bacteria to inhibit a specific pathogen. Lee *et al.* (2003) showed that LGG was able to inhibit strains of *S. typhimurium* for mucin adhesion, but failed to compete with *E. coli* O157.

The adhesion ability was found to be strain specific and the study indicates the diverse nature of *Lactobacillus* strains harboured in the gastrointestinal ecological niche. The selection of a lactobacilli strain on the basis of its ability to antagonize specific pathogen is an important step in the development of a probiotic product for prevention and treatment of infection caused by that pathogen. Together with other probiotic properties of the same *Lactobacillus* strains and present findings with adhesion ability indicates *L. plantarum* CS23 and *L. plantarum* CS24.2 as potential probiotic strains. Further *in vivo* evaluation is required for human consumption applicability. *L. delbrueckii* M also can be best utilized to eradicate *E. coli* adhesion to epithelial cells as the strain showed strong inhibitory activity. The high adhesion ability of *L. plantarum* CS24.2 with antagonistic effects on enteropathogenic *E. coli* O26:H11 adhesion to intestinal epithelial cell line suggests the applicability of this strain to replenish the protective microbes in pathogen associated gut diseases.

CHAPTER 4

IMMUNOMODULATORY POTENTIAL OF *LACTOBACILLUS* STRAINS CO-INCUBATED WITH INTESTINAL EPITHELIAL CELL LINES

"True wisdom comes to each of us when we realize how little we understand about life, ourselves, and the world around us."

- Socrates

Chapter 4

Immunomodulatory potential of *Lactobacillus* strains co-incubated with intestinal epithelial cell lines

4.1. Introduction

The intestinal mucosa is a physical barrier which protects the underlying cells from the direct exposure of the lumen which is in contact with an external environment (Louvard *et al.*, 1992). The intestinal mucosa is made up of intestinal epithelium covered with thick layer of mucus. The core of the intestinal epithelium is made up of mainly enterocytes and goblet cells. The former are specialized cells with absorptive and transport functions, while the latter are mucus secretory cells. The intestinal epithelium integrity is maintained by tight junction. The lumen contents are effectively restricted by this barrier and its efficiency is enhanced with the help of immune cells. As in the rest of the body, the intestinal epithelium is also provided with innate and adaptive immune response. And the response is mediated by the cells of intestinal epithelium and *lamina propria*, i.e. the layer located immediately underneath the epithelium. The intestinal epithelium is also consists of peneth and enteroendocrine cells along with above mentioned cell lineage. Peneth cells are located at the base of the crypt and secret antimicrobial peptides such as defensins which limit the bacterial growth and colonization in the crypt space (Ouellette and Selsted, 1996).

The intestinal epithelium is home for nearly trillion of micro-organisms which comprise the major population which resides in the human body. Despite such high bacterial load and variety in species present, the homeostasis is preserved at almost all times (McCracken and Lorenz, 2001). Among these bacteria, not all are pathogens but also consist commensals as well as beneficial microbes. There is a continuous cross talk between the gut microbes and intestinal epithelium which also helps in the

intestinal development and functions. Mucosal surfaces are the primary interaction sites between the host and its environment and they thus represent the major portal of entry for pathogens and vast majority of antigens. Under most circumstances, microbial attack and minor breaches in mucosa are handled by the innate and adaptive immune systems which fight against the invading micro-organisms with no or minor clinical symptoms (Lievin-Le Moal and Servin, 2006). Furthermore, the intestinal epithelium stays regularly exposed to commensal bacteria and become systemically tolerant to fed antigens and the phenomenon is known as oral tolerance (Strobel and Mowat, 1998). The enterocytes of the epithelial layer do act as immunocompetent cells and secrete various signalling molecules such as cytokines and chemokines upon adhesion and invasion by gut pathogens (Brandtzaeg, 2011). Upon stimulation, the specialized immune cells such as neutrophils and macrophages migrate to the site of infection and prevent pathogen entry. Under certain conditions, the inflammations become self-sustaining due to ineffective down regulation of pro-inflammatory molecules even after the elimination of the pathogens which leads to the inflammatory disorders (Dunne, 2001). Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-8 are reported to be highly expressed in Crohn's disease and acute colitis patients (Ganesan *et al.*, 2002; Banks *et al.*, 2003).

The probiotic lactobacilli do have the potential to interact with the mucosal layer. Several probiotic effects are mediated through immune regulation, particularly through establishing and maintaining a balance between pro- and anti- inflammatory cytokines (Isolauri *et al.*, 2001; Winkler *et al.*, 2007). That is why the study of immunomodulatory properties of the probiotics is also high on priority. Each strain of lactobacilli is bestowed with its own combination of pro- and anti-inflammatory potential. Thus, it becomes necessary to evaluate the potential of all putative probiotic strain to group them for particular immunological condition. Several *in vitro* and clinical trials have shown the effectiveness of *Lactobacillus* strains in modulating the expression of cytokines and chemokines (Rioux and Fedorak, 2006; Candela *et al.*, 2008; Chon and Choi, 2010; Bahrami *et al.*, 2011).

Under the present study, the ability of different *Lactobacillus* strains to modulate the cytokines and chemokines expression in Caco-2 cells was analysed. The set of pro and anti-inflammatory molecules expression was analysed at transcripts level in Caco-2 cells post stimulation with lactobacilli. Furthermore, the ability of *L.*

plantarum CS24.2 to normalize the expression level of TNF-α and IL-8 in HT-29 cells induced by adherent *E. coli* O26:H11 was also analyzed under different *in vitro* assays for possible application in the treatment of inflammatory bowel diseases.

4.2. Materials and methods

4.2.1. Stimulation of Caco-2 monolayers with lactobacilli

To obtain monolayer, 5×10^4 Caco-2 cells were seeded in T25 tissue culture flask (Corning Incorporated) and maintained for two weeks post confluent under the same condition as described in chapter 3 (Materials and methods; 3.2.1). The Caco-2 monolayers were stimulated with 1×10^8 cfu/ml of different lactobacilli and co incubated for 90 min in the absence of gentamycin at 37˚C in a CO_2 incubator (Morita *et al.,* 2002). Thereafter gentamycin (50 µg/ml) was added to prevent bacterial growth and further incubated under same condition for another 4 h and 30 min. At the end of incubation, culture supernatant was discarded and Caco-2 monolayers were lysed in the presence of guanidine thiocyanate which is included in the total RNA extraction kit (Bangalore Genei, Bangalore, India) employed. Further steps were performed as per manufacturer's instructions.

4.2.2. RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from control (to which no bacteria were added) Caco-2 cells and those co incubated with various lactobacilli using total RNA extraction kit (Bangalore Genei). The quality of the RNA samples was assessed by inspecting the 28S and 18S bands following agarose gel electrophoresis. Two µg of each RNA sample was used with Oligo (dT18) for cDNA synthesis in a 20 µl system using M-MuLV RT-PCR kit (Bangalore Genei, Bangalore, India) following manufacturer's instructions. Briefly, the RNA and Oligo (dT18) mixture was incubated at 65˚C for 10 min, centrifuged briefly and kept at room temperature for 2 min. Into this RNAsin, dithiothreitol, RT buffer, dNTP and M-MuLV reverse transcriptase were added, as instructed by the manufacturer and incubated further at 37˚C for 1 h, followed by 5 min incubation at 95˚C. Each of the cDNA preparations was then amplified for 30

cycles in a thermal cycler (Eppendorf, Hamburg, Germany) with β-actin-specific primers by taking 1 µl of the cDNA in a 12.5 µl system. This was used as control for synthesis of cDNA. For checking genomic DNA contamination, controls were set with amplification of the total RNA without reverse transcription which did not give any amplification (result not provided). PCR amplifications were performed with specific primers for cytokines in a 12.5 µl system with 1 µl of the first strand cDNA. This was run for 30 cycles after the addition 0.5 U of Taq polymerase. Amplification conditions were as follows: initial denaturation at 94˚C for 5 min, followed by 30 cycles of denaturation at 94˚C for 45 s, annealing for 30 s at 66˚C for β-actin, 60˚C for IL-6, IL-8, IL-12p35 and TGF-β, 62˚C for IL-12p40 and TNF-α and 56˚C for IL-15 followed by extension at 72˚C for 1 min and a final extension at 72˚C for 6 min. PCR products separated on a 2% agarose gel were stained with ethidium bromide (0.5 µg/ml) following which densitometry analysis was carried out employing AlphaEaseFC 4.0 software and the results are expressed as integrated density value divided by the selected area of band on the gel.

Primer sequences

	Primer sequences	**Amplicon size**	**NCBI Accession number**
β-actin	5’-AGC GGG AAA TCG TGC GTG ACA-3’ 5’-CGC AAC TAA GTC ATA GTC CG -3’	536 bp	NM_001101.3
IL-6	5’-CCC CAG GAG AAG ATT CCA A-3’ 5’-AAA GCT GCG CAG AAT GAG AT-3’	502 bp	JQ250825.1
IL-8	5’-CGG AAG GAA CCA TCT CAC TG-3’ 5’-GCT TGA AGT TTC ACT GGC ATC-3’	389 bp	NM_000584.3
IL-12p35	5'-TTC ACC ACT CCC AAA ACC TGC-3' 5'-GAG GCC AGG CAA CTC CCA TTA G-3'	226 bp	NM_000882.3
IL-12p40	5'-ATG TCG TAG AAT TGG ATT GGT ATC CG-3’ 5'-GTA CTG ATT GTC GTC AGC CAC CAG C-3’	358 bp	AF180563.1
TGF-β	5'-CTC CGA GAA GCG GTA CCT GAA C-3' 5'-CAC TTG CAG TGT GTT ATC CCT-3'	288 bp	NM_000660.4
TNF-α	5'-CAG AGG GAA GAG TTC CCC AG-3' 5'-CCT TGG TCT GGT AGG AGA CG-3'	324 bp	NM_000594.3
IL-15	5’-GTA GGA GGC ATC GTG GAT G-3’ 5’-GTC TAA GCA GCA GAG TGA TG-3’	759 bp	AF031167.1

Reaction system for semi-quantitative RT-PCR

Reaction Components	**Volume (µl)**
R.O water	7.25
10X Buffer for Taq DNA Pol	1.25
dNTP mix (2.5 mM each)	0.25
Forward primer (10 pmol/µl)	1.25
Reverse primer (10 pmol/µl)	1.25
Taq pol (1 U/µl)	0.25
First strand	1
Total volume	**12.5**

4.2.3. Stimulation of HT-29 cells with lactobacilli and *E. coli* O26:H11

To analyze the transcript level expression of TNF-α and IL-8 in HT-29 cells upon stimulation with lactobacilli and/or *E. coli* O26:H11 in co culture conditions, the different adhesion assays were performed as mentioned above. HT-29 cells were stimulated with 1×10^8 cfu/ml of lactobacilli and *E. coli* O26:H11 in competitive adhesion and co-incubated for 90 min at 37˚C in a CO_2 incubator. For adhesion inhibition and displacement assay, HT-29 cells were pre-incubated with lactobacilli and *E. coli* for 90 min, respectively. Thereafter, the unbound bacterial cells were removed by washing as mentioned in chapter 3 (Materials and methods; 3.2.3) and HT-29 cells were further incubated for 90 min with *E. coli* and lactobacilli in respective adhesion assays. To analyze the ability of lactobacilli and *E. coli* to modulate the expression of pro inflammatory molecules, HT-29 cells were co incubated with either lactobacilli or *E. coli* alone. After this initial incubation, the culture medium was replaced with gentamicin (50 µg/ml) containing media to prevent bacterial growth and further incubated under the same conditions for another 4 h and 30 min and 22 h and 30 min for transcript level analysis at 6 h and 24 h, respectively. At the end of incubation, culture supernatants were collected and stored at -80°C.

4.2.4. cDNA synthesis and qRT-PCR

Total RNA was isolated from control (to which no bacteria were added) HT-29 cells and those co incubated with lactobacilli and/or *E. coli* O26:H11. The quality of the RNA samples was analyzed as mentioned above (Materials and methods; 4.2.2). For cDNA synthesis, one µg of each total RNA sample was mixed with anchored oligo-dT in a 20 µl system using verso cDNA synthesis kit based on Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (Thermo Fisher Scientific, Surrey, UK) following manufacturer's instructions. Briefly, the RNA was mixed with oligo-dT, RT enhancer which contains DNAse I, dNTP mix and enzyme mix, followed by incubation at 50˚C for 30 min and then 95˚C for 2 min to inactivate the enzyme in a thermal cycler (Eppendorf, Hamburg, Germany). Quantitative PCR amplifications were then performed in CFX96TM real-time thermal cycler (Bio-Rad Laboratories, Hercules, USA) with specific primers for TNF-α and IL-8. The amplification conditions were as follows: initial denaturation at 94˚C for 3 min, followed by 45 cycles of denaturation at 94˚C for 10 s, annealing and extension for 30 s at 60˚C and the fluorescence was recorded after each cycle. Each sample was run in triplicate and cycle threshold (Ct) was used for gene expression analysis. The transcripts expression of TNF-α and IL-8 in each sample was normalized to a Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript expression of the same sample using the CFX manager software (Bio-Rad Laboratories). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The product specificity was confirmed by single peak in melt curve analysis (from 65°C to 95°C in 0.5°C/5 s increments). The negative controls were set with the total RNA without reverse transcription (data not provided).

Primer sequences

	Primer sequences	**Amplicons size**
GAPDH	3'- TGA GCA CCA GGT GGT CTC C -5' 3'- TAG CCA AAT TCG TTG TCA TAC CAG -3'	128 bp
TNF-α	3'- TCT TCT CGA ACC CCG AGT GA -5' 3'- CCT CTG ATG GCA CCA CCA G -5'	151 bp
IL-8	3'- GGC ACA AAC TTT CAG AGA CAG -5' 3'- ACA CAG AGC TGC AGA AAT CAG G -5'	153 bp

Reaction system for quantitative RT-PCR

Reaction Components	**Volume (µl)**
R.O water	3
2 × SsoFast EvaGreen supermix	5
Forward primer (10 pmol/µl)	0.5
Reverse primer (10 pmol/µl)	0.5
First strand	1
Total volume	**10**

4.2.5. IL-8 quantification in culture supernatants

IL-8 in supernatants was measured via ELISA using Mini ELISA Development Kit (PeproTech Asia, Rehovot, Israel). The 96-wells Maxisorp ELISA plates (Nunclon, Rochester, NY) were coated overnight at room temperature with capture monoclonal anti-IL-8 in PBS. pH 7.4. After washing (0.05% Tween 20 in PBS), plates were blocked adding PBS with 1% BSA at room temperature for 1 h and then washed again. Samples and standards were added to wells and incubated for 2 h at room temperature. Biotinylated anti-IL-8 antibody was added after washing and incubation was carried out for 2 h. After rinsing, streptavidinhorseradish peroxidase was added and incubated for 30 min followed by addition of 2, 2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich) and incubation for 30 min. The reaction was stopped with 1% sodium dodecyl sulphate, and plates were read immediately at 405 nm with an ELISA plate reader. ELISA was sensitive to concentrations <15 pg/ml IL-8. The standard plot was generated for quantifying IL-8 in sample using the known concentration of recombinant human IL-8.

4.2.6. Statistics

Values are given as mean along with the standard deviation (SD) from three experimental replicates. Significant ANOVAs were followed by Dunnett test in the case of different adhesion assays and compared with the respective control ($p<0.05$). The statistical analysis was conducted using SigmaStat 3.5 software.

4.3. Results

4.3.1. RNA isolation and semi-quantitative RT-PCR of Caco-2 exposed to different lactobacilli

The total RNA was isolated from the control and lactobacilli exposed Caco-2 cells. The presence of 18S and 28S rRNA on the agarose gel shows the integrity of RNA sample (Figure 4.1). The cDNAs prepared from these samples were further used to check the expression of cytokines and chemokines. The expression was measured semi-quantitatively by comparing the intensity of amplified product on gel using the densitometic analysis software.

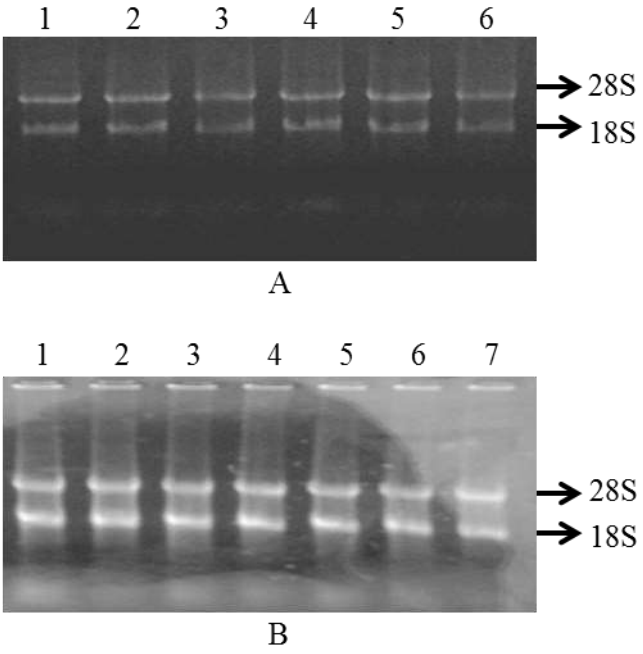


Figure 4.1. 0.8% agarose gel stained with ethidium bromide with the total RNA from Caco-2 cells co-incubated with different *Lactobacillus* strains.

Gel A

Lane 1: Control Caco-2 (No bacteria)

Lane 2: Caco-2 co-cultured with M

Lane 3: Caco-2 co-cultured with ATCC8014

Lane 4: Caco-2 co-cultured ASt1

Lane 5: Caco-2 co-cultured CS23

Lane 6: Caco-2 co-cultured CS25

Gel B

Lane 1: Control Caco-2 (No bacteria)

Lane 2: Caco-2 co-cultured CS24.2

Lane 3: Caco-2 co-cultured LGG

Lane 4: Caco-2 co-cultured CS5.2

Lane 5: Caco-2 co-cultured SCA

Lane 6: Caco-2 co-cultured SCB

Lane 7: Caco-2 co-cultured CD12D

Further, the amplification of individual cytokines and chemokines was performed using gene specific primers from cDNAs prepared for above mentioned samples. The expected size amplicons were observed for respective genes (Figure 4.2 and 4.3).

