CHAPTER 1

ANTI BACTERIAL PROPERTIES OF LACTOBACILLUS FERMENTUM

1.1 INTRODUCTION

1.1.1 LACTIC ACID BACTERIA
Lactic acid bacteria (LAB) are Gram-positive, non-sporing, non-motile, catalase-negative organisms that are devoid of cytochromes and of non aerobic habit, but are aerotolerant, fastidious, acid-tolerant, and strictly fermentative with lactic acid being the major end product of sugar fermentation [1, 2]. LAB produce lactic acid either through homofermentative or heterofermentative pathways. They occur as rods or coccobacilli in chains or pairs. LAB include the genus Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, and Vagococcus. The classification of lactic acid bacteria into different genera is based on morphology, mode of glucose fermentation, configuration of lactic acid produced, ability to grow at high salt concentrations, growth at different temperatures, and acid or alkali tolerance. LAB usually show a GC content of lower than 54 % [1, 3].

1.1.2 CLASSIFICATION
According to Taxonomic Outline of the Prokaryotes [4], the genus Lactobacillus belongs to the phylum Firmicutes, class Bacilli, order Lactobacillales, family Lactobacillaceae and its closest relatives, being grouped within the same family, are the genera Paralactobacillus and Pediococcus. The phylogenetically closest family appears to be the Leuconostocaceae family, which includes genera Leuconostoc, Oenococcus and Weissella. They are associated with habitats rich in nutrients such as various food products (milk, meat, vegetables), but some are also members of normal flora of mouth, intestine and vagina of mammals. They are nutritionally fastidious, requiring rich media to grow (carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins).
1.1.2.1 Classification at genus level

![Flowchart](image.png)

Figure 1.1 Flowchart describing the classification of LAB at the genus level

1.1.2.2 Classification at species level

The genus *Lactobacillus* is the largest of the genera included in LAB. It is also very heterogeneous, comprising of species with a large variety of phenotypic, biochemical and physiological properties. The heterogeneity is seen in the percentage of G+C content of the DNA. The classical ways of distinguishing among the species of lactobacilli have been carbohydrate fermentation patterns, hydrolysis of arginine, growth requirements, configuration of lactic acid produced and growth at certain temperatures. Complete classification may however require analysis of peptidoglycan, electrophoretic mobility of the LDH, percentage G+C content of the DNA and molecular level studies like DNA-DNA homology studies and 16S rRNA analysis.

1.1.3 MAJOR FERMENTATION PATHWAYS

There are two major pathways for hexose fermentation in LAB. The transport and phosphorylation of glucose may occur either by transport of free sugar and phosphorylation by an ATP dependent hexokinase or by the use of phosphoenolpyruvate-sugar phosphotransferase system, in which phosphoenolpyruvate is the phosphoryl donor.
Glycolysis, used by all LAB except leuconostocs and group III lactobacilli, is characterized by the formation of fructose-1,6-diphosphate (FDP), which is split by an FDP aldolase into dihydroxyacetonphosphate (DHAP) and glyceraldehydes-3-phosphate (GAP). GAP is then converted to pyruvate in a metabolic sequence including substrate level phosphorylation at two sites. Under normal conditions (excess sugar and limited access to oxygen), pyruvate is reduced to lactic acid by a NAD\(^+\)-dependent lactate dehydrogenase, thereby reoxidizing the NADH formed during the earlier glycolytic steps. A redox balance is thus obtained, lactic acid is the only end product, and the metabolism is referred to as homolactic fermentation and those LAB that follow this are called homofermentative LAB.

![Diagram of glycolysis and lactate fermentation](image)

**Figure 1.2 Homofermentative pathway of LAB**

The other fermentation pathway is the 6-phosphogluconate pathway. It is characterized by the initial dehydrogenation steps with the formation of 6-phosphogluconate, followed by decarboxylation. The remaining pentose-5-phosphate is split by phosphoketolase into GAP and acetyl phosphate. GAP is
metabolized in the same way as from the glycolytic pathway, resulting in lactic acid formation. When no additional electron acceptor is available, acetyl phosphate is reduced to ethanol via acetyl CoA and acetaldehyde. Since this metabolism leads to significant amounts of other end products (CO₂, ethanol) in addition to lactic acid, it is referred to as heterolactic fermentation and those LAB that follow this are called heterofermentative LAB.