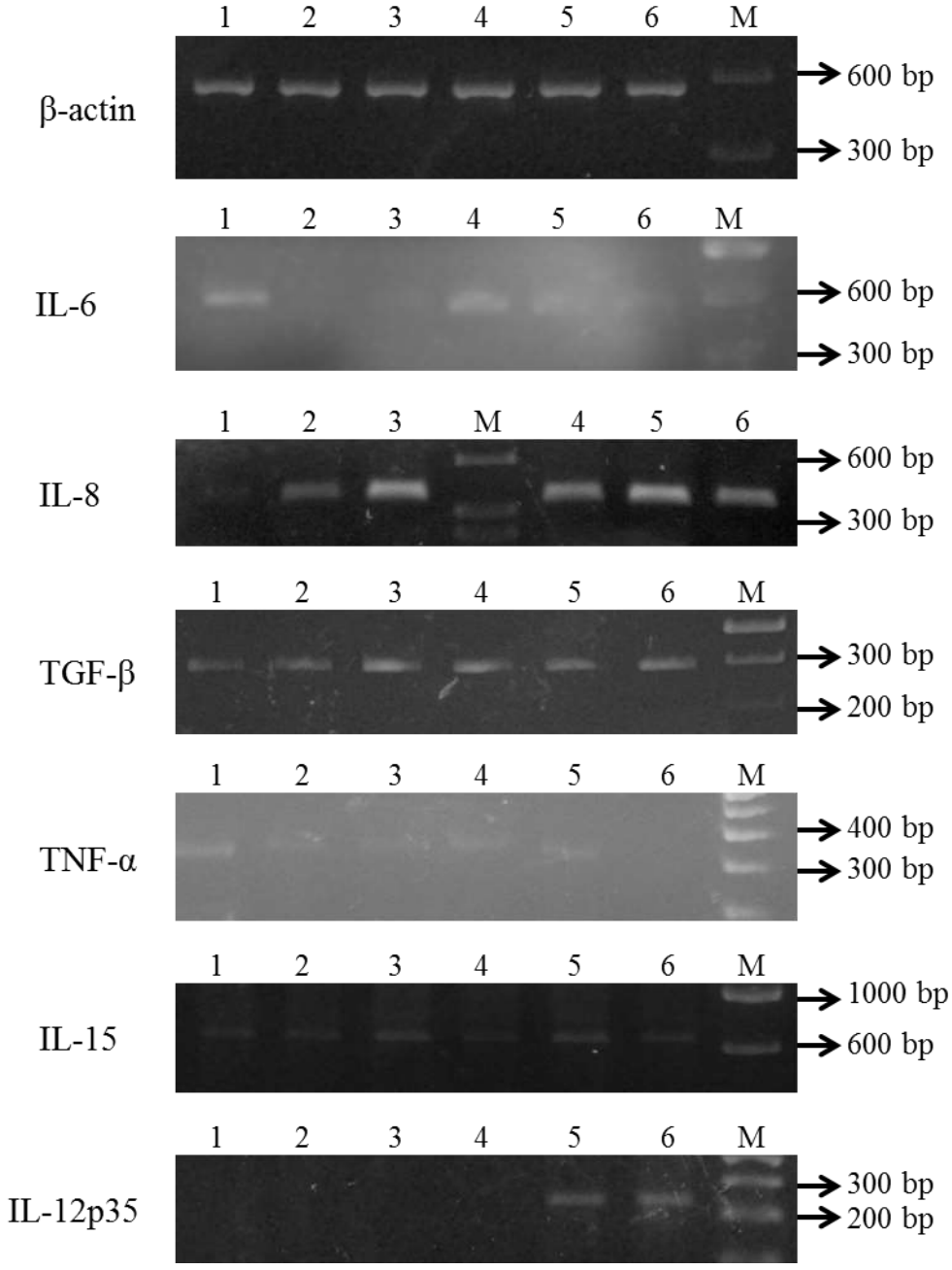


Figure 4.2. 1.5% agarose gel with cytokines and chemokines amplicons from cDNA of Caco-2 co-cultured with different *Lactobacillus* isolates

Lane 1: Caco-2 co-cultured with M
Lane 2: Caco-2 co-cultured with ATCC8014
Lane 3: Caco-2 co-cultured ASt1
Lane 4: Caco-2 co-cultured CS23
Lane 5: Caco-2 co-cultured CS25
Lane 6: Control Caco-2 (No bacteria)
Lane M: DNA marker

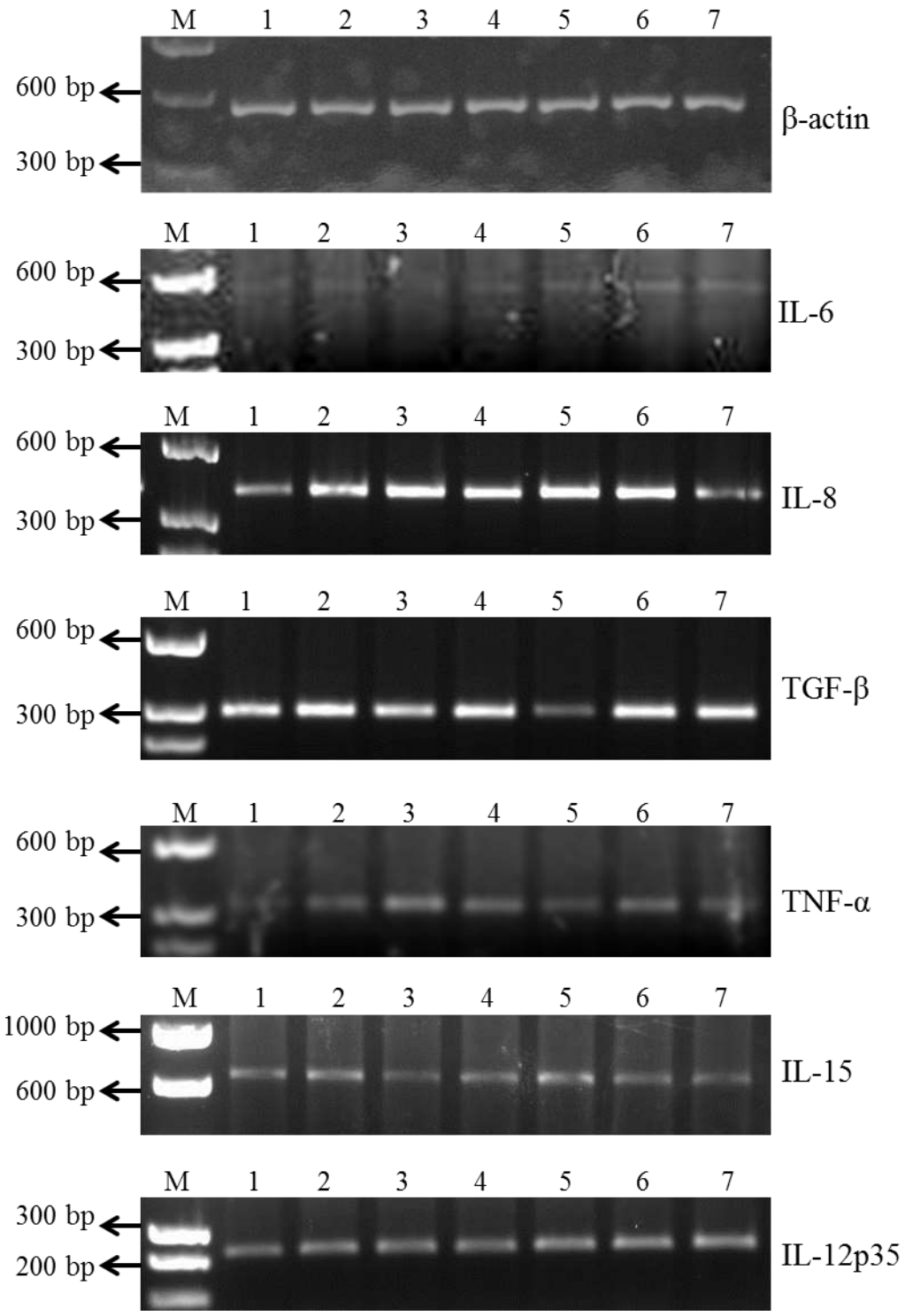
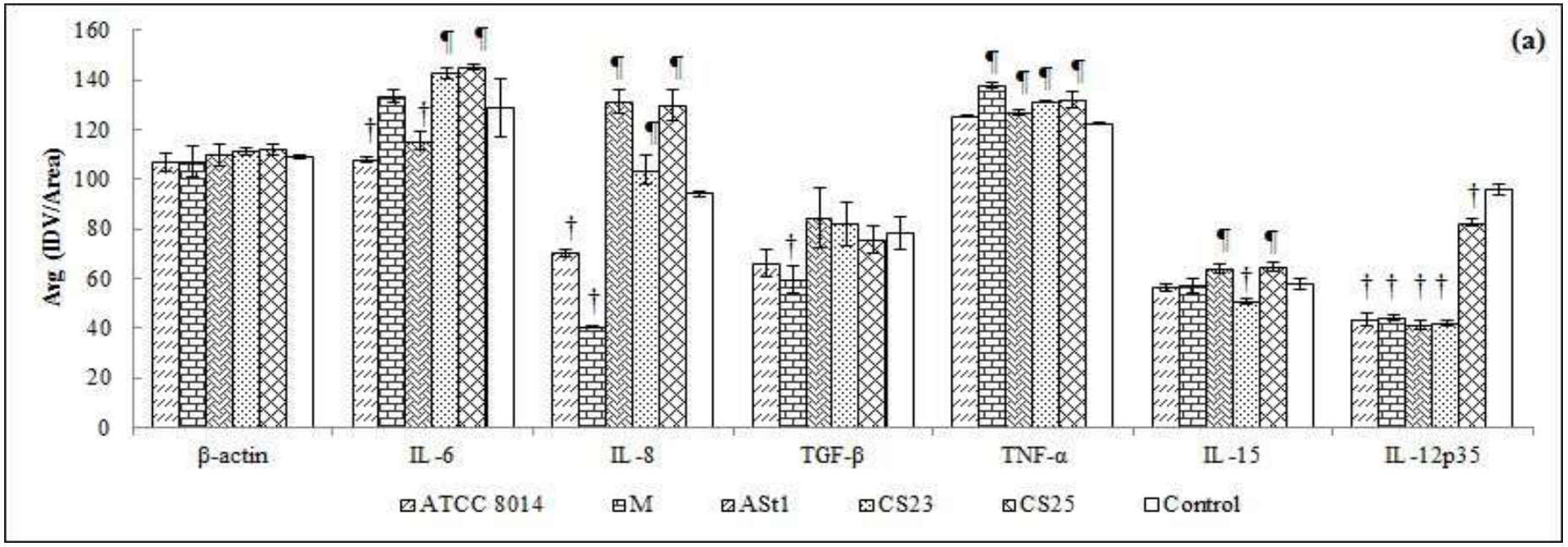


Figure 4.3. 1.5% agarose gel with cytokines and chemokines amplicons from cDNA of Caco-2 co-cultured with different *Lactobacillus* isolates

Lane M: Low range DNA marker
Lane 1: Control Caco-2 (No bacteria)
Lane 2: Caco-2 co-cultured CS24.2
Lane 3: Caco-2 co-cultured LGG
Lane 4: Caco-2 co-cultured CS5.2
Lane 5: Caco-2 co-cultured SCA
Lane 6: Caco-2 co-cultured SCB
Lane 7: Caco-2 co-cultured CD12D

The presence of transcripts of β-actin (Control gene) and a selected number of cytokine genes in Caco-2 cells co-cultured with different lactobacilli was analyzed employing semi quantitative RT-PCR. The intensity of band was measured using a densitometry tool. They were compared with control where none of the bacterial culture was added (Figure 4.4).



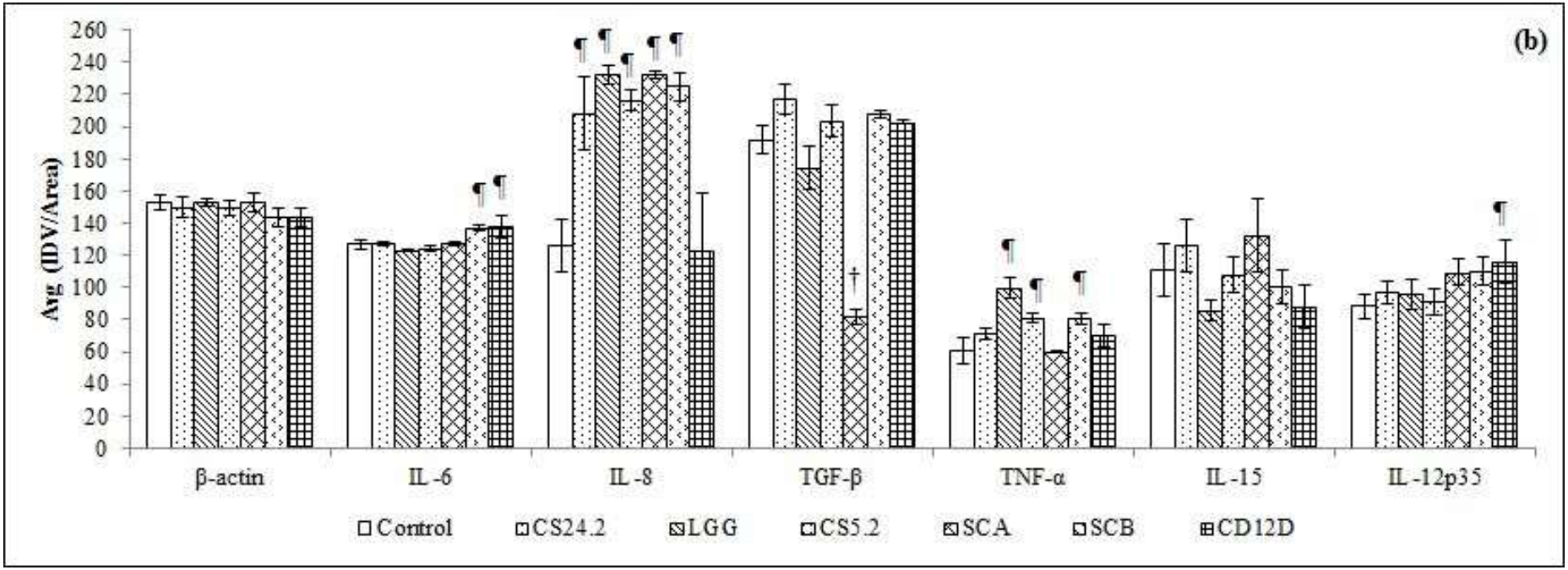


Figure 4.4. Cytokines and chemokine transcript level in Caco-2 cells co incubated with different lactobacilli (1×10^8 cfu/ml). Different lactobacilli used in study are **(a)** *L. plantarum* ATCC 8014, *L. delbrueckii* M, *L. fermentum* ASt1, *L. plantarum* CS23, *L. rhamnosus* CS25 and **(b)** *L. plantarum* CS24.2, LGG, *L. casei* CS5.2, *L. rhamnosus* SCA, *L. rhamnosus* SCB, *L. fermentum* CD12D and control to which no bacteria were added. The integrated density values of each cytokine or chemokine are used to express its transcript levels. Each bar represents mean value and standard deviation as error bar. Significant ANOVAs were followed by Dunnett test for multiple comparisons versus control group. † Within each cytokine or chemokine, mean value was significantly lower than that of the control ($P<0.05$). ¶ within each cytokine or chemokine, mean value was significantly higher than that of the control ($P<0.05$).

No amplification products were observed for IL-12p40 in Caco-2 cells.

Table 4.1. Summary of cytokines and chemokine transcript levels affected in Caco-2 cells co incubated with different lactobacilli *

***Lactobacillus* strains**	**Expression of cytokines/chemokine in Caco-2 cells**
L. delbrueckii M	(TNF-α)↑ (IL-8, TGF-β, IL-12p35)↓
L. plantarum ATCC 8014	 (IL-6, IL-8, IL-12p35,)↓
L. fermentum ASt1	(IL-8, TNF- α, IL-15)↑ (IL-6, IL12p35)↓
L. plantarum CS23	(IL-6, IL-8, TNF- α)↑ (IL-15, IL-12p35)↓
L. rhamnosus CS25	(IL-6, IL-8, TNF- α, IL-15)↑ (IL-12p35)↓
LGG	(IL-8, TNF- α)↑
L. plantarum CS24.2	(IL-8)↑
L. casei CS5.2	(IL-8, TNF- α)↑
L. rhamnosus SCA	(IL-8)↑ (TGF-β)↓
L. rhamnosus SCB	(IL-6, IL-8, TNF- α)↑
L. fermentum CD12D	(IL-6, IL-12p35) ↑

*Symbols refer to change in the expression level of cytokines and chemokine as compared to respective control: ↑, up regulation; ↓, down regulation. None inclusion of a cytokine means the cytokine message was not either detected or significantly affected in that particular co-cultivation.

4.3.2. Immunomodulatory effects of *L. plantarum* CS24.2 and LGG on HT-29 cells stimulated with *E. coli* O26:H11 under different adhesion assays

The total RNA was isolated from the control and *E. coli* O26:H11 and/or lactobacilli exposed to HT-29 cells. The presence of 18S and 28S rRNA on the agarose gel shows the integrity of RNA sample (Figure 4.5). Figure shows representative sample of total

RNA. The cDNAs prepared from these samples were further used to check the expression of TNF-α and IL-8 with quantitative real time PCR.

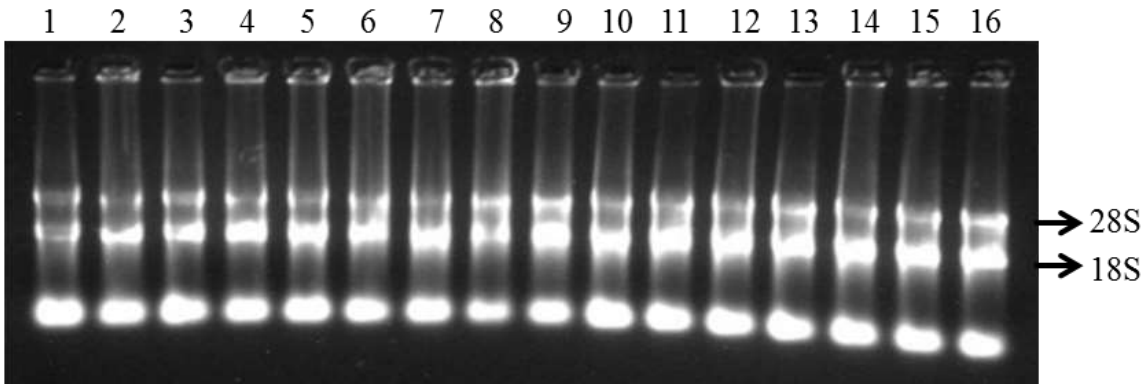


Figure 4.5. 0.8% agarose gel stained with ethidium bromide with the total RNA from HT-29 cells co-incubated with *E. coli* O26:H11 and/or *Lactobacillus* strains.

The ability of lactobacilli to modulate the transcript level expression of pro-inflammatory molecules in HT-29 cells stimulated with and without *E. coli* O26:H11 was analyzed under different adhesion assays at 6 h (Figure 4.6a and Figure 4.6b) and 24 h (Figure 4.6c and Figure 4.6d). There was no significant change in expression of TNF-α and IL-8 in HT-29 cells co cultured with lactobacilli at 6 h as well as after 24 h when compared with control to which no bacteria were added ($p<0.05$). The enteropathogenic *E. coli* strongly induced the TNF-α and IL-8 expression at 6 h with increase in fold expression to 1.8 and 2.4, respectively. The fold expression increased continuously with the time and reached to 6.1 and 9.0 at 24 h for TNF-α and IL-8, respectively.

In competitive adhesion assay, LGG was unable to prevent the up-regulation of TNF-α and IL-8 in HT-29 cells co-stimulated with *E. coli* and the level of expression was the same as observed with the stimulation by *E. coli*. Under similar assay conditions, *L. plantarum* CS24.2 significantly normalized the expression of both the gene till 6 h. The expression was increased with further incubation upto 24 h and the fold expression was 2.4 and 4.7 for TNF-α and IL-8, respectively. Interestingly, the expression was significantly low when compared to HT-29 cells stimulated with *E. coli* alone ($p<0.05$). In adhesion inhibition assay, both the lactobacilli effectively maintained the expression of TNF-α and IL-8 to the basal level upto 6 h upon

stimulation with *E. coli* after lactobacilli adhesion. There was increase in fold expression of both the genes at 24 h but the expression was significantly low compared to *E. coli* alone stimulated HT-29 cells, except the expression of TNF-α in case of LGG. The fold expression of TNF-α and IL-8 was 4.8 and 6.1 for LGG, while it was 3.3 and 4.6 for *L. plantarum* CS24.2, respectively. In the displacement assay, both the lactobacilli prevented the up-regulation of TNF-α and IL-8 upto 6 h in HT-29 cells pre incubated with *E. coli*. The fold expression of both the gene was significantly increased at 24 h in HT-29 cells except IL-8 in case of *L. plantarum* CS24.2 ($p<0.05$). There was no significant decrease in fold expression of both the genes at 24 h in case of LGG when compared with HT-29 cells stimulated with *E. coli* alone. *L. plantarum* CS24.2 significantly reduced the fold expression of TNF-α and IL-8 to 2.9 and 1.6, respectively when compared to *E. coli* induced expression at 24 h. The adhesion of *L. plantarum* CS24.2 to HT-29 cells pre-colonized with *E. coli* could protect the cells from up-regulation of IL-8 even after 24 h incubation.

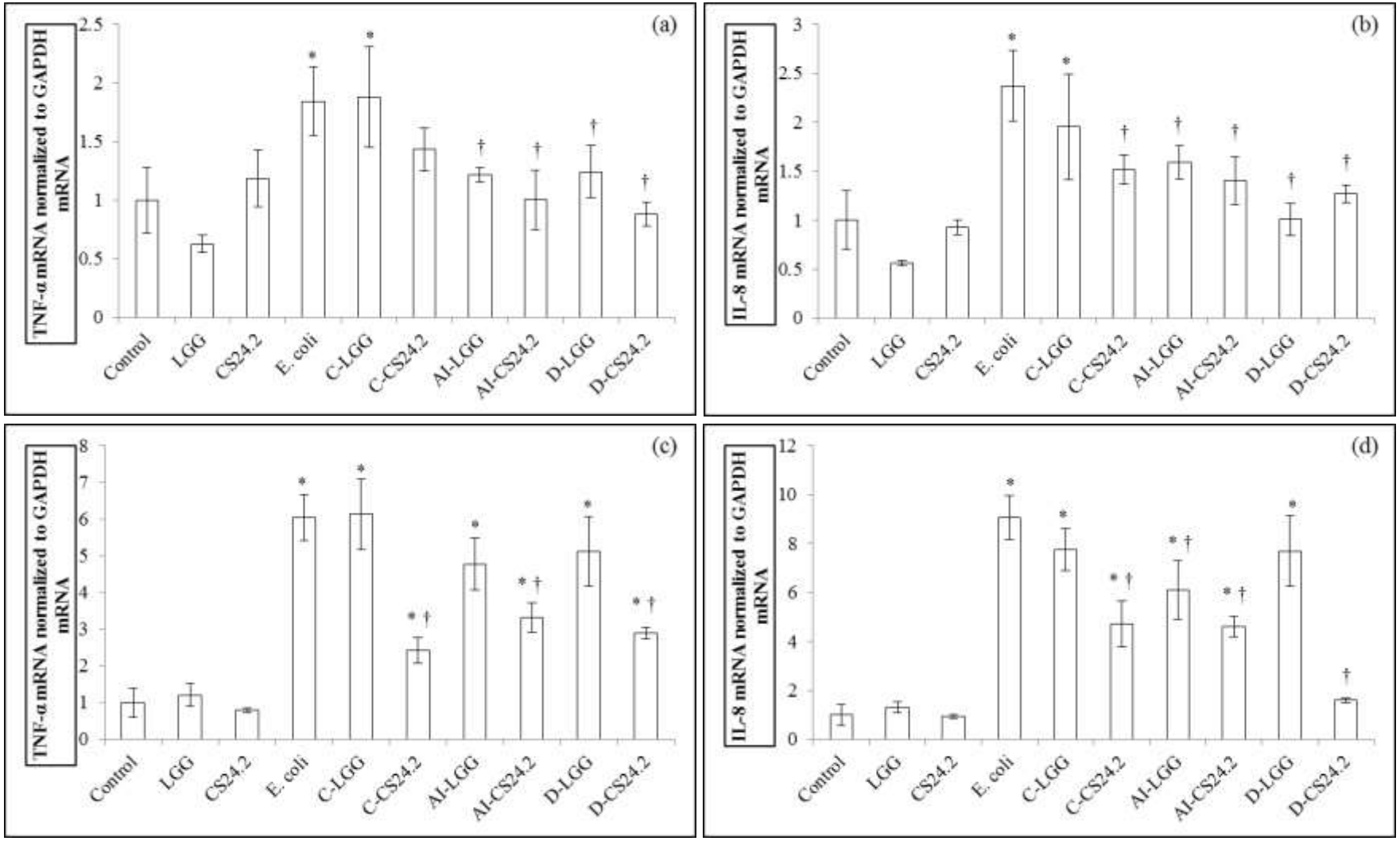


Figure 4.6. Comparison of effect of LGG and *L. plantarum* CS24.2 on mRNA expression of TNF-α and IL-8 in HT-29 cells upon stimulation with enteropathogenic *E. coli* O26:H11 under different adhesion assays. The adhesion assays were competitive inhibition (C), adhesion

inhibition (AI) and displacement (D). The transcript level expression was analysed at two time intervals in HT-29 cells at 6 h (a, b) and 24 h (c, d) by qRT-PCR. The expression of TNF-α and IL-8 was normalized to the internal reference GAPDH expression. The relative expression level in control (to which no bacteria added) was set to one for fold expression analysis in other experimental groups. All data are presented as the mean ± S.D of three independent experiments. Significant ANOVAs were followed by Dunnett's test for multiple comparisons *v.* control group. * the mean value of relative expression in all experimental group is significantly higher compared to that of the control ($p<0.05$). † the mean value of relative expression in different adhesion assays is significantly lower compared to that of *E. coli* ($p<0.05$).

4.3.3. Quantification of IL-8 in culture supernatant

The ability of lactobacilli to inhibit IL-8 secretion from HT-29 cells stimulated with *E. coli* O26:H11 was also confirmed at 24 h using ELISA (Figure 4.7). There was no significant difference in the secretion of IL-8 by stimulation with LGG (180±12 pg/ml) and *L. plantarum* CS24.2 (185±10 pg/ml) compared to control (148±10 pg/ml). *E. coli* significantly increased IL-8 secretion from HT-29 (786±102 pg/ml). LGG was unable to down-regulate IL-8 synthesis from HT-29 cells stimulated with *E. coli* under different competitive conditions except adhesion inhibition assay (662±17 pg/ml). *L. plantarum* CS24.2 significantly reduced the secretion of IL-8 in all adhesion assays compared to the same of HT-29 cells stimulated with *E. coli*. The level of IL-8 secretion from HT-29 cells was statistically similar to the control values under displacement assay with *L. plantarum* CS24.2 (187±36 pg/ml). Overall, the transcripts expression and secretion of IL-8 were comparable in all adhesion assays.

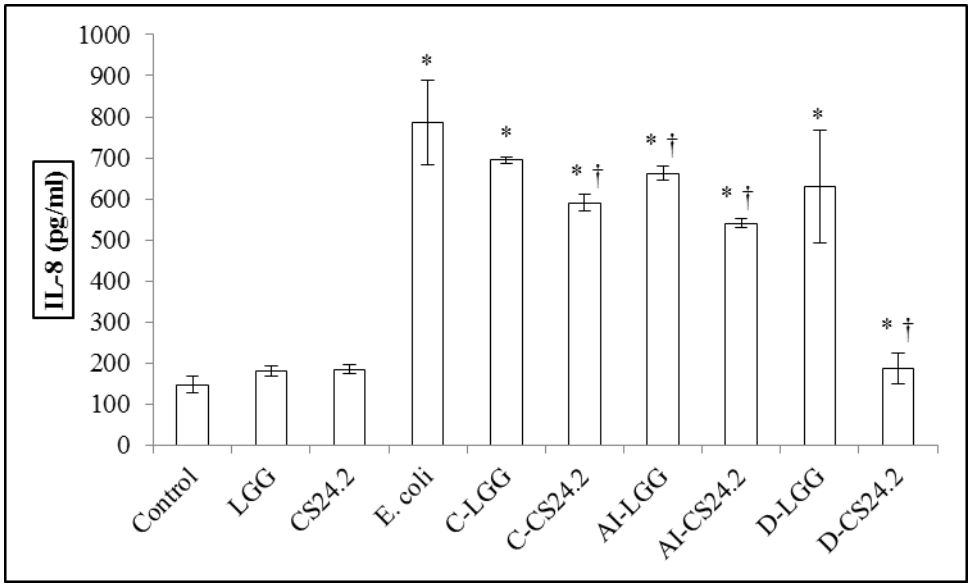


Figure 4.7. Comparison of effect of LGG and *L. plantarum* CS24.2 on IL-8 secretion from HT-29 cells upon stimulation with enteropathogenic *E. coli* O26:H11 under different adhesion assays at 24 h using ELISA. The adhesion assays were competitive inhibition (C), adhesion inhibition (AI) and displacement (D). All data are presented as the mean ± S.D of three independent experiments. Significant ANOVAs were followed by Dunnett's test for multiple comparisons v. control group. * the mean value of secreted IL-8 in all experimental group is significantly higher compared to that of the control ($p<0.05$). † the mean value of secreted IL-8 in different adhesion assays is significantly lower compared to that of *E. coli* ($p<0.05$).

4.4. Discussion

The interaction of the enterocytes with microorganisms is known to modulate the cytokine profile of the former. Certain cytokines in turn serve as chemo attractants and activators of other immunocompetent cells. The stimuli eventually have a potential to bring the immunocompetent cells from the gut-associated lymphoid population in direct contact of the intestinal microbiota, at least at the sites of injury. The bacteria on the other hand do have a potential to cross the epithelial barrier and thereafter come across gut associated immune cells (Perdigon *et al.*, 2002). It is important to study the interaction of probiotics with the enterocytes (Tannock, 1997).

In the present chapter, Caco-2 cells were used as *in vitro* models for studying the immunomodulatory capacity of different lactobacilli.

TNF-α and IL-6 are pro-inflammatory cytokines, which are produced by the host in response to bacterial colonization or invasion and hence are central to the host defense mechanism against pathogens (Solis-Pereyra *et al.*, 1997; Morita *et al.*, 2002). Though lipopolysaccharide of gram-negative bacteria is known to stimulate their production, Miettinen *et al.* (1996) have reported an increase in IL-6 and TNF-α production in human PBMCs exposed to lactobacilli and thereby suggested the use of probiotics as vaccine vectors and for the purpose of stimulating nonspecific immunity. In our study, most of the isolates have exhibited their ability to induce in Caco-2, a higher level of TNF- α transcript. Similarly, in the case of IL-6 in Caco-2 cells, isolates *L. plantarum* CS23, *L. rhamnosus* CS25, *L. rhamnosus* SCB and *L. fermentum* CD12D have a strong up regulatory potential compared to the down regulation that is induced by *L. plantarum* ATCC 8014 and *L. fermentum* ASt1. It should be noted here that IL-6 is known to have both pro- as well as anti-inflammatory properties. It has a very important role in resolving the initial inflammatory reaction which leads to the activation of the adaptive immune response. Therefore, it appears that while *L. plantarum* CS23, *L. rhamnosus* CS25 and *L. rhamnosus* SCB have the capacity to induce a strong pro-inflammatory environment, the presence of IL-6 may help them to convert an innate response to a more specific and sustained adaptive response against pathogens.

As is evident from the data, this possibility is strengthened all the more by the induction ability of *L. plantarum* CS23, *L. rhamnosus* CS25 and *L. rhamnosus* SCB towards the production by Caco-2 of IL-8, a chemokine that is responsible for recruiting elements of the innate immune system. Of all the organisms tested for effect on IL-8 transcript levels, isolate *L. rhamnosus* CS25 has a stronger up regulatory potential in Caco-2 cells. IL-8 has an important role in induction of neutrophil accumulation and activation (Godaly *et al.*, 1997). Zhang *et al*. (2005) have suggested that a higher dose of LGG (10^7 cfu/ml) induces an increased production of IL-8 in Caco-2, which is in agreement with our results wherein an increased transcription of IL-8 has been seen in Caco-2 stimulated by most of lactobacilli. The number of bacterial cells taken in our study is 10^8 cfu/ml with Caco-2. In spite of the high bacterial dose, a significant suppression of IL-8 transcription

has been observed in Caco-2 co-incubated with L. delbrueckii M and *L. plantarum* ATCC 8014. High expression level of IL-8 is associated with pathogenesis of several diseases including neonatal necrotizing enterocolitis and so the immunosuppressive effects of the above isolates in context of IL-8 transcription seems promising. However, further studies need to be conducted at the level of protein expression and *in vivo*.

IL-12 is a heterodimeric protein formed by a complex of p40 with p35, having an important role in IFN-γ production by T helper cells (Trinchieri, 2003). All isolates and standard strains demonstrated no change or suppression of IL-12p35 in Caco-2 cells except *L. fermentum* CD12D. IL-12 is a pro-inflammatory cytokine and plays an important role in the pathogenesis of inflammatory diseases such as Crohn's disease. In recent clinical, trials the use of antibody against IL-12 suggests a role for this cytokine in inflammatory bowel diseases (IBD; Mannon *et al.*, 2004) and hence the immunosuppressive property of the isolates in context of IL-12 can be appropriately utilized under similar circumstances.

IL-15 is a pleiotropic cytokine and regulates T cell and natural killer cell activation and proliferation (Ma *et al.*, 2000). It also has a role in maintaining immune homoeostasis in the mucosal environment by activating intraepithelial cells. It therefore appears from our data that *L. fermentum* ASt1 and *L. rhamnosus* CS25 are capable of functioning better at the mucosal interface. Isolates *L. fermentum* ASt1 and *L. rhamnosus* CS25 in Caco-2 have been observed to induce IL-15 at transcripts level. IL-15 plays a critical role in the maintenance of memory lymphocytes by supporting proliferation. Another cytokine that has also been looked at, TGF-β, is an anti-inflammatory immunoregulatory cytokine which regulates the production of IgA antibodies and important in early defense against intestinal infection (Sonoda *et al.*, 1989). In the present study, except *L. rhamnosus* SCA and *L. delbrueckii* M, none of our isolates modulated significant mRNA expression of TGF-β which is in support of earlier observations (Borruel *et al.*, 2003).

Bergonzelli *et al.* (2006) have hypothesized that the intestinal epithelial cells which sense the bacterial surface with pro inflammatory components like EF-Tu and GroEL and anti-inflammatory components like lipoteichoic acid, process the information and respond with a concurrent pro- or anti- inflammatory response. Among the isolates, *L.*

fermentum ASt1, *L. plantarum* CS23 and *L. rhamnosus* CS25 have shown strong and varied immunomodulatory characteristics that are reflected from the differences in the transcripts of different cytokines in Caco-2 cells co-incubated with these strains as compared to the respective controls.

The lipopolysacharides of gram negative bacteria are known to elevate the expression of TNF-α in gut mucosa (Papadakis and Targan 2000). IL-8 is a chemokine which acts as chemo attractant and brings neutrophils to the site of infection (Godaly *et al.* 1997). One of the key regulators of chronic inflammation in IBD was found to be an elevated expression of pro-inflammatory molecules including TNF-α and IL-8 (Ganesan *et al.* 2002; Banks *et al.* 2003). Under present study, LGG and adhesive isolate *L. plantarum* CS24.2 were further analysed to attenuate the pathogen induced pro-inflammatory molecules expression in HT-29. Both the *Lactobacillus* strains were unable to induce the expression of TNF-α and IL-8 in HT-29. However, the adhesive *E. coli* strongly induced the expression of both the pro-inflammatory molecules within 6 h and continuously increased with the time. Our genes expression studies with co-incubation of lactobacilli and pathogen to HT-29 cells showed the differential ability to regulate the expression of these molecules in different adhesion assays. When competing with *E. coli*, LGG did not maintain the basal degree of expression of TNF-α and IL-8 by HT‑29 cells. For the initial 6 h of the adhesion inhibition and displacement assays, both lactobacilli maintained expression of these inflammatory molecules to the degree found with un-stimulated HT‑29 cells. However, at 24 h, expression of these molecules increased in the case of LGG and reached the same degree as *E. coli* stimulated HT‑29 cells, the exception being IL‑8 in the adhesion inhibition assay. In a similar assay, Lopez *et al*. (2008) showed that pre-colonized LGG down-regulates flagellin-induced IL-8 production in Caco-2 cells. When competing with *E. coli* in the various assays, *L. plantarum* CS24.2 was unable to protect HT‑29 cells from induction of TNF-α and IL-8 at 24 h; however, the fold expression of both molecules was significantly less than that of *E. coli* stimulated HT-29 cells. Surprisingly, IL-8 expression was similar to that of the control after we added *L. plantarum* CS24.2 to HT-29 cells pre-stimulated with *E. coli*. We also observed the same effect in secretion of IL-8 from HT-29 cells.

The exact mechanisms of immunomodulation by probiotic bacteria are still under investigation; however several reports suggest that lactobacilli mediate anti-inflammatory effects by modulating NF-κB signalling pathways in the gut epithelium (Ko *et al.*, 2007; Wang *et al.*, 2011). Of note, in this study, the ability of *L. plantarum* CS24.2 to attenuate IL-8 secretion in displacement assay was markedly stronger than its abilities in the other two assay conditions. Suppression of activation of NF-κB and subsequent IL-8 expression by some secretory molecules of *L. plantarum* CS24.2 may explain this difference. The fact that, in the displacement assay, the unbound *E. coli* were removed before adding 1×10^8 cfu of *L. plantarum* CS24.2 to HT-29 cells may have led to increased accumulation of such anti-inflammatory molecules. In the competitive inhibition assay, equal numbers of lactobacilli and *E. coli* simultaneously stimulated the HT-29 cells, the balance being in favour of *E. coli* because it is a strong inducer of IL-8. Similarly, in the adhesion inhibition assay, the number of bound lactobacilli and molecules secreted by them would have been fewer when 1×10^8 cfu of *E. coli* was added after removal of unbound lactobacilli. These considerations explain why the activities in these two assays were not as strong as in the displacement assay. Additionally, in the displacement assay the number of lactobacilli added was large compared to the number of *E. coli* that remained adherent to the HT-29 cells after the initial wash step. The number of bacteria has been shown to play an important role in immunomodulation (Evrard *et al.*, 2011). However, for *L. plantarum* CS24.2, both the purported secretory molecules and the mechanisms involved have yet to be identified. Furthermore, both lactobacilli showed similar ability to inhibit adhesion of *E. coli* but different abilities to attenuate IL-8 expression in the displacement assay, suggesting that pathogen inhibition and immunomodulation are independent and strain specific properties. Although, LGG is reportedly effective in various infectious diseases, recent clinical data suggests that its efficacy in IBD is mixed (Bousvaros *et al.*, 2005).

As characterized in earlier chapters, the high adhesion index and ability of *L. plantarum* CS24.2 to inhibit the enteropathogenic *E. coli* O26:H11 adhesion to intestinal epithelial cell line suggests the applicability of this strain to replenish the protective microbes in pathogen associated gut diseases. Along with the antagonistic ability, the inability of *L. plantarum* CS24.2 to elicit the pro-inflammatory molecules expression and down-regulating the expression of TNF-α and IL-8 induced by *E. coli*

O26:H11 in HT-29 cells shows the applicability of this strain in maintaining protective immunity in healthy host and inflammatory disease patients, respectively. This primary study strongly suggests the efficacy of *L. plantarum* CS24.2 in the treatment of inflammatory diseases and need to be further confirmed with *in vivo* study.

CHAPTER 5

STUDY THE ROLE OF SELECTED ADHESINS IN THE DIFFERENT *LACTOBACILLUS* STRAINS AND THE INTERACTION STUDY WITH INTESTINAL EPITHELIAL CELL LINES

"We can choose to be affected by the world or we can choose to affect the world."

-Heidi Wills

Chapter 5

Study the role of selected adhesins in the different *Lactobacillus* strains and the interaction study with intestinal epithelial cell lines.

5.1. Introduction

Most lactobacilli found in GIT of animal and human are clearly autochthonous, since they are present throughout the life of the host and they can be cultured in large numbers, and they are present in all animals and humans (Walter, 2008). The different anatomical and physiological conditions present throughout the gut would require the distinct bacterial traits for colonization. Among the several factors which contribute to colonization, adhesion of bacteria in GIT is considered to be one of the key factors. Adherence of lactobacilli to the intestinal epithelium is an important characteristic as it promotes persistence time and colonisation, maintains and protect intestinal barrier by various mechanisms including antagonistic activities against pathogens and also stimulates host-microbe interactions through immunomodulation (Morelli, 2000; Servin, 2004). The adhesive properties of lactobacilli are directly linked to their surface properties which are influenced by the structure and composition of their cell wall. Several lactobacilli have been studied for their adherence ability to epithelial cells and components of intestinal mucosa *in vitro* and *in vivo* (Sghir *et al.*, 2000).

The adhesive mechanism of pathogenic bacteria is well studied however, the knowledge about surface molecules mediating adhesion of lactobacilli to the intestinal epithelium is scant, as only few of them have been identified and characterized. Both carbohydrates and proteins do have a role in adhesion of lactobacilli to intestinal epithelium. The adhesion proteins which have been characterized from different *Lactobacillus* strains include mucus binding protein (Mub / CyuL; *L. acidophilus* NCFM), mucus adhesion promoting protein (MapA; *L. reuteri* 104R), surface layer proteins (Slp; *L. helveticus* R0052) and elongation factor

Tu (EF-Tu; *L. johnsonii* NCC533) (Granato *et al.*, 2004; Buck *et al.*, 2005; Miyoshi *et al.*, 2006; Johnson-Henry *et al.*, 2007). Genomics-based approaches have also revealed some bacterial cell-surface-associated proteins that bind to mucus and intestinal cells (Buck *et al.*, 2005). EF-Tu is a G-protein which facilitates the transfer of aminoacyl-tRNA to the acceptor site of the ribosome. Despite being a cytoplasmic and an anchorless protein, EF-Tu has also been found at the surface of *L. johnsonii* NCC 533 (La1) and it mediates the colonization of the bacteria by attachment to mucus and intestinal epithelium (Granato *et al.*, 2004). However, the mechanism by which EF-Tu interacts with intestinal cell is not well understood. The presence of EF-Tu at the surface of pathogenic *E. coli* and periplasm of *Neisseria gonorrhoeae*, also has been reported (Jacobson and Rosenbusch, 1976; Porcella *et al.*, 1996). Similarly, MapA is also anchored less surface protein which mediates the adhesion of *L. reuteri* 104R to Caco-2 cells (Miyoshi *et al.*, 2006). MapA shares a homology with the collagen binding protein, CnBP of *L. reuteri* NCIB 11951 and the bacterial surface protein, BspA of *L. fermentum* (Roos *et al.*, 1996; Turner *et al.*, 1999)

In present chapter, the role of *Lactobacillus* adhesins – EF-Tu and MapA was established in different *Lactobacillus* strains. The applicability of EF-Tu in preventing adhesion of pathogenic bacteria to gut epithelium was also examined by competitive exclusion of pathogens from Caco-2 cells and immobilized mucin, using partially purified recombinant EF-Tu derived from *L. plantarum* CS24.2. Further, the change in expression of EF-Tu gene in selected *Lactobacillus* strains was analysed in the presence of mucin. The pull down assay was carried out with Caco-2 lysate to identify the EF-Tu interacting receptors.