![Diagram of the heterofermentative pathway of LAB](image)

**Figure 1.3 Heterofermentative pathway of LAB**

### 1.1.4 MICROBES IN THE GUT

The gastrointestinal tract (GIT) of all humans becomes colonized with bacteria shortly after birth. These include transient microorganisms and the autochthonous bacteria, which develop later into
relatively stable populations that are characteristic of the species. pH and retention time of digested food affect the number and species of microorganisms in a given gut segment. The low pH of gastric contents and rapid transit of the gut digesta through the small intestine of most species tend to inhibit the growth of many bacteria. The relatively neutral pH and prolonged retention of food in the large intestine of all terrestrial vertebrates are associated with an increase in the number and variety of bacteria and, in some species, the additional presence of protozoa and even fungi [5]. However, it is difficult to distinguish true autochthonous lactobacilli from transiently passing lactobacilli, originating from fermented foods or from the oral cavity, which is habitat to a considerable amount of lactobacilli. *L. gasseri, L. reuteri, L. crispatus, L. salivarius,* and *L. ruminis* appear to be predominant autochthonous *Lactobacillus* species. *L. acidophilus, L. fermentum, L. casei, L. rhamnosus, L. johnsonii, L. plantarum, L. brevis, L. delbrueckii, L. curvatus,* and *L. sakei* can also be found in the human GIT at fluctuating levels. Although less accessible, lactobacilli are commonly identified in biopsy samples from stomach, small intestine, and colon but in variable and usually rather low numbers [6].

Bacteria native to the gastrointestinal tract ferment dietary and endogenous carbohydrates into short-chain fatty acids (SCFA) that provide energy for the gut epithelium and other tissues and facilitate the absorption of sodium and water. They also convert dietary and endogenous nitrogenous compounds into ammonia and microbial protein and synthesize B vitamins[5].

### 1.1.4.1 Source of Original Microbiota

The basis of the healthy gut microbiota is derived from the mother. The mother’s microbiotic composition and the original inoculum provided at birth depend on poorly understood genetic factors. Diet, environment, and stress factors during pregnancy and birth influence the mother’s fecal microbiota composition and, consequently, the inoculum transferred to the infant at birth [7, 8]. The microbiota of a newborn develops rapidly after birth and is initially strongly dependent on feeding practices and the newborn infant’s hygienic environment [7, 9].

### 1.1.4.2 Succession of Microbial Communities

Establishment of the gut microbiota is characterized by early colonization at birth with facultative anaerobes. Then enterobacteria, coliforms, lactobacilli, and streptococci, colonize the intestine, followed in rapid succession by anaerobic genera, such as *Bifidobacterium, Bacteroides, Clostridium,* and *Eubacterium* [6, 9, 10]. In comparison to the adult microbiota, the infant microbiota
is highly unstable but contains lactobacilli in variable amounts. The number of *Lactobacillus* cells in neonates was found to be in the range of $10^5$ cfu / g of feces, while in infants 1 month of age and older, the counts ranged from $10^6$ to $10^8$ cfu / g of feces. Breast-feeding promotes a strong bifidobacterial presence in the infant gut by providing oligosaccharides that act as favorable substrates for bifidobacteria. Introduction of solid foods, as well as antimicrobial treatment periods, interrupt the constant supply of oligosaccharides and microbes from the mother, introducing new microbial genera and species into the GI tract. This process facilitates the development of adult-type mature microbiota.

1.1.4.3 Fate of ingested bacteria in the gastrointestinal tract

It is commonly suggested that bacteria must “persist and multiply” in the target ecosystem to be effective. A number of studies with a variety of bacterial strains have been conducted to determine the extent to which microbes “colonize” or, more correctly, transiently persist in the intestine. The combined results demonstrate conclusively that ingested strains do not become established members of the normal microbiota but persist only during periods of dosing or for relative short periods thereafter. There is also evidence that common microfloral strains differ in their degree of persistence. This may reflect in part their capacity to resist the harsh conditions encountered in the upper digestive tract [10].

1.1.5 CELL SURFACE STRUCTURES OF LACTOBACILLI

Specific metabolic and physiological characteristics of lactobacilli that play a key role in the adaptation to the host and the production and availability of probiotic factors are discussed below. The typical cell surface structures of lactobacilli is described, since these structures are in direct contact with the environment and can function as both key adaptation and probiotic factors.  

In Gram-positive bacteria, the cell wall consists of several characteristic structures: a thick, multilayered peptidoglycan (PG) sacculus impregnated with proteins, teichoic acids, and polysaccharides and, in some species, surrounded by an outer shell of proteins packed in a paracrystalline layer (S layer). These molecular structures provide the bacteria with species- and strain-specific properties.
1.1.5.1 Peptidoglycan

Like most Gram-positive species, the cell wall of lactobacilli is characterized by a thick PG layer. This 20- to 100-nm-thick PG multilayer, sometimes referred to as the murein sacculus, plays a key role in structural integrity and protects the cell against lysis. Cell wall PG is further covalently and noncovalently impregnated with teichoic acids, polysaccharides, and proteins [11]. PG is composed of glycan chains of repeating β-1,4-linked N-acetylg glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues extensively cross-linked by two pentapeptide side chains. The chemistry of the glycan chains vary only slightly among different bacteria, but there is considerable variation in the compositions of stem peptides, which are linked to the carboxyl group of MurNAc [12, 13]. In LAB, the consensus sequence for the pentapeptide is L-alanine–D-glutamate–meso–diaminopimelic acid or L-lysine–D-alanine–D-alanine, with a preference for L-lysine [11]. In several lactobacilli, like *L. casei* and *L. plantarum*, the C-terminal D-alanine residue of the muramyl pentapeptide is replaced by D-lactate, referred to as a pentadepsipeptide. This pentadepsipeptide confers resistance to the glycopeptide antibiotic vancomycin [11].

The biosynthesis of PG involves the synthesis of the nucleotide sugar-linked precursors UDP-MurNAc-pentapeptide and UDP-GlcNAc in the cytoplasm, followed by the assembly of the PG subunits at the cytoplasmic membrane, lipid anchored to undecaprenol (lipids I and II) [12, 13]. The building blocks are translocated across the cytoplasmic membrane. Polymerization and cross-linking occur on the external face of the cytoplasmic membrane. This is achieved mainly through the action of the so-called penicillin-binding proteins, which catalyze the transglycosylation and transpeptidation reactions responsible for the formation of the glycosidic and peptide bonds of the PG, respectively [11, 12]. Although this overall scheme of PG biosynthesis is quite similar in all LAB, many variations in the PG structure that may affect interactions of lactobacilli with the environment and the host can be introduced [11]. Following polymerization and incorporation into the cell wall PG, GlcNAc and/or MurNAc may undergo different modifications, such as the O-acetylation of the cell wall MurNAc residues in *L. casei* [14]. Additionally, the peptide cross-links can differ considerably. Frequently, D-asparagine is used as a cross-bridge between D-alanine and L-lysine in LAB [14], and this residue may also be amidated [11].
1.1.5.2 Teichoic acids

Teichoic acids (TAs) are the second major components of the cell walls of most Gram-positive bacteria. These anionic cell wall polymers are generally made of polyglycerol phosphate or polyribitol phosphate repeating units covalently anchored to either PG (wall TAs [WTAs]) or attached to the cytoplasmic membrane (lipoteichoic acids [LTAs]) [15]. Both WTA and LTA are often substituted with glycosyl or D-alanyl (D-Ala) esters. The D-Ala ester substitution of LTA (and WTAs) requires four proteins that are encoded by the *dlt* operon. Two of these proteins are the D-alanyl, D-alanyl carrier protein ligase (Dcl, encoded by *dltA*), which activates D-alanine by use of ATP, and the D-alanyl carrier protein (Dcp), which is encoded by *dltC*. DltB is a transport protein predicted to be involved in the passage of the activated D-alanyl–Dcp complex across the glycerol phosphate backbone of LTA, while the DltD membrane protein facilitates the binding of Dcp for ligation with D-Ala and has thioesterase activity for removing mischarged D-alanyl carrier proteins [11, 15]. Substitution contributes greatly to the functionality of these anionic polymers.