5.2. Materials and methods

5.2.1. Bacterial strains

Lactobacillus strains**:** LGG, *L. plantarum* ATCC 8014, *L. delbrueckii* M, *L. fermentum* ASt1, *L. plantarum* CS23, *L. plantarum* CS24.2, *L. rhamnosus* CS25 and *L. casei* CS5.2

E. coli DH5α [F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–]

E. coli BL21(DE3) [F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])]

5.2.2. PCR profiling of *Lactobacillus* strains for EF-Tu and MapA gene

The presence of known adhesins was detected in *Lactobacillus* strains by utilizing gene specific primers for elongation factor Tu (EF-Tu) and mucus adhesion promoting protein (MapA) by colony PCR. The PCR primers EF-Tu F: 5'ATGGCAAAAGAACATTAT3' and R: 5'GTCATCAATTTCTGAAAC3' were designed using sequence of EF-Tu gene (lp_2119; 1188 bp) from *L. plantarum* WCFS1 (NC_004567). The amplification of MapA gene was carried out with PCR primers MapA F: 5' ATGAAAAAATGGCTCAAAG 3' and MapA R: 5' TTTGTTTGTGACGTTACC 3' designed from a gene (AL935261) reported earlier (Ramiah *et al.*, 2007). The PCR products were resolved by separation on a 1% agarose gel and stained with ethidium bromide for visualization under an UV-transilluminator.

Reaction system for EF-Tu and MapA gene amplification

Reaction Components	**Volume (µl)**
R.O water	16.9
10X Buffer for Taq DNA Pol (Sigma-Aldrich)	2.5
dNTP mix (2.5 mM each)	2.0
Forward primer (100pmol/µl)	0.8
Reverse primer (100pmol/µl)	0.8
Taq pol (2.5 U/µl)	1.0
Colony suspension	2
Total volume	**25**

PCR condition for amplification of EF-Tu and MapA gene

Steps		Temperature (˚C)		Time	No. of cycles
		EF-Tu	MapA		
1	Pre-cycle denaturation	94	94	5 min	1
2	Denaturation	94	94	30 sec	30
3	Primer annealing	44.9	46.4	30 sec	
4	Primer extension	72	72	1.2 min for EF-Tu 1 min for MapA	
5	Post-cycle elongation	72	72	10 min	1

5.2.3. Cloning and expression of EF-Tu and MapA in pET expression system

The EF-Tu and MapA genes were amplified from *L. plantarum* CS24.2 by colony PCR. The PCR primers EF-Tu (F: 5’ GTCAGCATATGATGGCAAAAGAACATTAT 3’ and R: 5’ GTATAGGATCCGTCATCAATTTCTGAAAC 3’) and MapA (F: 5’ GTCAGCATATGATGAAAAAATGGCTCAAAG 3’ and R: 5’ GTATAGGATCCTTTGTTTGTGACGTTACC 3’), containing recognition sequences (underlined) for restriction enzymes *Nde*1 and *Bam*H1 sites, respectively were designed using sequence of EF-Tu and MapA gene of *L. plantarum* WCFS1. Amplifications were performed using AccuTaq (Sigma-Aldrich) under the conditions as mentioned above. The expected 1.2 Kb and 0.8 Kb amplicons for EF-Tu and MapA genes were observed on agarose gel electrophoresis. The amplicons were cloned initially into pJET1.2 blunt end cloning vector (Fermentas, Maryland, USA).

Ligation system

Component	**Volume (µl)**
2X reaction buffer	10
Eluted PCR product	02
Water, nuclease free	05
DNA blunting enzyme	01
Total volume:	18

Vortexed briefly, centrifuged for 3-5 s, incubated at 70°C for 5 min, held briefly on ice and the following components added:	
Component	**Volume (µl)**
pJET1.2/blunt cloning vector (50ng/ µl)	1
T4 DNA ligase (5 U/µl)	1
Total volume:	20
Incubated at 22°C for 2 hr.	

The ligation products were used for transforming *E. coli* DH5α cells following the transformation protocol described by Sambrook *et al*. (1989). Transformants were screened on the basis of ampicillin (100 µg/ml) resistance. The cloned EF-Tu and MapA genes were excised from pJET1.2 using restriction enzymes *Nde*1 and *Bam*H1 and subcloned by directional cloning into expression vector pET30(a) and pET28(c) (Novagen, EMD Biosciences, Darmstadt, Germany) giving rise to pET-EFTu and pET-MapA, respectively. These clones were confirmed by sequencing. For expression analysis, *E. coli* BL21(DE3) transformed with either pET-EFTu or pET-MapA was inoculated with overnight grown culture in 5 ml of Luria broth containing 1% glucose and 40 µg/ml of kanamycin and incubated at 37ºC till log phase was achieved. Likewise, *E. coli* BL21(DE3) transformed with control vector, pET30(a) was also inoculated and used as a negative control. Isopropyl-β-D-thiogalactopyranoside (IPTG; Fermentas) was added to a final concentration of 5 mM and culture was further incubated at 37ºC and aliquots were withdrawn at different time point to check for optimal expression of recombinant protein. Induced cells of *E. coli* BL21(DE3) transformed with pET-EF-Tu and pET30(a) were harvested by centrifugation at 10,000 *g* × 10 min, 4ºC and pellet was resuspended in 1 × gel loading buffer, boiled for 5 min and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. Protein bands were visualized by staining the gel with Coomassie brilliant blue R-250 (Sigma-Aldrich).

Reagents for Glycine SDS PAGE

Gel stock solution (30%, 100ml)

Acrylamide	29 g
Bis acrylamide	1 g
Distilled Water (DW)	100 ml

Resolving gel

DW	1.9 ml
30% gel stock	1.7 ml
1.5 M Tris HCl (pH 8.8)	1.3 ml
10% SDS (w/v)	50 µl
10% APS (w/v)	50 µl
TEMED	7.5 µl

Stacking gel

DW	1.4 ml
30% gel stock	0.33 ml
1 M Tris HCl (pH 6.8)	0.25 ml
10% SDS (w/v)	20 µl
10% APS (w/v)	20 µl
TEMED	5 µl

Sample buffer (5X)

250 mM Tris HCl (pH 6.8)
500 mM β-Mercapthoethanol
10% SDS (w/v)
50% Glycerol (v/v)
0.5% Bromophenol Blue

Tank buffer (5X, 1 L). Use as 1X

Tris base	15.1 g
Glycine	94 g
SDS	10 g

5.2.4. Purification of recombinant EF-Tu and MapA

Two different methods were employed to purify the recombinant proteins

1) Partial purification of EF-Tu with gel elution method

The cells were grown in 25 ml Luria broth containing 1% glucose and 40 µg/ml of kanamycin and induced with 5 mM IPTG at log phase. They were harvested after 14 h incubation by centrifugation at 10,000 *g* for 10 min at 4ºC. The cells were resuspended in 3 ml PBS, and lysed by sonication (9.9 sec ON, 9.9 sec OFF, 3 min 30 sec). The lysate was centrifuged at 10,000 *g* for 10 min at 4ºC. The pellet obtained therefrom was resuspended in 3 ml PBS. EF-Tu was purified from semi denaturing SDS-PAGE using an in house electro elution apparatus. Briefly, post-sonication resuspended pellet was incubated overnight at 37ºC with gel loading buffer without β-mercaptoethanol and thereafter resolved on 10% SDS-PAGE. The band corresponding to 50 kDa was excised from the gel and was electro eluted into a dialysis membrane by overnight transfer (50 V, 4°C) in transfer buffer containing Tris-glycine. The eluted protein was dialyzed twice against 2.5% Triton-X 100 in 50 mM Tris (pH 8.8) and twice against 50 mM Tris (pH 8.8). Subsequently, the eluted sample was run on 10% SDS-PAGE along with various concentrations of bovine serum albumin (BSA). The gel was then stained with silver nitrate and EF-Tu estimated by densitometry analysis employing AlphaEaseFC 4.0 software (data not shown). The electroeluted sample from the corresponding region of the SDS-PAGE gel lane bearing induced *E. coli* BL21(DE3) transformed with pET30(a) vector was taken as negative control for the effect of EF-Tu.

2) Purification of His tagged recombinant proteins by Ni-affinity chromatography

The EF-Tu and MapA were expressed with C-terminal and N-terminal His-tag, respectively. The pellet obtained post IPTG induction for 4 hrs was resuspended in 1 ml of lysis buffer containing sarcosine. The sample was sonicated as mentioned above and the supernatant obtained thereafter was used. The hundred microliter of Ni-Cl agarose beads were washed twice with 1.5 ml of distilled water each. The beads were collected at the bottom of the tube at every step by centrifuging at 5000 rpm for 5 min. The beads were kept in equilibration buffer for 15 min before it was loaded with cell lyste. The beads were incubated with equal volume of cell lysate for 2 hrs at

37°C. The unbound fraction was collected and beads were washed five times with wash buffer. The his-tagged recombinant proteins were separated from beads by incubation with elution buffer for 30 min at 37°C.

The purified recombinant proteins from both the methods were analysed on 10% glycine and tricine SDS-PAGE for EF-Tu and MapA, respectively. The purified recombinant proteins by Ni-affinity chromatography in each preparation were estimated by Bradford method using bovine serum albumin (BSA) as a standard.

Reagents of Ni-affinity purification

Lysis buffer

1% N-lauryl sarcosine (w/v)
25 mM Triethanol amine
0.5 % Triton X-100 (w/v)
300 mM NaCl
50 mM Phosphate buffer pH-8.0

Equilibration buffer

300 mM NaCl
50 mM Phosphate buffer pH-8.0
5 mM Imidazole

Wash buffer

300 mM NaCl
50 mM Phosphate buffer pH-8.0
20 mM Imidazole

Elution buffer

300 mM NaCl
50 mM Phosphate buffer pH-8.0
250 mM Imidazole

Reagents for Tricine SDS-PAGE:

Gel buffer (3X)

3 M Tris Cl
1 M HCl
0.3% SDS (w/v)
pH 8.45

Resolving gel

DW	1.9 ml
30% gel stock	1.7 ml
Gel buffer (3X)	1.3 ml
Glycerol	50 µl
10% APS (w/v)	50 µl
TEMED	7.5 µl

Stacking gel

DW	1.4 ml
30% gel stock	0.33 ml
Gel buffer (3X)	0.25 ml
10% APS (w/v)	20 µl
TEMED	5 µl

Cathode buffer (10X)

3 M Tris Cl
1 M Tricine
1% SDS (w/v)
pH ~8.25

Anode buffer (10X)

3 M Tris Cl
0.225 M HCl
pH 8.9

Sample buffer (4X)

12% SDS (w/v)
6% mercaptoethanol (v/v)
30% glycerol (w/v)
0.05% Coomassie blue G-250 (w/v; Sigma)
150 mM Tris/HCl (pH 7.0)

5.2.5. Adhesion of *Lactobacillus* strains and enteropathogens to Caco-2 cells in the presence of recombinant EF-Tu

Post confluent Caco-2 cells in 24 wells tissue culture plate were pre-incubated with 50 µl of recombinant EF-Tu (8 µg/ml) and 900 µl of DMEM to give a final concentration of 400 ng/ml EF-Tu. The cells were incubated for 15 min at 37ºC followed by addition of 50 µL suspension of lactobacilli and pathogenic strains in respective wells to get 1×10^8 cfu/well. The electroeluted sample from the corresponding region of the SDS-PAGE gel lane bearing induced *E. coli* BL21(DE3) transformed with pET30(a) vector was taken as negative control for the effect of EF-Tu. After 90 min incubation in a 5% CO_2 – 95% air atmosphere, adhered lactobacilli and pathogenic strains from respective wells were counted by plating on MRS and Luria agar plates respectively. The experiment was carried out in duplicate in two successive passages.

5.2.6. Preparation of polyclonal antibody against EF-Tu

Immunization of rabbit with EF-Tu was done as described previously (Bellis *et al.*, 1994). Briefly, a part of the pellet containing EF-Tu was run on 10% SDS-PAGE, stained with coomassie brilliant blue and the band containing EF-Tu was excised from the gel and emulsified with Freund's incomplete adjuvant (FIA) (Bgenei). The rabbit was administered with emulsified suspension via subcutaneous route twice with a 3 week interval between immunizations. One microgram of partially purified EF-Tu by electro-elution was emulsified with Freund's complete adjuvant (FCA) (Bgenei) and used for booster immunization. Blood sample was collected one week after booster immunization for serum preparation.

5.2.7. Western Blot analysis with anti-EF-Tu antibodies

The eluted protein was confirmed by Western blot analysis using rabbit anti-EF-Tu polyclonal antibody. A partially purified EF-Tu, the cell lysate of pET-EF-Tu transformed *E. coli* BL21(DE3) as positive control and the cell lysate of pET30(a) transformed *E. coli* BL21(DE3) as negative control were resolved on 10% SDS-PAGE and transferred onto a nitrocellulose membrane by electro blotting (16 h, 25 V, 4˚C). After transfer, membrane was blocked with 2% skimmed milk in PBS (w/v) for 1 h. Subsequently, the membrane was washed with PBS and incubated with rabbit anti-EF-Tu polyclonal antibody in PBS for 1 h. After incubation, the membrane was washed thrice for 1 min each with 0.2% Tween20 in PBS (v/v). The membrane was further incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG followed by washing as above and developed with substrate (0.05% Diaminobezidine,0.02 % nickel chloride and 0.003% H_2O_2 in PBS).

Reagents for Western Blot analysis

Transfer buffer for Western blot (600 ml)

Tris base	1.81 g
Glycine	8.64 g
DW	480 ml
Methanol	120 ml

Substrate for Western Blot

Diamino Benzidine Tetrahydrochloride (DAB)	5 mg
1% NiCl (w/v)	200 µl
20 mM PBS	10 ml
H_2O_2	10 µl

5.2.8. Antibody mediated adhesion inhibition of *Lactobacillus* strains

The antibody mediated inhibition assay was performed with Caco-2 monolayers in 24 well tissue culture plate according to method previously described (Chen *et al.*, 2007). Different concentrations of rabbit anti EF-Tu polyclonal antibody (2 mg/ml) – 5%,

10% and 15% in DMEM were initially used for adhesion inhibition. Based on the initial observation, 0.5 ml of DMEM containing 0% and 5% rabbit anti-EF-Tu polyclonal antibody and approximately 0.5 $\times 10^8$ cfu of lactobacilli were added to Caco-2 monolayers. Caco-2 monolayers incubated with negative serum and lactobacilli were used as controls. After 90 min incubation in a 5% CO_2 – 95% air atmosphere, adhered lactobacilli from respective wells were counted by plating on MRS agar plate. The experiment was carried out in duplicate in two successive passages.

5.2.9. Adhesion of lactobacilli and enteropathogens to mucin in the presence of EF-Tu

The lactobacilli and enteropathogens were assayed for adhesion to mucin in the presence of EF-Tu. The mucin coated wells were pre-incubated with 190 µl of recombinant EF-Tu for 15 min at 37 °C at different concentrations (0.5 µg, 1 µg and 2 µg per well). Ten microlitres of each of the lactobacilli and enteropathogens were added thereafter to respective wells to get 2×10^7 cfu per well and further incubated for 90 min. After the incubation, the wells were processed by Triton X-100 as described above after washing with sterile PBS. The negative controls were set with bovine serum albumin (BSA; 1 µg per well) instead of recombinant EF-Tu.

5.2.10. Mucin exposure of lactobacilli

The strains which showed presence of EF-Tu in the genome with gene specific primers were further analysed for change in expression of EF-Tu gene in response to mucin exposure. The strains of *L. plantarum* and *L. delbrueckii* M were incubated in MRS broth containing 0.05% (w/v) mucin as described earlier with some modifications (Ramiah *et al.* 2007). Briefly, lactobacilli grown in MRS broth for 18-20 h at 37 °C were transferred to fresh MRS broth with an OD_{600} of 1.8 and incubated for 4 h at 37 °C. After the incubation, the cells were harvested by centrifugation at 10,000 *g* for 2 min, and washed twice in sterile PBS. The washed cells were further incubated in MRS broth containing mucin with an OD_{600} of 1.8. Controls were set

with culturing the lactobacilli in MRS broth without mucin. The resuspended cells were incubated at 37 °C and the samples were drawn for RNA extraction after 3 h.

5.2.11. cDNA synthesis and RT-qPCR

The cells pellet obtained from approx 1×10^8 cfu (OD_{600} of 1.0) was resuspended in 200 µl of Tris–HCl buffer (10 mM, pH 8.0). Cells were lysed by incubation with lysozyme (12 µl of 50 mg/ml lysosyme) for 20 min at 37 °C (Ramiah et al. 2007). RNA was isolated with the total RNA extraction kit (Bangalore Genei) which contains guanidine thiocyanate. RNA was resuspended in 30 µl of DEPC-treated water. The quality of the RNA sample was determined by inspecting the integrity of 23S and 16S bands on agarose gel electrophoresis. The total RNA in each sample was quantified on NanoPhotometer® Pearl and one µg of total RNA was used for cDNA synthesis. The RNA sample was mixed with anchored oligo-dT in a 20 µl system using verso cDNA synthesis kit based on Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (Thermo Fisher Scientific, Surrey, UK) following manufacturer's instructions. Briefly, the RNA was mixed with oligo dT, RT enhancer which contains DNAse I, dNTP mix and enzyme mix, followed by incubation at 50˚C for 30 min and then 95˚C for 2 min to inactivate the enzyme in a thermal cycler (Eppendorf, Hamburg, Germany). Quantitative PCR amplifications were then performed in CFX96^{TM} real-time thermal cycler (Bio-Rad Laboratories, Hercules, USA) with specific primers for EF-Tu. The amplification conditions were as follows: initial denaturation at 94˚C for 3 min, followed by 45 cycles of denaturation at 94˚C for 10 s, annealing and extension for 30 s at 60˚C and the fluorescence was recorded after each cycle. Each sample was run in triplicate and cycle threshold (Ct) was used for gene expression analysis. The transcripts expression of EF-Tu in each sample was normalized to internal reference 16S rRNA transcript expression of the same sample using the CFX manager software (Bio-Rad Laboratories). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers were designed from the conserved region of EF-Tu gene and 16S rRNA gene of different *Lactobacillus* strains. The sequence of the both the genes were obtained from all the available whole genome sequenced lactobacilli in NCBI sequence database. The bioinformatics tool ClustalW for multiple sequence alignment was used to identify the conserved region among different sequences obtained for each gene. The primers sequences were EF-Tu RT F: 5'-AGCCGGCGATAACGTTGGT-3' and R: 5'-TCAGTGGTGTGGAAGTAGAA-3'

(201 bp) and 16S rRNA F: 5'-TTATGACCTGGGCTACACAC-3' and R: 5'-GTGTGTACAAGGCCCGGGA-3' (189 bp). The relative expression of EF-Tu in control for each strain was set to one and relative fold change for each mucin exposed sample was reported. The product specificity was confirmed by single peak in melt curve analysis (from 65°C to 95°C in 0.5°C/5 s increments). The negative controls were set with the total RNA without reverse transcription (data not provided).

Reaction system for EF-Tu and 16S rRNA gene amplification

Reaction Components	**Volume (µl)**
R.O water	3
2 × SsoFast EvaGreen supermix	5
Forward primer (10 pmol/µl)	0.5
Reverse primer (10 pmol/µl)	0.5
First strand	1
Total volume	**10**

5.2.12. Preparation of Caco-2 protein

Crude Caco-2 protein extracts were obtained from T-75 flasks grown to confluence as described earlier (Wampler *et al.,* 2004). Cells were detached with 2 ml of Triton X-100 lysis buffer (300 mM NaCl, 50 mM Tris-HCl [pH 7.6], 0.5% Triton X-100) and 200 µl of 10X Protease Inhibitor Cocktail (Sigma), with rocking of the flasks on ice for 45 min. The lysate was centrifuged at 10,000 *g* for 15 min at 4°C, and the supernatant was collected and stored at -80°C until used.