There are considerable differences between the WTA and LTA molecules of different *Lactobacillus* strains, especially in the degree of substitution and chain length [16, 17] and the quantity, which may account for 20 to 50% of the dry weight of the cell wall of lactobacilli, depending on the species or strain, growth stage, pH of the medium, carbon source, and availability of phosphate, etc [11]. Moreover, although all lactobacilli have TAs in their cell walls, not all *Lactobacillus* cell walls seem to contain WTA. For example, the cell walls of many *L. rhamnosus* and *L. casei* strains appear to contain only LTA [18, 19], in contrast to most *L. plantarum* strains, which contain the two types of TAs [17]. Additionally, a fraction of LTA may be released through deacylation (removal of the lipid anchor) or the inside-to-outside expansion of PG without the removal of the lipid anchor [11], indicating that LTAs of certain *Lactobacillus* strains can sometimes act as soluble factors, which is important for the interpretation of some functional studies.

1.1.5.3 Exopolysaccharides

Polysaccharides are also ubiquitous components of the cell envelope of lactobacilli, in addition to PG and TA [11]. Conceptually, a distinction is made between the capsular polysaccharides, which form the thick outermost shell intimately associated with, and often covalently bound to, the cell wall, and cell-bound polysaccharides, which are loosely associated with it. Some extracellular polysaccharides are also released into the medium [20, 21]. For some members of the *Bacillaceae*, a
different class of “nonclassical” secondary cell wall polymers has been identified, which is involved in the anchoring of S layer proteins to the bacterial cell surface [22]. In the case of Lactobacillus buchneri, it was shown that hydroxyl groups of a neutral cell wall polysaccharide are responsible for the attachment of the S-layer protein to the cell wall [23]. However, differentiation between these various classes of cell wall polysaccharides is often difficult. For lactobacilli, the term exopolysaccharides (EPSs) is preferred and relates to extracellular polysaccharides that can be attached to the cell wall or be secreted into the surroundings. Like polysaccharides in general, EPSs of lactobacilli are complex structures that differ not only in the nature of sugar monomers but also in their modes of linkage, branching, and substitution, greatly contributing to the structural variety of the Lactobacillus cell wall [24]. EPSs of lactobacilli are generally heteropolysaccharides (HePSs) composed of different sugar moieties (glucose, galactose, rhamnose, GlcNAc, and N-acetylglactosamine) [24, 25]. Residues such as glucuronic acid and glycerol-3-phosphate can also be present, as can phosphate groups, acetyl, and pyruvyl groups although only in a subset of Lactobacillus strains [25]. Information about the structures and biosynthesis pathways of these HePSs in lactobacilli is incomplete. The genes encoding HePS biosynthesis are typically located in a gene cluster of 12 to 25 kb depending on the complexity of the HePS [11, 24, 26, 27]. The gene organization of these clusters seems to be highly conserved: a central region with similarity to glycosyltransferases is flanked by regions with similarity to genes involved in polymerization, export, and regulation. Based on homology, it is assumed that in lactobacilli, the repeating units of EPSs are synthesized in a stepwise manner that involves the intracellular formation of sugar nucleotides and the assembly of the subunit on undecaprenol at the cytoplasmic membrane by specific glycosyltransferase [11, 24, 26, 27]. This final subunit is translocated across the membrane, followed by the linkage of the repeat units into long-chain polysaccharides. In lactobacilli, most EPSs are secreted or remain weakly bound to the cell wall by electrostatic interactions (ionic, hydrogen bonds, or hydrophobic interactions) [11, 24, 26-28]. EPS production is also highly dependent on the environmental conditions, and the regulation of EPS production and chain length occurs, at least in part, through the action of a phosphoregulatory system that includes an autophosphorylating tyrosine kinase [29-32]. Nevertheless, much remains to be learned about the specific aspects of the biosynthetic pathway to EPSs in lactobacilli. More knowledge is needed about the exact locations, compositions, polymer sizes, and conformation properties of surface
polysaccharides on lactobacilli. It was shown by the application of single-molecule force spectroscopy that the probiotic strain *L. rhamnosus* GG has two major classes of surface polysaccharides: long galactose rich EPS molecules and shorter glucose-rich (and/or mannose rich) polysaccharides of unknown nature [33]. Preliminary experiments indicate that some polysaccharide chains could be present as glycoproteins, adding an extra level of complexity to the *Lactobacillus* cell wall architecture. In contrast to typical heteropolymeric EPS molecules, some *Lactobacillus* strains can also synthesize homopolysaccharides, glucans or fructans, from sucrose by the single action of extracellular glycosyltransferases, termed glucosyltransferases or fructosyltransferases, respectively. These glycosyltransferases use the energy of the osidic bond of sucrose to catalyze the transfer of a glycosyl moiety of sucrose to H₂O, an acceptor carbohydrate, or a growing polymer chain [34]. As for HePSs, these HoPSs can show a high degree of diversity in polymer length, linkages, and branching, etc. Because the biosynthesis of these HoPSs does not involve transport processes or the use of activated carbohydrate precursors, there is no direct (P) energy requirement for the producer organisms other than the biosynthesis of the glycosyltransferase enzymes[34].