5.2.13. Pull down assay to identify Caco-2 receptor interacting to EF-Tu

The experiment was designed to identify receptor like molecules interacting with EF-Tu. A two hundred microliters whole cell lysate aliquot of *E. coli* BL21(DE3) expressing His-tagged EF-Tu was mixed with 200 µl of Ni-Cl agarose beads and allowed to bind for 2 h at the 37°C. Following incubation, the unbound proteins were removed and agarose beads were washed seven times using wash buffer with intervals of 15 min between each wash. Thereafter, two hundred microliters of Caco-

2 protein extracts was loaded on EF-Tu bound Ni-Cl agarose beads and incubated in rotary shaker at 50 rpm, for 2 h at 37°C. After incubation, the column was again washed seven times with wash buffer. The protein-protein complex of EF-Tu and interacting molecule from cell lysate was eluted from the Ni-Cl agarose beads by incubating the beads with 100 µl of elution buffer for 30 min. A negative control was set up by incubating the cell lysates directly with Ni-Cl agarose beads. The eluted samples were analysed on 10% glycine SDS-PAGE and stained with silver nitrate.

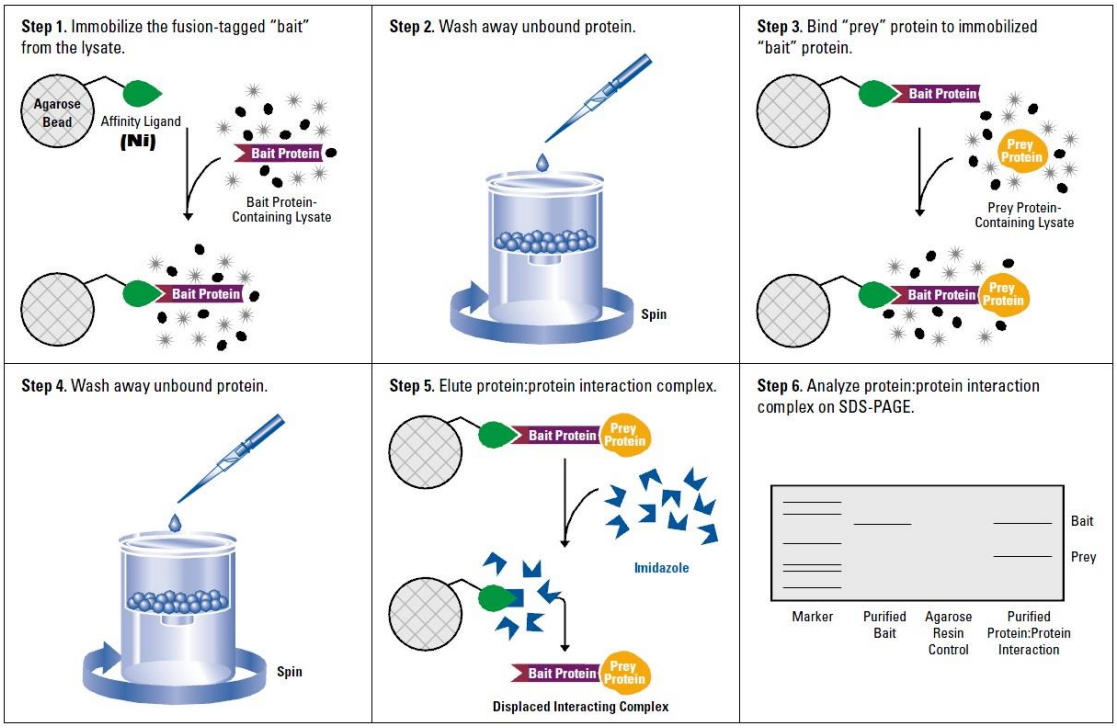


Figure 5.1. Schematic representation of pull down assay.

5.2.14. Confirmation of EF-Tu – Caco-2 receptor like molecules interaction with cross-linking agent

Post-confluent Caco-2 cells in 24-wells tissue culture plate were washed three times with PBS (pH8.0). The cells were co-incubated with different concentrations of purified recombinant EF-Tu (3 µg, 5 µg or 10 µg/ml PBS) in duplicate wells for one hour. Following incubation, wells were washed with PBS and non-cleavable homofunctional cross-linker BS^3 (bis[sulfosuccinimidyl] suberate; Sigma) was added to a final concentration of 2 mM or 5 mM in respective wells. The reaction was allowed to continue for 30 min at room temperature. The reaction was stopped with

Quench solution, 1M Tris (pH 7.5) at a final concentration of 20 mM by incubating for 15 min at room temperature. The cell monolayer was lysed using lysis solution. Caco-2 cells exposed to only BS^3 was used as a negative control. Subsequently, the analysis of cross-linking was carried out by western blotting using anti-EF-Tu antibodies.

5.2.15. Adhesion of lactobacilli to mucin and Caco-2 in the presence of MapA

The *Lactobacillus* strains were also assayed for the adhesion to immobilized mucin and Caco-2 cells in the presence of recombinant MapA. The assay was performed similarly as mentioned above for EF-Tu. One microgram of recombinant MapA was used in all the assays.

5.2.16. Statistics

Values are given as mean along with the standard deviation (SD) from two independent experiments in duplicate. Significant ANOVAs were followed by Dunnett test in the case of different adhesion assays and compared with the respective control ($p<0.05$). The statistical analysis was conducted using SigmaStat 3.5 software.

5.3. Results

5.3.1. Adhesin profiling of *Lactobacillus* strains

The PCR amplification products from *L. plantarum* ATCC 8014, *L. delbrueckii* M, *L. plantarum* CS23 and *L. plantarum* CS24.2 were of the expected 1.2 Kb size which suggests the presence of adhesive EF-Tu gene in these strains. Further confirmation was carried out by sequencing and subsequent sequence homology search using BLAST tool which showed 99% identity with EF-Tu gene of *L. plantarum* WCFS1. The expected 0.8 Kb product of MapA gene was found in all *Lactobacillus* strains which were further confirmed by sequencing and homology search (Figure 5.2).

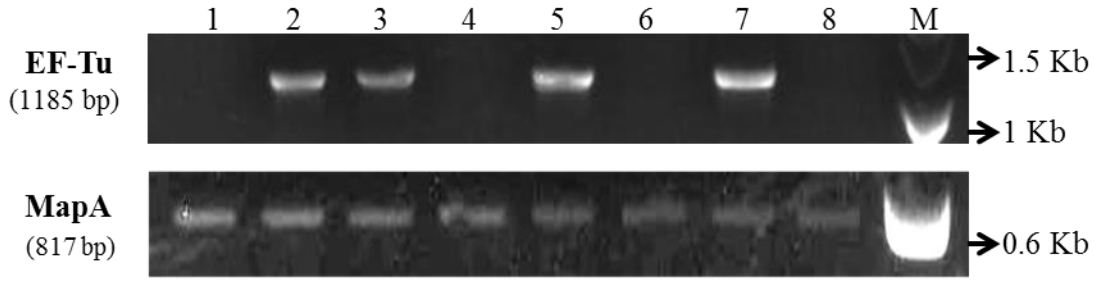


Figure 5.2. 1% agarose gel stained with ethidium bromide for PCR based profiling of *Lactobacillus* strains for adhesins- EF-Tu and MapA.

Lane 1: *L. rhamnosus* CS25
Lane 2: *L. plantarum* CS24.2
Lane 3: *L. plantarum* CS23
Lane 4: *L. casei* CS5.2
Lane 5: *L. delbrueckii* M
Lane 6: *L. fermentum* ASt1
Lane 7: *L. plantarum* ATCC8014
Lane 8: LGG
Lane M: Low range DNA marker

5.3.2. Cloning of EF-Tu and MapA gene in pET expression vector

The EF-Tu and MapA gene were amplified from *L. plantarum* CS24.2 with proofreading DNA polymerase (Figure 5.3) and cloned into blunt end cloning vector pJET1.2. The transformants were confirmed by colony PCR with gene specific primers and inserts release using *Nde*1 and *Bam*H1 restriction enzymes. The restriction digestion of clones showed the expected size insert release with 1.2 Kb and 0.8 Kb for EF-Tu and MapA gene, respectively (Figure 5.4 and Figure 5.5). The gel eluted released inserts of EF-Tu and MapA were then ligated into pET30(a) and pET28(c) digested with same restriction enzymes, respectively. The ligated products were initially transformed in *E. coli* DH5α. The transformants were screened with colony PCR and confirmed constructs were then transformed in expression host *E. coli* BL21(DE3) (Figure 5.6). Further confirmation was obtained from the sequencing of recombinant plasmids using gene specific primer. The blast analysis of the sequencing results confirmed the inserts as the gene coding for EF-Tu and MapA for pET30(a)-EF-Tu and pET28(c)-MapA, respectively.

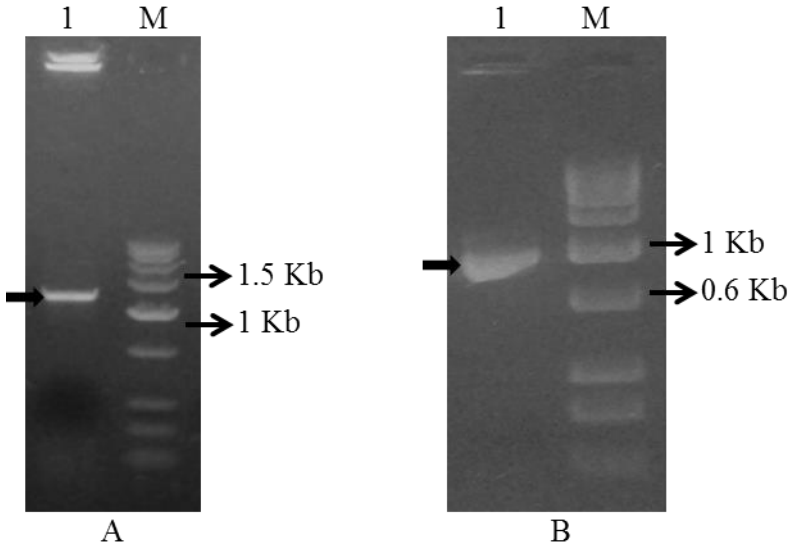


Figure 5.3. 0.8% agarose gel stained with ethidium bromide with the colony PCR sample of *L. plantarum* CS24.2 for **A)** EF-Tu and **B)** MapA gene using gene specific primers. The arrow indicates the expected size amplicon.

Gel A

Lane 1: EF-Tu amplicon

Lane M: Low range DNA marker

Gel B

Lane 1: MapA amplicon

Lane M: Low range DNA marker

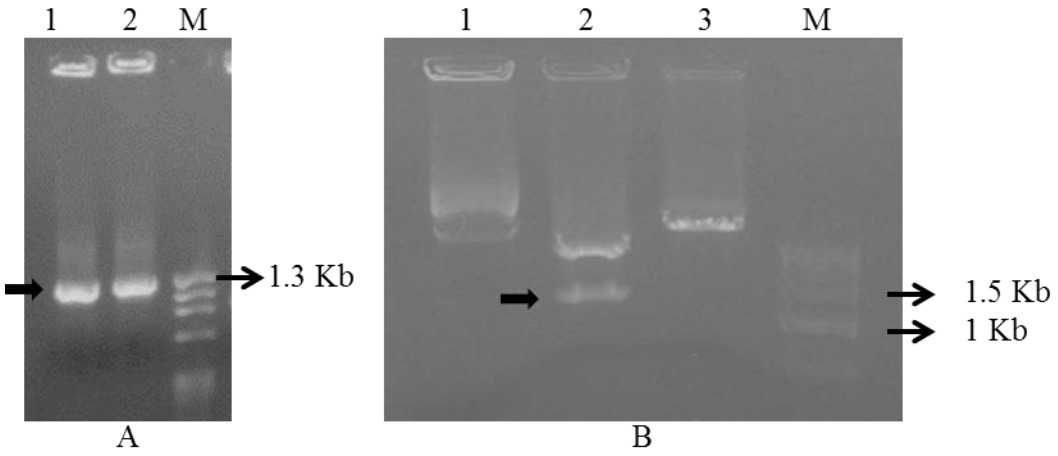


Figure 5.4. 0.8% agarose gel for confirmation of EF-Tu gene cloning in pJET1.2- A) colony PCR with EF-Tu gene specific primers and B) insert release with restriction enzymes digestion.

Gel A

Lane 1: Putative clone-1

Lane 2: Putative clone-2

Lane M: ΦX174 DNA / *Hae*III

Gel B

Lane 1: Undigested pJET-EF-Tu (Clone-1)

Lane 2: pJET-EF-Tu / *Nde*1, *Bam*H1 (Clone-1)

Lane 3: pJET-EF-Tu / *Nde*1, *Bam*H1 (Clone-2)

Lane M: Low range DNA marker

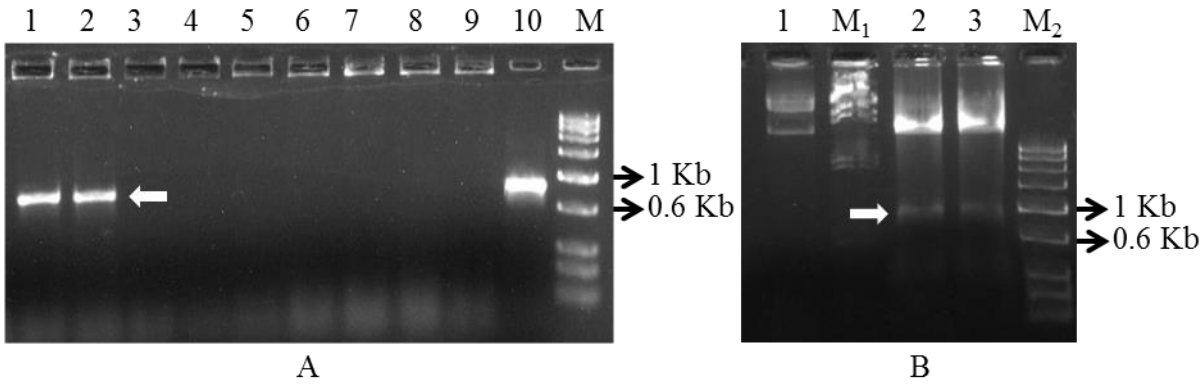


Figure 5.5. 0.8% agarose gel for confirmation of MapA gene cloning in pJET1.2- A) colony PCR with MapA gene specific primers and B) insert release with restriction enzymes digestion.

Gel A

Lane 1-9: Putative clones-1-9

Lane 10: Positive control

Lane M: Low range DNA marker

Gel B

Lane 1: Undigested pET28(c)-MapA (Clone-1)

Lane M_1: λ DNA / *Hind*III digest

Lane 2: pET28(c)-MapA / *Nde*1, *Bam*H1 (Clone-1)

Lane 3: pET28(c)-MapA / *Nde*1, *Bam*H1 (Clone-2)

Lane M_2: Low range DNA marker

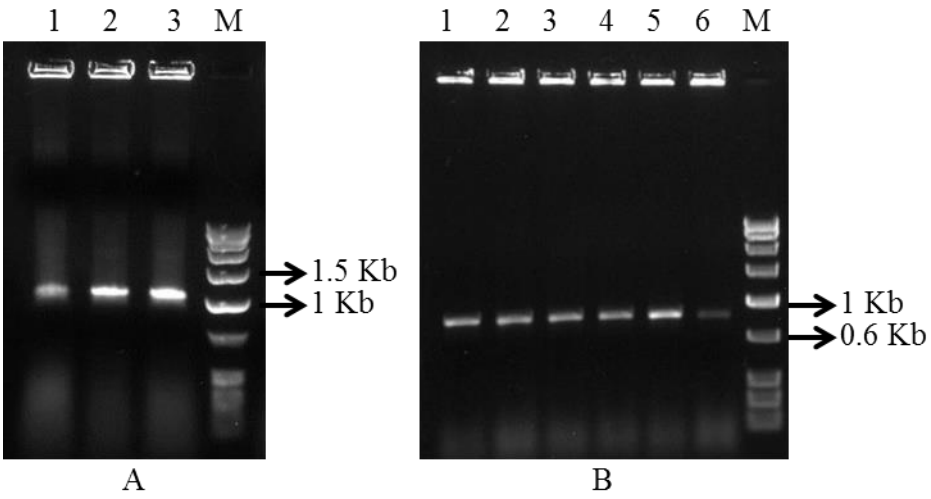


Figure 5.6. 0.8% agarose gel stained with ethidium bromide with the colony PCR sample for confirmation of *E. coli* BL21(DE3) transformed with A) pET30(a)-EF-Tu and B) pET28(c)-MapA using gene specific primers.

Gel A	Gel B
Lane 1-3: Putative transformants-1-3	**Lane 1-6:** Putative transformants-1-6
Lane M: Low range DNA marker	**Lane M:** Low range DNA marker

5.3.3. Expression and purification of recombinant EF-Tu and MapA

The expression of EF-Tu was induced with IPTG at log phase and cells were harvested at 4 h, 14 h and 24 h. The cells were lysed in Laemmli sample buffer and expression was analysed on glycine SDS-PAGE. Optimum time for the induction of EF-Tu in *E. coli* BL21(DE3) was observed to be 14 h. A 50-kDa band corresponding to EF-Tu was observed in the lane with *E. coli* BL21(DE3) transformed with recombinant pET30(a)-EF-Tu plasmid in contrast to that in the negative control that contained the similarly induced sample of *E. coli* BL21(DE3) transformed with control plasmid pET30(a) alone (Figure 5.7). The initial construct of EF-Tu was not having N-terminal his-tag so the protein was purified with gel elution method. The partially purified EF-Tu preparation was analyzed on 10% SDS-PAGE employing silver staining (Figure 5.8). Another construct of EF-Tu with C-terminal His-tag showed the high expression at 4 h. The recombinant EF-Tu was purified with Ni-affinity chromatography from whole cell extract of *E. coli* BL21(DE3) expressing

pET30(a)-EF-Tu. The partially purified EF-Tu preparation was analyzed on 10% SDS-PAGE employing silver staining (Figure 5.9) and further confirmed through western blot analysis (Figure 5.10).

The expression of MapA was induced similarly with IPTG and 27-kDa induced band corresponding to MapA was observed on gel compared to un-induced and negative control. The recombinant MapA was purified with Ni-affinity chromatography utilizing the N-terminal His-tag. The purified recombinant MapA was analysed on 10% tricine SDS-PAGE (Figure 5.11).

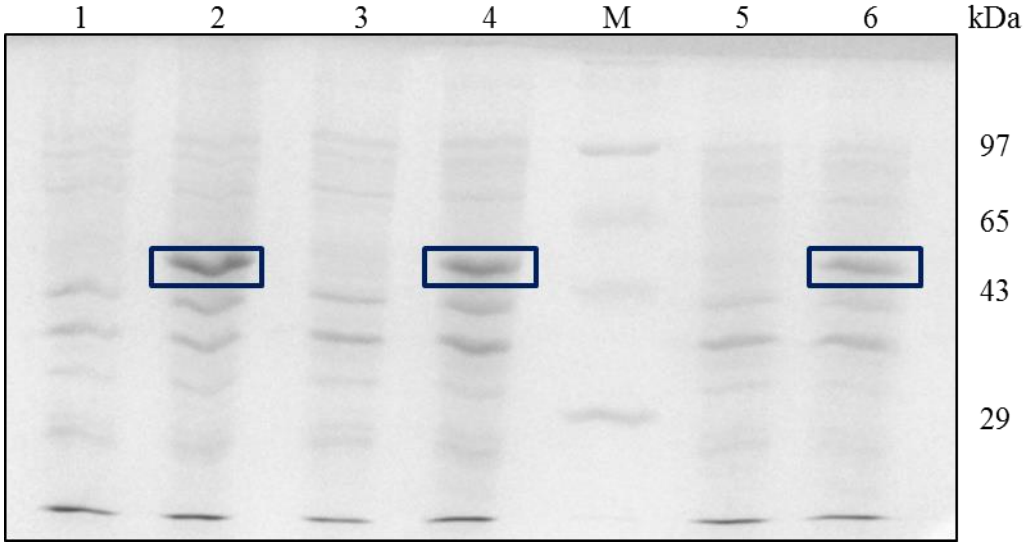


Figure 5.7. Coomassie stained 10% glycine SDS-PAGE with whole cell extracts of *E. coli* BL21(DE3) transformed with pET30(a) or pET30(a)-EF-Tu post 5mM IPTG induction.