### 1.1.5.4 Surface associated proteins

The bacterial envelope of lactobacilli comprises of different cell wall-associated proteins, which are often large proteins consisting of repeating modules or particular domains. Cell surface proteins can be anchored to the cell wall by different mechanisms [35]: by single N- or C-terminal transmembrane anchors, lipoprotein anchors, LPxTGtype anchors, or other cell wall-binding (repeated) domains such as LysM domains or glycine-tryptophan dipeptide motifs [36, 37]. Other proteins are secreted in the surroundings, mediating interactions with the environment independent from direct cell contact. Some secreted proteins have also been shown to reassociate to the cell wall by electrostatic interactions [36, 38]. Sortase-dependent proteins (SDPs) and S-layer proteins are best characterized in lactobacilli and will be further discussed below.

SDPs are an important group of cell surface proteins suggested to play a crucial role in *Lactobacillus*-host interactions [39]. These SDPs are characterized by the presence of a cell wall-sorting signal at the C terminus that comprises a short pentapeptide motif (LPxTG) followed by a stretch of hydrophobic side chains and a positively charged tail. After transfer of the surface protein precursor to the plasma membrane and cleavage of the signal peptide, the SDP is retained within the cell wall by the cleavage of the sorting signal at the pentapeptide motif by a membrane-associated
sortase. Sortase A (SrtA) cleaves between the threonine and glycine residues and then covalently links the threonine residue to an amino group of PG cross-bridges [40]. Gram-positive bacteria also utilize sortase-dependent mechanisms to assemble pili or fimbriae where sortase cross-links individual pilin monomers and ultimately joins the resulting covalent polymer to the cell wall PG [41]. The occurrence of pili in lactobacilli does not seem to be a general rule, but they have been suggested to occur, for instance, in L. johnsonii [42]. Some specific lactobacillus strains are surrounded by a superimposed surface layer, the S layer, made of protein subunits packed into a paracrystalline hexagonal or tetragonal monolayer [38]. Examples of S-layer-containing lactobacilli are L. acidophilus [43], L. gasseri, L. johnsonii [44], L. brevis [45], L. helveticus [46], and L. crispatus [47]. S-layer proteins are usually small and highly basic proteins of 40 to 60 kDa with generally highly stable tertiary structures. These proteins are noncovalently bound to the cell wall, mostly to secondary cell wall polymers (LTA, WTA, and neutral polysaccharides), and assemble into surface layers with high degrees of positional order, often completely covering the cell wall. Although glycan structures have been identified in the S-layer proteins of several Gram-positive bacteria, most appear to be nonglycosylated in lactobacilli [38]. A detailed glycan structure on S layer proteins has been reported only for L. buchneri [38].