Lane 1: pET30(a), 4hr sample
Lane 2: pET30(a)- EF-Tu, 4hr sample
Lane 3: pET30(a), 14hr sample
Lane 4: pET30(a)- EF-Tu, 14hr sample
Lane M: Protein high mol wt marker
Lane 5: pET30(a), 24hr sample
Lane 6: pET30(a)- EF-Tu, 24hr sample

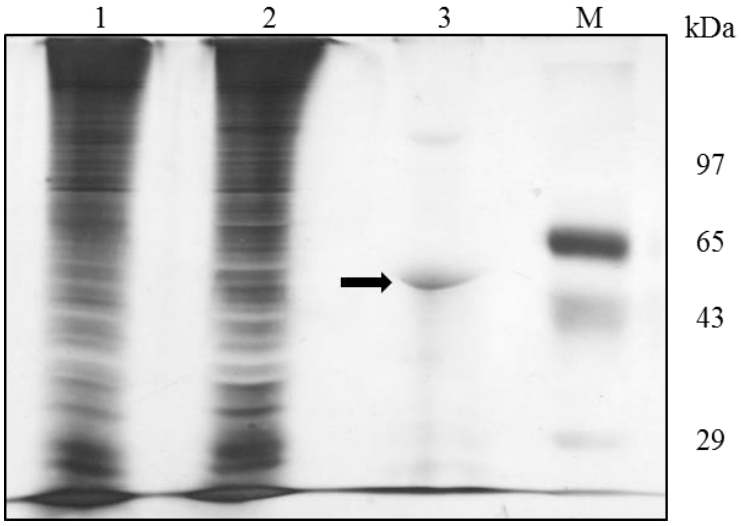


Figure 5.8. Silver stained 10% glycine SDS-PAGE with partially purified EF-Tu from gel elution method.

Lane 1: pET30(a), 14hr sample
Lane 2: pET30(a)-EF-Tu, 14hr sample
Lane 3: Partially purified EF-Tu
Lane M: Protein high mol wt marker

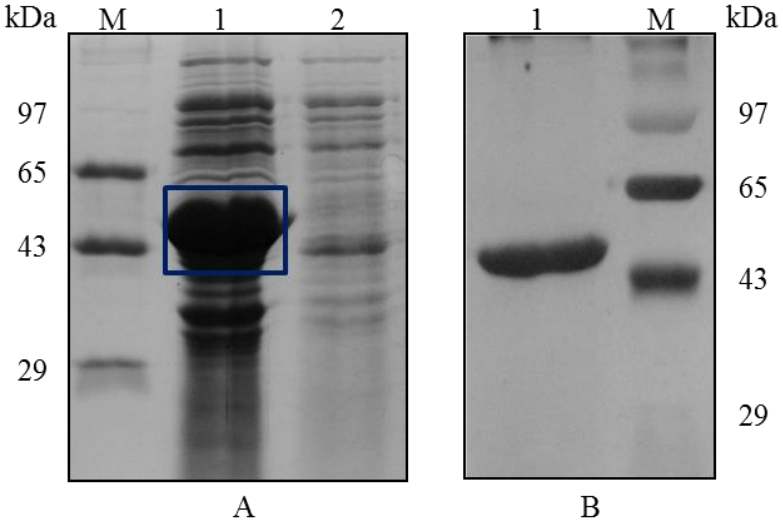


Figure 5.9. A) Coomassie stained 10% glycine SDS-PAGE with whole cell extracts of *E. coli* BL21(DE3) transformed with pET30(a) or pET30(a)-EF-Tu post 5mM IPTG induction and B) silver stained purified EF-Tu with Ni-affinity chromatography.

Gel A
Lane M: Protein high mol wt marker
Lane 1: pET30(a)-EF-Tu, 4hr sample
Lane 2: pET30(a), 4hr sample

Gel B
Lane 1: Purified EF-Tu
Lane M: Protein high mol wt marker

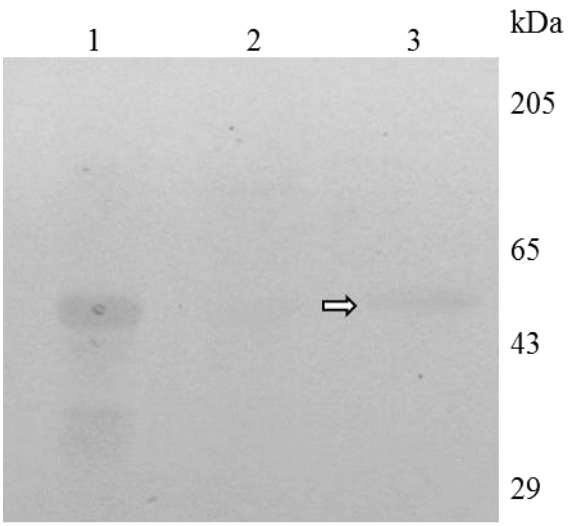


Figure 5.10. Western blot analysis of partially purified EF-Tu from gel elution method.

Lane 1: Whole cell lysate *E. coli* BL21(DE3) pET30(a)-EF-Tu, 4hr sample

Lane 2: Whole cell lysate *E. coli* BL21(DE3) pET30(a), 4hr sample

Lane 3: Purified EF-Tu

⇨ Denotes band of 50 kDa EF-Tu

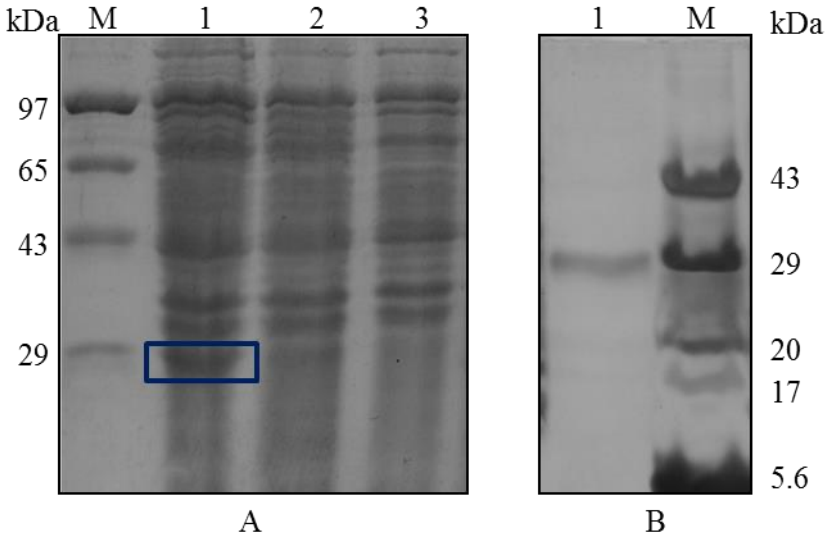


Figure 5.11. A) Coomassie stained 10% glycine SDS-PAGE with whole cell extracts of *E. coli* BL21(DE3) transformed with pET30(a) or pET28(c)-MapA post 5mM

IPTG induction and B) silver stained 10% tricine SDS-PAGE of purified MapA with Ni-affinity chromatography.

Gel A

Lane M: Protein high mol wt marker

Lane 1: pET28(c)-MapA, 4hr sample

Lane 2: pET28(c)-MapA, 4hr sample (Un-induced)

Lane 3: pET30(a), 4hr sample

Gel B

Lane 1: Purified MapA

Lane M: Protein low mol wt marker

5.3.4. Adhesion of *Lactobacillus* strains to Caco-2 cells and mucin in the presence of EF-Tu

To evaluate the contribution of EF-Tu in adhesion of *Lactobacillus* strains to Caco-2 cells, competition assays were performed in the presence of purified recombinant EF-Tu preparation (Figure 5.12). In competition assay, there was significant reduction ($p<0.05$) in adhesion of the strains of *L. plantarum*, *L. delbrueckii* M and *L. rhamnosus* CS25 to Caco-2 cells, when the EF-Tu was co-incubated with the bacterial cells. Adhesion of *L. plantarum* ATCC 8014, *L. plantarum* CS23, *L. plantarum* CS24.2, *L. delbrueckii* M and *L. rhamnosus* CS25 was inhibited by 23.8%, 30.5%, 50.9%, 23.5% and 17.5%, respectively.

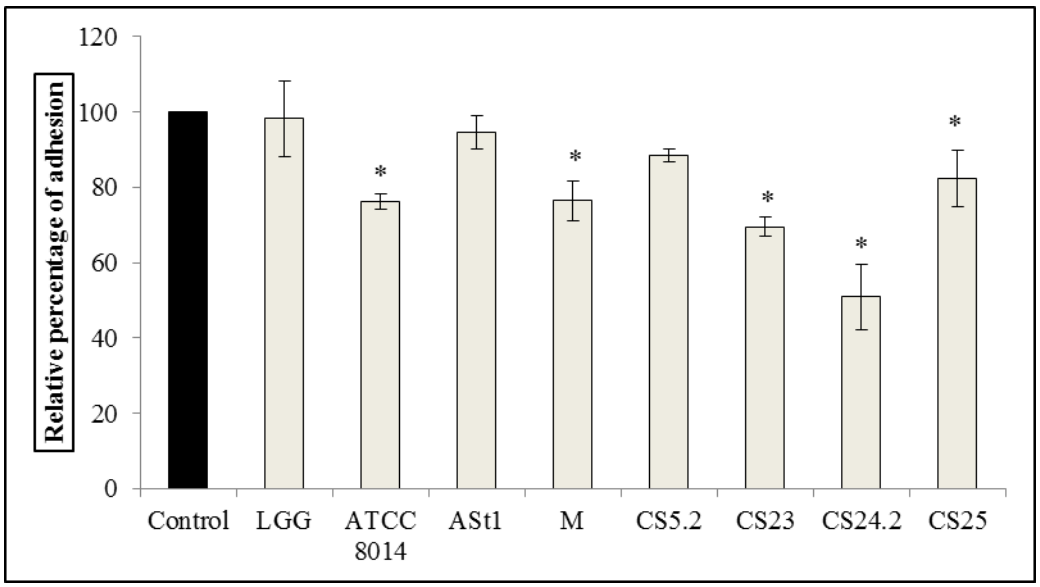


Figure 5.12. Adhesion of *Lactobacillus* strains to Caco-2 cells in the presence of purified recombinant EF-Tu. Adhesion of lactobacilli in the absence of EF-Tu is

denoted as control. Each bar represents mean value and standard deviation as error bar. Significant ANOVA was followed by Dunnett test for comparison versus control group. * mean value of adhesion was significantly lower than that of control (P<0.05).

Further, the *Lactobacillus* strains were also analysed for adhesion to mucin in the presence of EF-Tu (Figure 5.13). There was significant reduction in adhesion of same *Lactobacillus* strains to immobilized mucin also as observed with Caco-2 cells (p<0.05). The reduction in adhesion was ranged between 18.4% to 70.0%. With both adhesion model, *L. rhamnosus* CS25 showed poor adhesion inhibition compare to other strains.

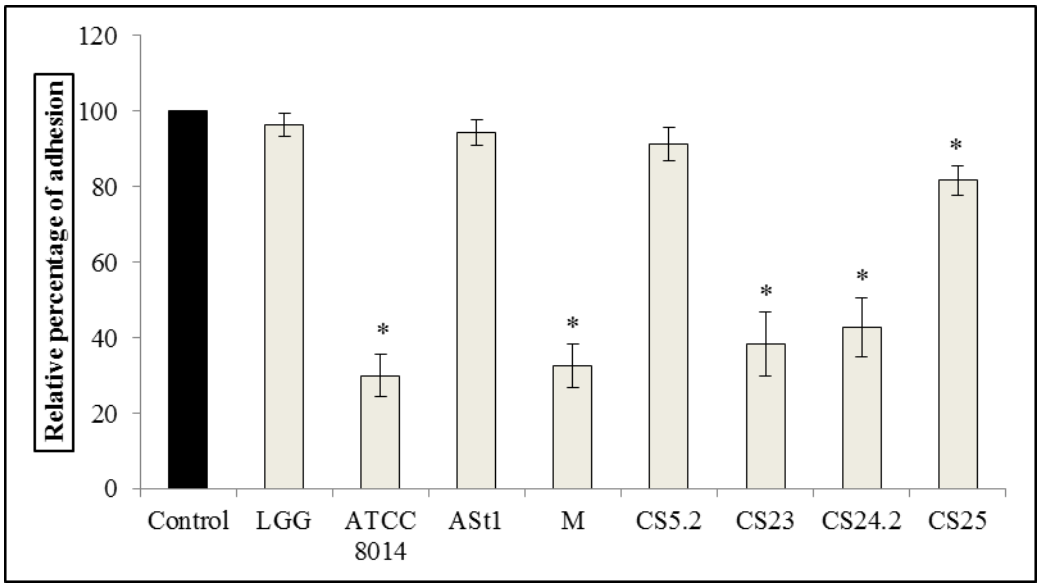


Figure 5.13. Adhesion of *Lactobacillus* strains to immobilized mucin in the presence of purified recombinant EF-Tu. Adhesion of lactobacilli in the absence of EF-Tu is denoted as control. Each bar represents mean value and standard deviation as error bar. Significant ANOVA was followed by Dunnett test for comparison versus control group. * mean value of adhesion was significantly lower than that of control (P<0.05).

5.3.5. Confirmation of EF-Tu mediated adhesion inhibition of *Lactobacillus* strains with anti-EF-Tu antibodies

To further confirm the role of EF-Tu in adhesion, antibody mediated adhesion inhibition assay was performed in the presence of rabbit anti-EF-Tu polyclonal antibody (Figure 5.14). Co-incubation with negative serum showed no effect on adhesion of lactobacilli compared with control where no serum was added. There was significant decrease ($p<0.05$) in adhesion of *L. rhamnosus* CS25, *L. delbrueckii* M and the strains of *L. plantarum* by addition of 5% polyclonal antibody. Adhesion of *Lactobacillus* strains was reduced by 9% - 36.5% in the presence of polyclonal antibody when compared with control.

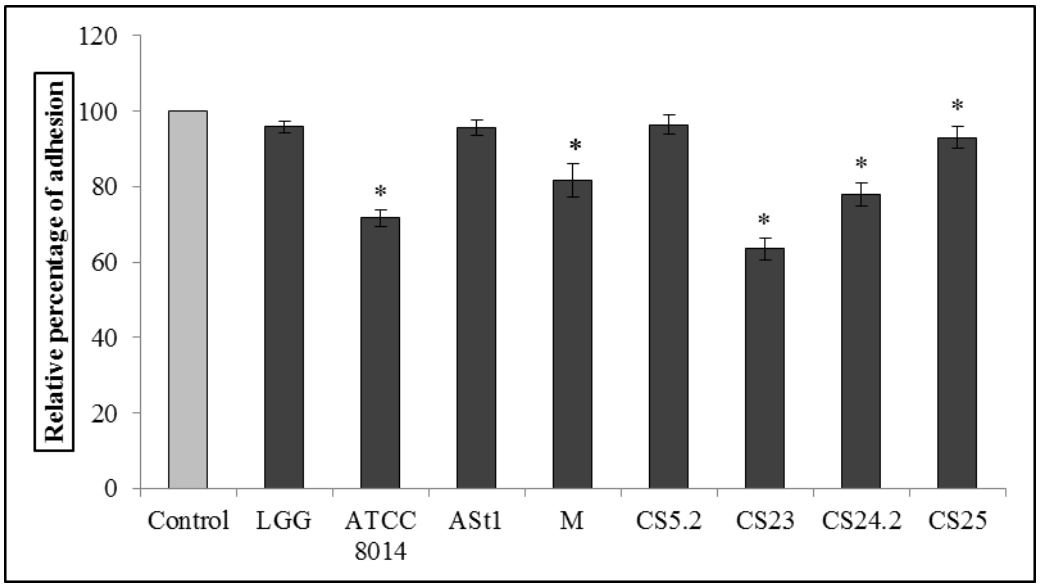


Figure 5.14. Effect of EF-Tu on the adhesion of *Lactobacillus* strains to Caco-2 cells was determined by antibody mediated adhesion inhibition assay. Adhesion of lactobacilli in the absence of rabbit anti-EF-Tu polyclonal antibody is considered as absolute binding and denoted as control. Each bar represents mean value and standard deviation as error bar. Significant ANOVA was followed by Dunnett test for comparison versus control group. * mean value of adhesion was significantly lower than that of control ($P<0.05$).

5.3.6. Competition assays between EF-Tu and enteropathogens for adhesion to Caco-2 cells

To study the effect of EF-Tu on the adhesion of enteropathogens to Caco-2 cells, the bacterial cells were co-incubated with partially purified recombinant EF-Tu preparation of *L. plantarum* CS24.2 in competitive adhesion assays as described above. There was significant decrease ($p<0.05$) in adhesion of both enteropathogens in the presence of EF-Tu compared to control. The effect of EF-Tu addition to assay was more prominent on the adhesion of *S.* Typhi compared to that of *E. coli*. Adhesion of *E. coli* and *S.* Typhi to Caco-2 cells was strongly inhibited by addition of recombinant EF-Tu, as it was reduced by 35.3% and 47.7% respectively (Figure 5.15).

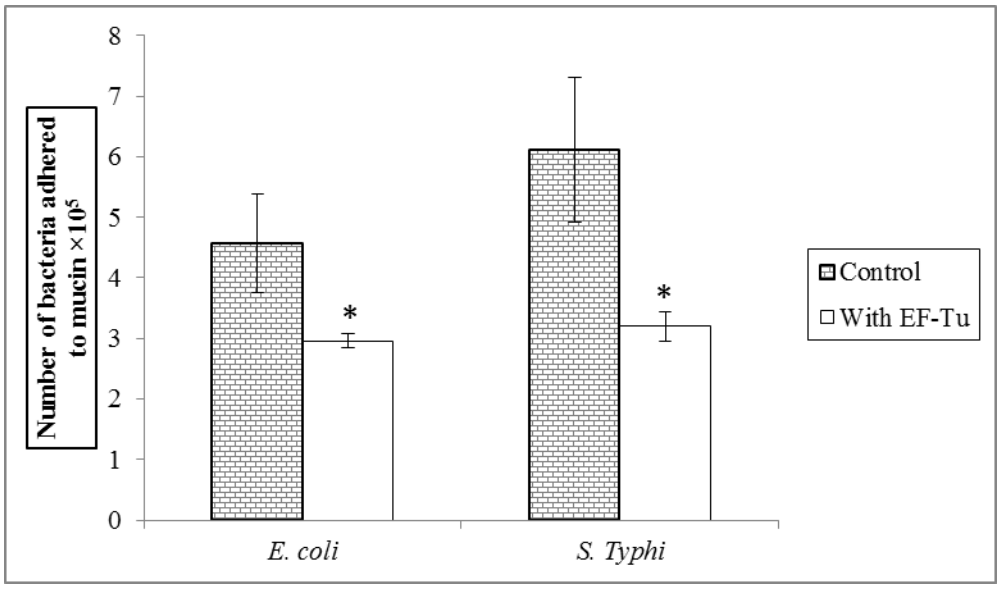


Figure 5.15. Adhesion of enteropathogens *E. coli* O26:H11 and *S.* Typhi MTCC 733 to Caco-2 cells in the presence of the recombinant EF-Tu. Adhesion of enteropathogens in the absence of EF-Tu is considered as absolute binding and denoted as control. Each bar represents mean value and standard deviation as error bar of three independent experiments. Significant ANOVA was followed by Dunnett test to compare with respective control group. * mean value was significantly lower than that of control ($p<0.05$).

Further, the adhesion of *E. coli* and *S.* Typhi to mucin was strongly inhibited by addition of 2 µg of recombinant EF-Tu and it was reduced by 46.1% and 40.6%,

respectively. There was no significant reduction in adhesion of *S.* Typhi with 0.5 µg of recombinant EF-Tu but adhesion of *E. coli* was reduced significantly by 26.0% at the same concentration of EF-Tu ($p<0.05$). There was no significant difference in adhesion when BSA was used instead of recombinant EF-Tu (Figure 5.16).