1.1.6 PROBIOTICS
1.1.6.1 History of the term “Probiotic”
The role of fermented milk in human diet was known since olden times. However, the scientific interest in this area boosted after the publication of the book entitled The Prolongation of Life by Ellie MetchinkoffI in 1908. He suggested that people should consume fermented milk containing lactobacilli to prolong their lives. Bulgarian peasants who were subjected to the experiments on longevity had consumed large quantities of sour milk. Since then, researchers started investigations relating to the role of lactic acid bacteria in human and animal health [48]. The term “probiotic” is derived from the Greek language, meaning “for life”. It was first used by Lilly and Stillwell in 1965 to describe “substances secreted by one microorganism which stimulates the growth of another” and thus was contrasted with the term antibiotic. In 1974, Parker was the first to use the term probiotic in the sense that it is used today. He defined probiotics as “organisms and substances which contribute to intestinal microbial balance.” In 1989 Fuller attempted to improve
Parker’s definition of probiotic with the following distinction: “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.” This revised definition emphasizes the requirement of viability for probiotics and introduces the aspect of a beneficial effect on the host, which was, according to his definition, an animal [49]. Today the working group (2002) of WHO / FAO defines probiotics as “live micro-organisms which when administered in adequate amounts confer a health benefit on the host.”

A good probiotic agent needs to be non-pathogenic, nontoxic, resistant to gastric acid and bile, adhere to gut epithelial tissue and produce antibacterial substances. It should persist, for short periods in the gastro-intestinal tract influencing metabolic activities like cholesterol assimilation, lactose activity and vitamin production. The survival of probiotic organisms in the gut depends on the bacterial colonization factors, possession of probiotic bacterial organelles which enable them to resist the antibacterial mechanisms that operate in the host gut. In addition to the antibacterial mechanisms, they need to avoid the effects of peristalsis, which tend to flush out bacteria with food [48].

1.1.6.2 Beneficial effects of lactobacilli on the host

Diverse studies over the years have shown that lactobacilli exert health benefits when applied under various conditions. The best evidence exists for the treatment and prevention of enteric infections and postantibiotic syndromes. Several meta-analyses have established the efficacy of some lactobacilli in acute infectious diarrhea and the prevention of antibiotic-associated diarrhea [50]. Certain lactobacilli have been shown to reduce the recurrence of *Clostridium difficile* associated diarrhea [51] and prevent necrotizing enterocolitis in preterm neonates [52]. Some promising results have also been obtained for the prevention and treatment of inflammatory bowel disease (IBD) [53], prevention of colorectal cancer [54], and treatment of irritable bowel syndrome [55]. Although the gastrointestinal tract is the site where probiotic lactobacilli are believed to exert most health-modulating activities, probiotic applications with some *Lactobacillus* strains at other sites of the body have shown positive effects, such as the prevention and treatment of urogenital diseases and bacterial vaginosis in women [56], the prevention of atopic disease and food hypersensitivity [43], and the prevention of dental caries [57]. Probiotic lactobacilli have a high safety profile, recognized by a “generally-regarded-as-safe” status, and the tolerance is usually excellent. However, in rare cases, reports of infections presumably caused by probiotic lactobacilli in immuno compromised
patients or patients with severe underlying disease have been published [58, 59]. It is also fair to state that many clinical studies did not result in positive outcomes. On the other hand, studies that did report health benefits of lactobacilli often lacked control groups, blinding, validated outcomes, or standards for reporting adverse events [60].

1.1.7 MECHANISMS OF SURVIVAL AND PERSISTENCE OF LACTOBACILLI IN THE HOST: ADAPTATION FACTORS
Diverse mechanisms are adopted by probiotic lactobacilli to encounter various environmental conditions upon ingestion by the host and during transit in the gastrointestinal tract.