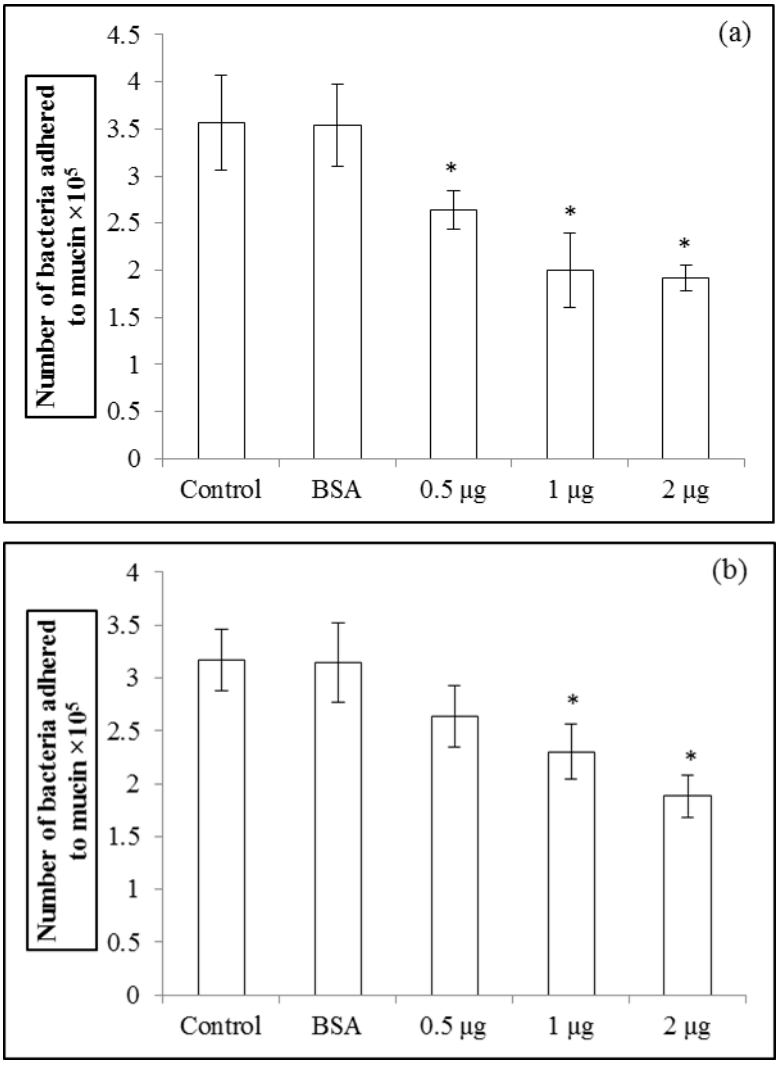


Figure 5.16. Adhesion of enteropathogens (**a**) *E. coli* O26:H11 and (**b**) *S.* Typhi MTCC 733 to mucin in the presence of the recombinant EF-Tu with different concentrations (0.5 µg, 1 µg and 2 µg per well). Adhesion of enteropathogens in the absence of EF-Tu is considered as absolute binding and denoted as control while adhesion in the presence of BSA (1 µg per well) was taken as negative binding control. Each bar represents mean value and standard deviation as error bar of three independent experiments. Significant ANOVA was followed by Dunnett test to compare with respective control group. * mean value was significantly lower than that of control ($p<0.05$).

5.3.7. Expression of EF-Tu gene in response to mucin exposure

The total RNA was isolated from the control and mucin exposed lactobacilli. The presence of 16S and 23S rRNA on the agarose gel shows the integrity of RNA sample (Figure 5.17). Figure shows representative sample of total RNA. The cDNAs prepared from these samples were further used to check the expression of EF-Tu and 16S rRNA gene with quantitative real time PCR.

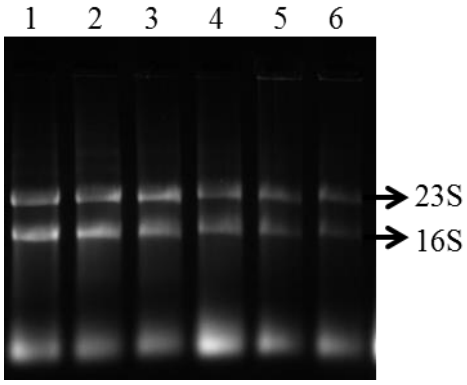


Figure 5.17. 0.8% agarose gel stained with ethidium bromide with the total RNA from control and mucin exposed lactobacilli.

The fold change of EF-Tu gene expression in response to mucin exposure in the strains of *L. plantarum* and *L. delbrueckii* M is shown in Figure 5.18. The relative expression of EF-Tu gene of each strain without mucin exposure was considered as control and set to one. There was a significant up-regulation of EF-Tu gene in all the strains when exposed to mucin for 3 h. The effect of mucin was strain specific and fold change was found to be 9.15, 2.96 and 5.69 for *L. plantarum* ATCC 8014, *L. plantarum* CS23 and *L. plantarum* CS24.2, respectively. The fold change of EF-Tu gene in *L. delbrueckii* M when exposed to mucin was very low and found to be 1.52.

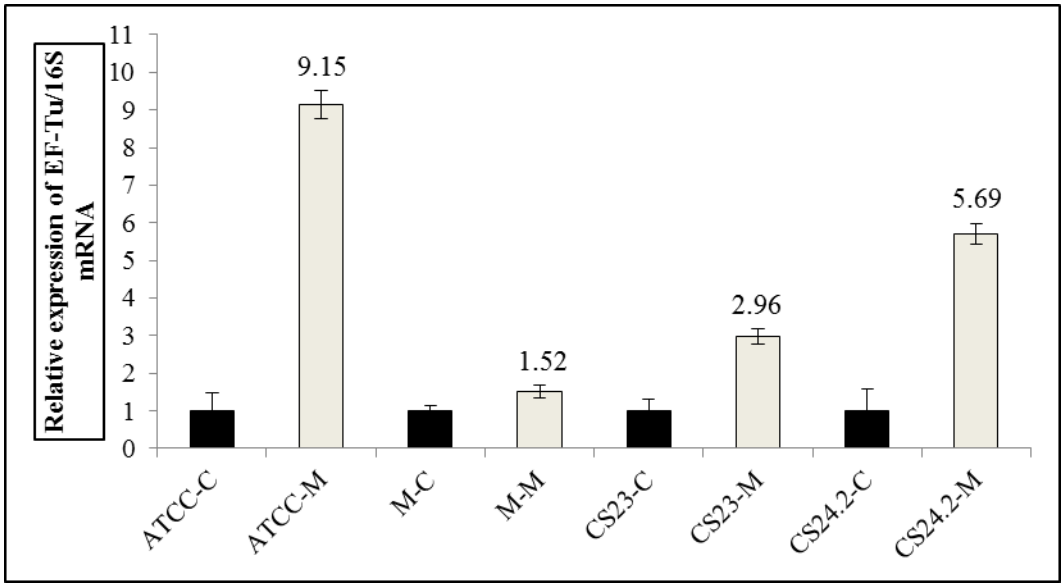


Figure 5.18. The effect of mucin on the expression of EF-Tu gene in the strains of *L. plantarum* and *L. delbrueckii* M. The expression of EF-Tu gene was normalized to the internal reference 16S rRNA gene expression. The relative expression level in control (without mucin exposure) was set to one for fold expression analysis in other experimental groups. Each bar represents mean value and standard deviation as error bar. Significant ANOVAs were followed by Dunnett's test for multiple comparisons *v.* control group ($p<0.05$).

5.3.8. Adhesion of *Lactobacillus* strains to mucin in the presence of MapA

To evaluate the role of MapA in adhesion of *Lactobacillus* strains to Caco-2 cells, competition assays were performed in the presence of purified recombinant MapA (Figure 5.19). In competition assays, there was significant reduction ($p<0.05$) in adhesion of *Lactobacillus* strains except *L. fermentum* ASt1 and *L. casei* CS5.2 to Caco-2 cells, when the MapA was pre-incubated with the bacterial cells. The adhesion inhibition of *Lactobacillus* strains was ranged between 14% - 45% in the presence of recombinant MapA.

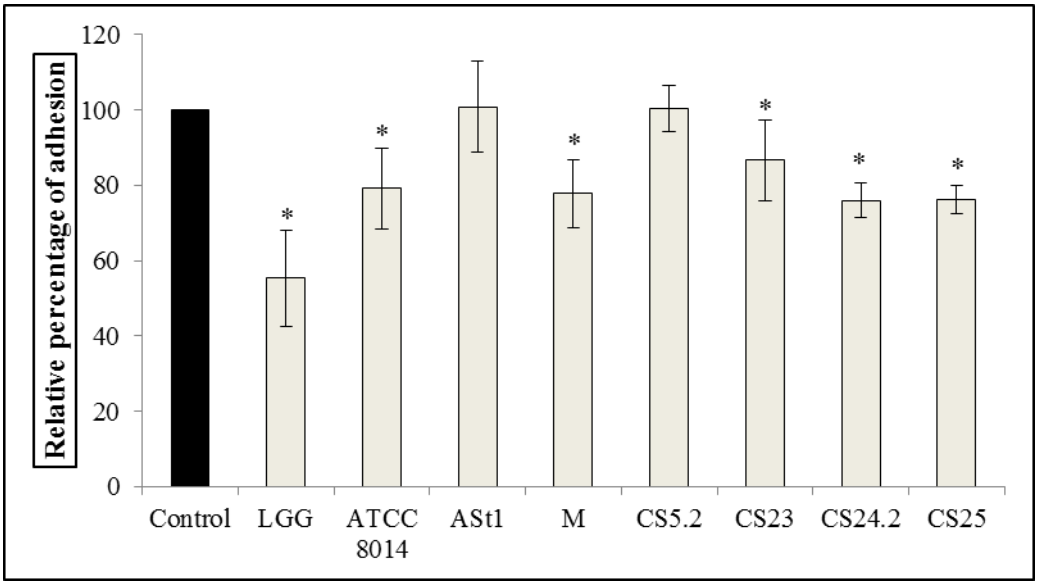


Figure 5.19. Adhesion of *Lactobacillus* strains to Caco-2 cells in the presence of purified recombinant MapA. Adhesion of lactobacilli in the absence of MapA is denoted as control. Each bar represents mean value and standard deviation as error bar. Significant ANOVA was followed by Dunnett test for comparison versus control group. * mean value of adhesion was significantly lower than that of control ($P<0.05$).

Further, the *Lactobacillus* strains were also analysed for ability to adhere immobilized mucin in the presence of recombinant MapA (Figure 5.20). As observed in above experiments, the same strains also showed reduction in adhesion to mucin in the presence of recombinant MapA. The adhesion inhibition was ranged between 20% - 39% in the presence of recombinant MapA.

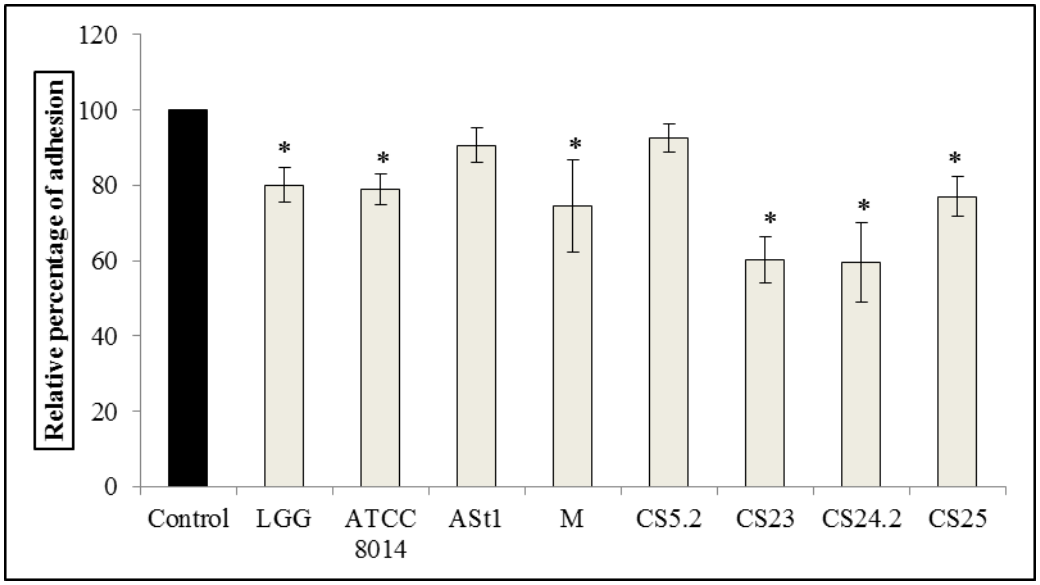


Figure 5.20. Adhesion of *Lactobacillus* strains to immobilized mucin in the presence of purified recombinant MapA. Adhesion of lactobacilli in the absence of MapA is denoted as control. Each bar represents mean value and standard deviation as error bar. Significant ANOVA was followed by Dunnett test for comparison versus control group. * mean value of adhesion was significantly lower than that of control ($P<0.05$).

5.3.9. Pull down assay

EF-Tu interacting receptor like protein from Caco-2 cells was identified in the pull down assay. The pull down elution fraction of EF-Tu co-incubated with Caco-2 whole cell lysate showed the presence of an additional ~ 60 kDa size protein band along with 50 kDa of recombinant EF-Tu. This distinct protein band was not present in Caco-2 lysate elution fraction and purified EF-Tu elution fraction (Figure 5.21; Gel A- Lane 3 & 4). To further confirm the interaction, the non-cleavable cross-linker BS^3 was incorporated in the reaction mix containing live Caco-2 cells and recombinant EF-Tu. The cross-linker is expected to cross link relatively all surface molecules leading to the formation of stable complexes. Western bolt analysis of cross-linked interaction mixture detected the presence of ~ 110 kDa protein band with anti-EF-Tu antibodies which correspond to 50 kDa of EF-Tu and ~60 kDa of Caco-2 surface receptor like molecules interacting with EF-Tu (Figure 5.22; Gel A- Lane 2 and Gel B- Lane 1 & 2), thus confirming the presence of a ~60 kDa Caco-2 surface protein which serves as a putative receptor for EF-Tu.

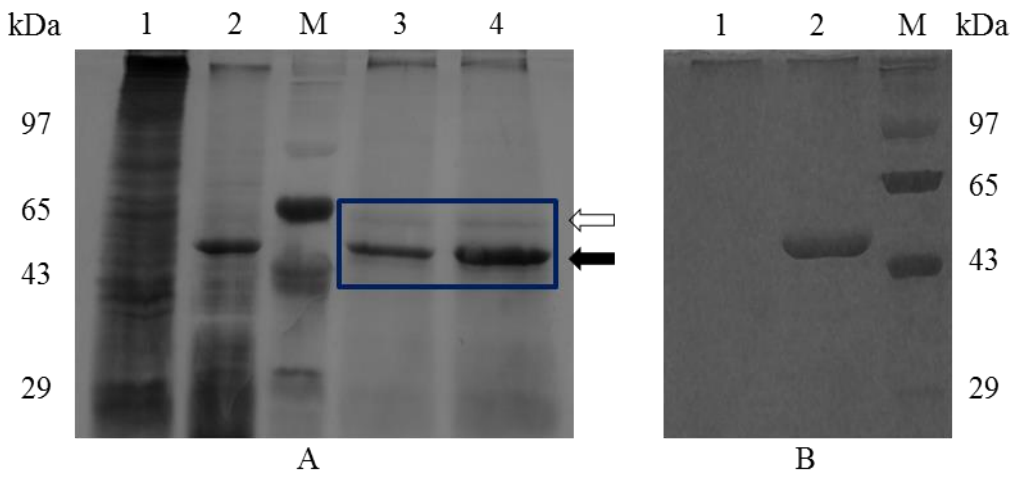


Figure 5.21. Silver stained 10% glycine SDS-PAGE with EF-Tu interacting Caco-2 receptor like molecule in pull down assay.

Gel A

Lane 1: Caco-2 cell lyste

Lane 2: Whole cell lyste expressing EF-Tu

Lane M: Protein high mol wt marker

Lane 3: Pull down assay extract (4°C)

Lane 4: Pull down assay extract (37°C)

Gel B

Lane 1: Negative control of Caco-2 lyste

Lane 2: Purified EF-Tu

Lane M: Protein high mol wt marker

⇨ indicates the presence of ~60 kDa protein band of Caco-2 receptor molecule

➡ indicates the presence of ~50 kDa protein band of EF-Tu

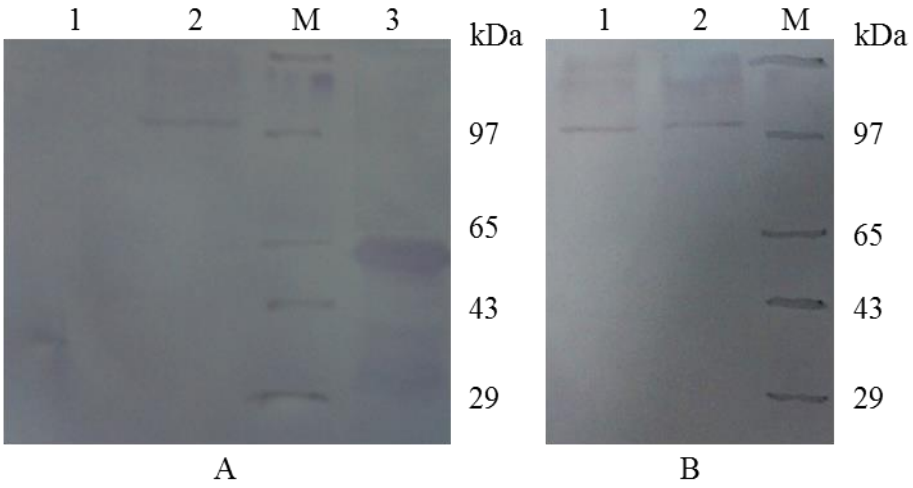


Figure 5.22. Western blot analysis of cross linked EF-Tu interacting receptor like Caco-2 surface molecule using anti-EF-Tu antibodies.

Gel A

Lane 1: Caco-2 cell lyste (Negative control)

Lane 2: EF-Tu - Caco-2 complex crosslinked with BS^3 (10 µg EF-Tu; 5mM BS^3)

Lane M: Protein high mol wt marker

Lane 3: Purified EF-Tu

Gel B

Lane 1 & 2: EF-Tu - Caco-2 complex crosslinked with BS^3 (5 µg EF-Tu; 5mM BS^3 & 10 µg EF-Tu; 5mM BS^3, respectively)

Lane M: Protein high mol wt marker

5.4. Discussion

Most lactobacilli established as probiotic have been selected by their superior phenotypic properties such as adhesion to Caco-2 cells, bile and acid tolerance and/or antimicrobial activity (Doron *et al.*, 2005; Goossens *et al.*, 2005; Swetwiwathana *et al.*, 2008). However, studying the underlying molecular mechanisms for these properties can assist in exploring the respective molecules for direct application. A variety of adhesive molecules have been identified from different strains of lactobacilli. In case of *L. plantarum* and *L. rhamnosus* strains, few of the known

adhesive molecules are alfa-enolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and mucus via mannose binding (Msa) and modulator of adhesion and biofilm (MabA) and secreted LPXTG-like pilin (SpaC), respectively (Kleerebezem *et al.*, 2003; Kinoshita *et al.*, 2008; Castaldo *et al.*, 2009; Kankainen *et al.*, 2009; Velez *et al.*, 2010). Some highly conserved cytoplasmic proteins such as EF-Tu, GAPDH and GroEL, which lack typical signal sequence and membrane anchoring mechanism for surface expression, have been characterized (Granato *et al.*, 2004; Bergonzelli *et al.*, 2006; Kinoshita *et al.*, 2008). These proteins are normally referred as anchorless multifunctional proteins or moonlighting proteins. The published genome database of the lactobacilli denotes the presence of EF-Tu gene in all those *Lactobacillus* strains which is not surprising since the elongation factor Tu (EF-Tu) is a G-protein which plays an important role in protein synthesis. Granato *et al.*, (2004) had first demonstrated the presence of EF-Tu on the surface of *L. johnsonii* NCC 533 (La1) and reported it as an adhesive molecule mediating attachment to mucin and intestinal epithelial cells.