1.1.7.1 Stress resistance mechanisms in the host
Humans secrete approximately 2.5 liters of gastric juice each day, generating a fasting pH of 1.5, increasing to pH 3 to 5 during food intake [61]. Lactobacilli need to survive these harsh conditions of the stomach. Lowering the intracellular pH reduces the transmembrane pH difference, which determines the proton motive force used as an energy source in numerous transmembrane transport processes. Internal acidification also reduces the activity of acid-sensitive enzymes and results in damage to proteins and DNA [62].

The liver excretes bile into the small intestine, and exposure to bile represents another challenge for bacteria entering the GIT [63]. Although the toxicity of bile acids for bacterial cells is not well understood, bile acids are surface active, amphipatic molecules with potent antimicrobial activity and act as detergents, disrupting biological membranes. These acids can passively diffuse in the undissociated form through the cell membrane (or via a transporter) and, after entry into the cytoplasm, rapidly dissociate into protons and charged derivatives to which the cell membrane is impermeable [62].

1.1.7.2 Maintaining integrity of the cell envelope
The macromolecules constituting the cell membranes and cell walls of lactobacilli have been shown to contribute to maintaining cell integrity during stress. A low pH caused a shift in the fatty acid composition of the cell membrane of an oral strain of *L. casei* [64]. Similarly, bile salts have been shown to induce changes in the lipid cell membrane of *L. reuteri* CRL1098 [65]. Genes involved in PG biosynthesis and organization have been identified in various screens for acid responses in lactobacilli. D-Ala esters of LTA and WTA have been suggested to be necessary for the proper
functioning of cell integrity at low pH and in the presence of bile [15]. Moreover, a genetic characterization of the bile salt response in *L. plantarum* WCFS1 by screening of a promoter probe library also resulted in the identification of several genes involved in cell envelope functions, including genes encoding muramidases [66].

### 1.1.7.3 Adaptation to the host nutritional environment

In addition to the survival capacity of lactobacilli under various stress conditions, the capacity to adapt to the special nutritional conditions is of utmost importance for their residence time and survival in the various microhabitats of the GIT. Important sources of carbon and energy for bacteria growing in the gut are simple sugars that are readily utilized in the upper GIT and non-digestible complex carbohydrates that remain abundant in the lower part of the GIT and originate from the diet or from host mucins. Thus, bacteria such as *Bifidobacterium longum*, which contain numerous genes which are known to be involved in polysaccharide degradation, are probably better suited to reside in the colon, whereas species containing various sugar transporters, such as most intestinal lactobacilli, seem better adapted to the proximal compartments of the GIT [67].

### 1.1.8 MECHANISMS OF HEALTH-PROMOTING EFFECTS OF LACTOBACILLI: PROBIOTIC FACTORS

#### 1.1.8.1 Production of antimicrobial compounds

Lactobacilli are known to produce a variety of compounds that exert a direct antimicrobial action toward competing bacteria and viruses. Antimicrobial substances produced by lactic acid bacteria can be divided into two main groups: low molecular mass substances with molecular mass <1 kDa and high molecular mass substances with molecular mass >1 kDa, such as bacteriocins. All non-bacteriocin antimicrobial substances from LAB are of low molecular mass [68].

#### 1.1.8.1.1 Low molecular mass antimicrobials

The production of antimicrobial metabolites by LAB are dependent on the species of LAB and chemical composition of the growth media. Most of the heterofermentative species have flavoprotein oxidases, which catalyse the reduction of oxygen, resulting in the accumulation of hydrogen peroxide. During heterofermentations, accumulation of products such as formic acid, acetoin, acetaldehyde and diacetyl, occurs, which possess antimicrobial activity. Malic, lactic and citric acid, may be further metabolised to other antimicrobial products such as acetic acid, formic acid and CO₂
The main low molecular mass metabolites of LAB and their antimicrobial spectra are shown in Table 1 [69-74].