To assess the role of EF-Tu as an adhesive molecule, we carried out heterologous expression of EF-Tu from *L. plantarum* CS24.2 into *E. coli*, followed by partial purification. The sequencing of cloned EF-Tu gene has shown 99% identity with EF-Tu gene of *L. plantarum* WCFS1. The amino acid sequence deduced from the nucleotide sequence was analyzed using Pfam database for adhesive domain search. The analysis showed that the *L. plantarum* CS24.2 EF-Tu has three adhesive domains that are an EF-Tu GTP-binding domain (PF00009), an EF-Tu domain 2 (PF03144), and an EF-Tu C-terminal domain (PF03143). Adhesion of *L. delbrueckii* M and strains of *L. plantarum* to Caco-2 cells and mucin in the presence of partially purified recombinant EF-Tu was significantly reduced which supports the adhesive role of EF-Tu in these strains. Similarly, Granato *et al.*, (2004) had also shown that La1 EF-Tu could prevent up to 40% adhesion of La1 bacteria to mucin. It is also interesting to note here that the same strains also showed the presence of EF-Tu in the genome by PCR using gene specific primers. This suggests the role of EF-Tu in the adhesion of these strains to mucin. The unequivocal contribution of EF-Tu as adhesin, atleast in case of adhesive strains have been reiterated by the experiment carried out using polyclonal antibody to EF-Tu. *L. rhamnosus* CS25 which was negative for the presence of adhesive EF-Tu gene with gene specific primers, showed the adhesion

inhibition to mucin in the presence of recombinant EF-Tu. However, the adhesion inhibition was less compared to strains with adhesive EF-Tu. This could be possible with the presence of adhesin on *L. rhamnosus* CS25 which has adhesive domains similar to that of the adhesive EF-Tu. Under the present study, there was no effect on the adhesion of LGG to Caco-2 cells and mucin which suggests that EF-Tu may not have an exclusive role in adhesion of LGG. It may be interesting to note that the recently published genome sequence of LGG reveals the presence of EF-Tu (YP_003171088) and shows 95.7% similarity to La1 EF-Tu. The sequence information also suggests the presence of all 3 adhesive domains reported for La1 EF-Tu. However, it has been reported by others that in the case of fibronectin binding protein of isogenic mutants, two lactobacilli strains with similar fibronectin binding protein didn't show similar results in adhesion to Caco-2 cells. *L. acidophilus* NCFM fbpA^{-} isogenic mutant showed reduction in adhesion to Caco-2 cells as compared to wild type strain, while such mutation in *L. casei* didn't show any reduction in adhesion to Caco-2 cells (Buck *et al.,* 2005; Munoz-Provencio *et al.,* 2009). MapA was first reported on the surface of *L. reuteri* 104R with role in adhesion to Caco-2 cells (Miyoshi *et al.,* 2006). In our studies, it was found in all the strains at the genetic level but its role in adhesion could be established in the strains except *L. casei* CS5.2 and *L. fermentum* ASt1.

The bacterial genes are known to express differentially under environmental stress conditions (Boor 2006). In the case of *Lactobacillus*, Ramiah *et al.* (2007) first reported the change in expression of mucin adhesion genes of *L. plantarum* 423 when exposed to mucin. Recently, Izquierdo *et al.* (2009) also found the overexpression of the some adhesion related proteins including EF-Tu in the cell wall proteomic analysis of highly adhesive strain *L. plantarum* WHE 92. In our study, we analysed the fold change of EF-Tu gene in the mucin exposed selected *Lactobacillus* strains which showed the presence of adhesin EF-Tu with PCR profiling. *L. plantarum* ATCC 8014 showed the highest fold change with 9.15 compared to control and adhesive strain *L. plantarum* CS24.2 showed 5.69 fold change. There was a difference in the fold change of EF-Tu gene among the strains of the same species which suggests the differential gene regulation at the strain level. Duary *et al.* (2012) showed the relative expression of 14.04 and 42.84 for EF-Tu gene of *L. plantarum* Lp9 and *L. plantarum* Lp91 in the presence of 0.05% mucin where the relative

expression in the control was 11.79 and 17.17, respectively. The presence of EF-Tu and the change in expression with response to mucin clearly indicates the significant role of EF-Tu in the adhesion ability of these strains.

Moonlighting function for many housekeeping proteins of pathogens is well documented and many of them act as enhancers of pathogen virulence. For example, when GAPDH is displayed on streptococcal surface, it has been reported to function as fibronectin binding protein (Pancholi and Fischetti, 1992). Nesser *et al.*, (2000) have shown that *L. johnsonii* La1 bind to intestinal cell membrane molecules through mechanisms that are shared by pathogenic bacteria. The data presented in this study shows that EF-Tu as an adhesive molecule of lactobacilli, provides beneficial effects against enteropathogen infection *in vitro*. The competitive adhesion analysis using recombinant EF-Tu against enteropathogens demonstrated a reduction in pathogen adhesion by 35-50%. However, it is also necessary to know whether pathogen exclusion is mediated through steric hindrance or by interacting with specific receptors. The role of EF-Tu in pathogen virulence is not yet studied but the presence of EF-Tu at the surface of pathogenic *E. coli* and membrane association is well documented (Jacobson and Rosenbusch, 1976). Bioinformatics analysis of EF-Tu from several pathogenic strains of *E. coli* and *S.* Typhi using NCBI and Pfam databases shows around 83% similarity with La1 EF-Tu and the presence of all three adhesive domains (PF00009, PF03144 and PF03143) reported for La1 EF-Tu. So the role of this EF-Tu like molecules in the adhesion of pathogen can be further investigated to establish EF-Tu mediated inhibition of enteropathogens by lactobacilli. Additionally, the identification of putative Caco-2 receptor interacting with EF-Tu was carried out with pull down assay. However, the further characterization is needed for understanding the molecular interactions.

The adhesion inhibition study with recombinant EF-Tu showed the significant role of EF-Tu in adhesion of the *Lactobacillus* strains. A similar assay with the enteropathogens demonstrated possible molecular mechanism underlying adhesin mediated inhibition of enteropathogens by the lactobacilli. With the earlier reports and present observation, it could be said that the adhesins on the surface and its expression profile do have a major role to play in the colonization of *Lactobacillus* strains in the intestinal tract. In conclusion, this is the first report stating that the moonlighting protein, EF-Tu from probiotic lactobacilli antagonizes the adhesion of enteropathogens to intestinal cells.

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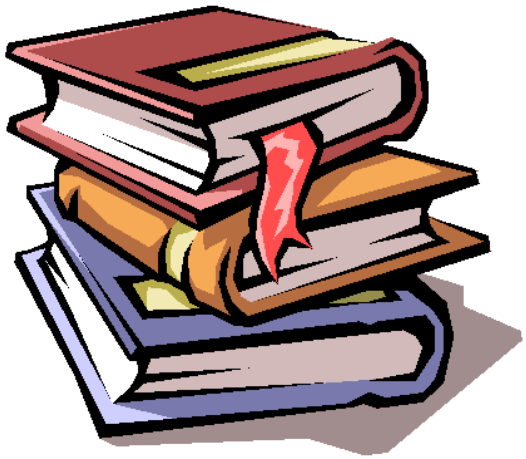

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ABSTRACT CUM SUMMARY OF THE THESIS

Thesis title: The interaction of *Lactobacillus* strains with intestinal epithelial cell lines

The human body contains diverse groups of commensal microbiota which includes both aerobes as well as anaerobes. The largest population amongst that resides in the gastrointestinal tract which is colonized by more than 400 bacterial species in the adult. The gut commensal bacteria do regulate the intestinal epithelial development and function and any interruption of these interactions may result in disease condition. The beneficial effects of the gut bacteria are attributed to probiotics which are defined as 'Live microorganisms that when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001).

Lactobacillus species are Gram positive, non-pathogenic and desirable members of intestinal tract. The health promoting effects of lactobacilli have been widely explored and include stabilization of indigenous microbial population, protection against intestinal infection, alleviation of lactose intolerance, increased nutritional value of foods, reduction of serum cholesterol levels and non-specific enhancement of the immune systems. Several lactobacilli which act as probiotic bacteria are currently being explored as novel biological therapeutic agents. Since not all lactobacilli possess ability of health benefits to host, it becomes necessary to screen and characterize numerous strains in order to obtain ideal probiotics. The colonization of the gastrointestinal tract is desirable for any probiotic which depends on several factors including the ability of the bacteria to tolerate acidic pH of the stomach and bile and on the adhesion of bacteria to intestinal cells and mucus. Isolation from humans, capability to tolerate acidic pH and bile, antimicrobial activity and good adhesion ability are principle desirable properties in potential probiotics.

The adherence of bacteria to the surface of intestinal mucosa is a key process for colonization and persistence in gastrointestinal tract (GIT). Therefore, adhesion is an important property for both pathogenic bacteria as well as bacteria

belonging to normal gut microbiota. Several *in vitro* models have been established to study bacterial adhesion and competitive inhibition ability to screen probiotic strains. The mucin is majorly synthesized by goblet cells and helps in colonization of bacteria by providing a site for attachment. The human intestinal epithelial cell lines - Caco-2 and HT-29 have been extensively used to study adhesion ability of lactobacilli. Bacterial adhesion is reported to be a non-specific physical interaction between two surfaces which is followed by specific interaction between surface adhesion molecules of bacteria and complementary receptors present on the mucus layer and the underlying gut epithelial cells. Physiochemical properties, especially cell surface hydrophobicity do have a role in aggregation and adhesion ability of bacteria. Autoaggregation ability of probiotic strains is reported to help in adhesion to intestinal epithelium and may subsequently help colonization by forming a biofilm like structure. Additionally, probiotic strains have been shown to aggregate with enteropathogen and interfere with their adhesion to the gut epithelium. Autoaggregation feature has also been correlated with adhesion ability by several investigators.

Several diseases of GIT are caused by *Salmonella enterica* serotype Typhimurium and *Escherichia coli*. To cause infection, adhesion to epithelium is essential; preventing pathogenic bacterial adhesion to the epithelial cells is an effective strategy to reduce pathogen associated illness. The epithelium of GIT can be protected from colonization of pathogen by a number of mechanisms including antibiotic treatment. Probiotic therapy is seen as potential attractive alternative to antibiotic treatment to overcome problems such as emergence of multidrug-resistant bacteria and imbalance of resident gut microbiota. It is believed while inhibition of pathogens is through soluble effectors; competitive exclusion of pathogenic bacteria is through direct competition for common attachment sites on gut epithelium. However, the molecular mechanisms of pathogens inhibition by lactobacilli are still under investigation.

Mucosal surfaces are the primary interaction sites between the host and its environment and they thus represent the major portal of entry for pathogens and vast majority of antigens. Under most circumstances, microbial attack and minor

breaches in mucosa are handled by the innate and adaptive immune systems which fight against the invading micro-organisms with no or minor clinical symptoms. Furthermore, the intestinal epithelium stays regularly exposed to commensal bacteria and become systemically tolerant to fed antigens and the phenomenon is known as oral tolerance. The enterocytes of the epithelial layer do act as immunocompetent cells and secrete various signalling molecules such as cytokines and chemokines upon adhesion and invasion by gut pathogens. Upon stimulation, the specialized immune cells such as neutrophils and macrophages migrate to the site of infection and prevent pathogen entry. Under certain conditions, the inflammations become self-sustaining due to ineffective down regulation of pro-inflammatory molecules even after the elimination of the pathogens which leads to the inflammatory disorders. Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-8 are reported to be highly expressed in Crohn's disease and acute colitis patients. The probiotic lactobacilli do have the potential to interact with the mucosal layer. Several probiotic effects are mediated through immune regulation, particularly through establishing and maintaining a balance between pro- and anti- inflammatory cytokines. That is why the study of immunomodulatory properties of the probiotics is also high on priority. Each strain of lactobacilli is bestowed with its own combination of pro- and anti-inflammatory potential. Thus, it becomes necessary to evaluate the potential of all putative probiotic strain to group them for particular immunological condition.

Most lactobacilli established as probiotic have been selected by their superior phenotypic properties such as adhesion to Caco-2 cells, bile and acid tolerance and/or antimicrobial activity. However, studying the underlying molecular mechanisms for these properties can assist in exploring the respective molecules for direct application. The adhesive mechanism of pathogenic bacteria is well studied however, the knowledge about surface molecules mediating adhesion of lactobacilli to the intestinal epithelium is scant, as only few of them have been identified and characterized. The adhesion proteins which have been characterized from different *Lactobacillus* strains include mucus binding protein (Mub / CyuL; *L. acidophilus* NCFM), mucus adhesion promoting protein

(MapA; *L. reuteri* 104R), surface layer proteins (Slp; *L. helveticus* R0052) and elongation factor Tu (EF-Tu; *L. johnsonii* NCC533). Some highly conserved cytoplasmic proteins such as EF-Tu, GAPDH and GroEL, which lack typical signal sequence and membrane anchoring mechanism for surface expression, have been also characterized. These proteins are normally referred as anchorless multifunctional proteins or moonlighting proteins. The published genome database of the lactobacilli denotes the presence of EF-Tu gene in all those *Lactobacillus* strains which is not surprising since the elongation factor Tu (EF-Tu) is a G-protein which plays an important role in protein synthesis. However, the mechanism by which EF-Tu interacts with intestinal cell is not well understood.

The primary goal of the study was to isolate and characterize *Lactobacillus* strains of human origin which has an ability to survive in gastrointestinal conditions and shows probiotic attributes. Under the present study, lactobacilli were isolated from different sources, characterized biochemically and confirmed by 16S-23S rRNA gene intergenic region amplification and subsequent sequencing. The organisms were identified by BLAST analysis and the sequences were submitted to Genbank. These isolates were analyzed for various probiotic and cell surface properties which include bile and acid tolerance, antimicrobial activity, cell surface hydrophobicity, autoaggregation and coaggregation with pathogens. To evaluate the ability, the established probiotic strain *L. rhamnosus* GG (LGG) was incorporated in the study. The isolates were analyzed for adhesion ability to intestinal epithelial cell lines (Caco-2 and HT-29) and immobilized mucin. Among the isolates, *L. plantarum* CS23, *L. plantarum* CS24.2 and *L. rhamnosus* CS25 showed significantly comparable and/or higher ability compared to LGG under all these different *in vitro* assays to characterize a strain as a probiotic.

To mimic *in vivo* conditions, the different competitive adhesion assays were designed such as competitive inhibition, adhesion inhibition and displacement assay between lactobacilli and *E. coli* O26:H11 with intestinal epithelial cell lines. From the adhesion study, we could identify two strains (*L. plantarum*

CS23 and *L. plantarum* CS24.2) which were able to survive and colonize under *in vitro* conditions better than LGG. These strains also showed the ability to antagonize enteropathogens adhesion in cell cultures. The primary mode of inhibition was found to be production of lactic acid by lactobacilli.

Thereafter, we analyzed the change in transcripts expression of various pro- and anti-inflammatory cytokines in Caco-2 cells upon stimulation with lactobacilli using semi-quantitative Reverse transcription polymerase chain reaction (RT-PCR). Based on the expression profile of different cytokines and chemokine, the isolates could be grouped into pro- or anti-inflammatory. TNF-α and IL-8 are reported to be highly expressed in Crohn's disease and acute colitis patients. Under such circumstances, the probiotic lactobacilli which have ability to attenuate the pro-inflammatory molecules expression can be used. Thus, we analyzed the ability of highly adhesive *Lactobacillus* isolate - *L. plantarum* CS24.2 and LGG to modulate the Pro-inflammatory molecules expression in HT-29 cells stimulated with *E. coli* under the different competitive adhesion assays by qRT-PCR. The isolate *L. plantarum* CS24.2 was found to attenuate the expression of the both the molecules in HT-29 cells stimulated with *E. coli* upto 24 h which was better than LGG. The results were also confirmed with ELISA.

Apart from the above, one part of work was to understand the host-microbial interaction at the molecular level. In this context, the known *Lactobacillus* adhesins- Elongation factor-Tu (EF-Tu) and Mucus adhesion promoting protein (MapA) were amplified from isolate *L. plantarum* CS24.2 and expressed heterologously in *E. coli* using pET expression system. The Ni-affinity purified His-tagged EF-Tu and MapA were then used to establish its role in adhesion of different *Lactobacillus* strains under competitive adhesion assays. There was a significant reduction in adhesion of strains of *L. plantarum* and *L. delbrueckii* M to Caco-2 cells in the presence of recombinant EF-Tu. Similar results were obtained when cells were pre-incubated with anti-EF-Tu antibodies before adding to Caco-2 cells. MapA was found to have adhesive role in all the *Lactobacillus* strains, except *L. fermentum* ASt1 and *L. casei* CS5.2.

Furthermore, the EF-Tu was also found to have a role in inhibition of enteropathogens adhesion to Caco-2 cells. This was the first report stating that the moonlighting protein, EF-Tu from probiotic lactobacilli antagonizes the adhesion of enteropathogens to intestinal cells. The quantitative expression analysis of EF-Tu gene by qRT-PCR in the selected *Lactobacillus* strains also showed the up-regulation of EF-Tu gene in the presence of mucin. Furthermore, the identification of putative approximately 60 kDa Caco-2 receptor interacting with EF-Tu was also carried out with pull down assay.

Conclusions

Among the nine *Lactobacillus* strains isolated from various sources, *L. plantarum* CS23 and *L. plantarum* CS24.2 showed significantly comparable and/or higher probiotic attributes compared to the established probiotic strain LGG. Further, *L. plantarum* CS24.2 was able to antagonize the adhesion of enteropathogens to HT-29 cells and also down-regulated pro-inflammatory molecules induced by *E. coli* O26:H11 in HT-29 cells. The *Lactobacillus* adhesin-EF-Tu was found to antagonize the adhesion of enteropathogens to Caco-2 cells. The expression of EF-Tu was also found to be up-regulated in the highly adhesive strains in exposure to mucin. From these primary observations, it could be said that the adhesins on the surface and its expression profile do have a major role to play in the colonization of *Lactobacillus* strains in the intestinal tract. The findings need further confirmation by an *in vivo* study.

L. plantarum CS24.2 was identified as a novel probiotic strain with high *in vitro* colonization ability and anti-inflammatory properties.

LIST OF PUBLICATIONS

1. **Dhanani, A.S.** and Bagchi, T. (2013) The expression of adhesin EF-Tu in response to mucin and its role in *Lactobacillus* adhesion and competitive inhibition of enteropathogens to mucin. *Journal of Applied Microbiology* **115:** 546-554.

2. **Dhanani, A.S.** and Bagchi, T. (2013) *Lactobacillus plantarum* CS24.2 prevents *Escherichia coli* adhesion to HT-29 cells and also down-regulates enteropathogen-induced tumor necrosis factor-α and interleukin-8 expression. *Microbiology and Immunology* **57:** 309–315.

3. **Dhanani, A.S.**, Gaudana, S.B. and Bagchi, T. (2011). The ability of *Lactobacillus* adhesin EF-Tu to interfere with pathogen adhesion. *European Food Research and Technology* **232:** 777-785.

4. Gaudana, S.B., **Dhanani, A.S.** and Bagchi, T. (2010). Probiotic attributes of *Lactobacillus* strains isolated from food and of human origin. *British Journal of Nutrition* **103:** 1620-1628.

LIST OF PRESENTATIONS

1. **Dhanani, A.S.** and Bagchi, T. **'Evaluating adhesion and immunomodulatory potential of *Lactobacillus* strains isolated from food and human origin'**, presented at The 7th International Yakult Symposium at The Queen Elizabeth II Conference Centre, London, UK. April 22-23, 2013.

2. **Dhanani, A.S.** and Bagchi, T. **'Expression analysis of *Lactobacillus* adhesins under mucin exposure and its role in adhesion of different *Lactobacillus* strains'**, presented at 1st annual conference of Probiotic Association of India (PAi) held at India Habitat Centre, New Delhi, India. August 27-28, 2012. **(Awarded 2nd Prize)**

3. **Dhanani, A.S.** and Bagchi, T. **'Analysis of bile salt hydrolases and adhesion factors of *Lactobacillus* strains'**, presented at 52nd annual conference of Association of Microbiologists of India (AMI) held at Punjab University, Chandigarh, India. November 3-6, 2011.

4. **Dhanani, A.S.** and Bagchi, T. **'Studies of cell surface properties of *Lactobacillus* strains isolated from different sources and their adhesion to mucin'**, presented at 79th annual conference of Society of Biological Chemists, India (SBCi) held at Indian Institute of Science, Bangalore, India. December 13-15, 2010.

5. **Dhanani, A.S.**, Gaudana, S.B. and Bagchi, T. **'Study of lactobacilli surface proteins involved in binding to Caco-2 cells'**, presented at 50th annual conference of Association of Microbiologists of India (AMI) held at National Chemical Laboratory (NCL), Pune, India. December 15-18, 2009.