### Table 1.1 Low molecular mass antimicrobial metabolites of lactic acid bacteria

<table>
<thead>
<tr>
<th>Compound</th>
<th>Microorganisms producers</th>
<th>Antimicrobial spectrum</th>
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<tbody>
<tr>
<td>lactic acid</td>
<td>all LAB</td>
<td>Gram-negative bacteria</td>
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<td>Gram-positive bacteria</td>
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<td>Yeasts</td>
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<td>acetic acid</td>
<td>heterofermentative LAB</td>
<td>Gram-negative bacteria</td>
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<td>Gram-positive bacteria</td>
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<td>Yeasts</td>
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<td>diacetyl acetaldehyde acetoin</td>
<td>variety of genera of lactic acid</td>
<td>Gram-negative bacteria</td>
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<td>including: Lactococcus,</td>
<td>Gram-positive bacteria</td>
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<td>Leuconostoc, Lactobacillus</td>
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<td>and Pediococcus</td>
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<td>hydrogen peroxide</td>
<td>heterofermentative LAB</td>
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<td>Gram-positive bacteria</td>
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<td>Yeasts</td>
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<td>carbon dioxide</td>
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<td>Most groups of microorganisms</td>
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<td>Lactobacillus reuteri</td>
<td>Gram-positive and Gram-negative bacteria, Fungi, Protozoa</td>
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<tr>
<td></td>
<td>Leuconostoc mesenteroides</td>
<td></td>
</tr>
<tr>
<td>3-hydroxy fatty acids</td>
<td>Lactobacillus plantarum</td>
<td>Fungi</td>
</tr>
<tr>
<td>benzoic acid methylhydantoin</td>
<td>Lactobacillus plantarum</td>
<td>Fungi</td>
</tr>
<tr>
<td>mevalonolactone</td>
<td></td>
<td>Gram-negative bacteria</td>
</tr>
</tbody>
</table>
1.1.8.1.1a Organic acids
Lactic and acetic acid are the most important and best characterised antimicrobials produced by LAB. The subsequent microbial activity in the fermented material is influenced by the amount and type of acids produced during fermentation. Acetic acid, for example, is more antagonistic against yeasts compared to lactic acid. The inhibitory effect of organic acids is mainly caused by undisassociated form of the molecule, which diffuses across the cell membrane towards the more alkaline cytosol and interferes with essential metabolic functions. The toxic effects of lactic and acetic acid include the reduction of intracellular pH and dissipation of the membrane potential [69-74].

1.1.8.1.1b Diacetyl, acetaldehyde and acetoin
Acetaldehyde is formed by decarboxylation of pyruvate during heterofermentative LAB metabolism. This then condenses with pyruvate, forming α -acetolactate and it is converted to diacetyl by α-acetolactate synthases. The product of decarboxylation of α-acetolactate and reduction of diacetyl is acetoin [68, 75]. Acetaldehyde, usually present in fermented dairy products in concentrations smaller than necessary for inhibition of undesired microorganisms, also plays a role in controlling the growth of contaminants, together with other antimicrobial metabolites of lactic acid bacteria [70].

1.1.8.1.1c Carbon dioxide
Excluding its own antimicrobial activity, CO₂ creates an anaerobic environment by replacing the existent molecular oxygen. The influence of carbon dioxide on product preservation is thus twofold. The antifungal activity of CO₂ is due to the inhibition of enzymatic decarboxylations and to its accumulation in the membrane lipid bilayer resulting in dysfunction in permeability [69].

1.1.8.1.1d Reuterin and reutericyclin
Isolates of *Lactobacillus reuteri* produce two compounds, reuterin and reutericyclin, both active towards Gram-positive bacteria. Reutericyclin is a tetramic acid derivative and reuterin is a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of β-hydroxypropionaldehyde with a broader spectrum of inhibitory activity, including Gram-negative bacteria, fungi and protozoa [76, 77].
1.1.8.1.1e Hydrogen peroxide (H₂O₂)
Production of H₂O₂ by lactobacilli has been suggested to be an important antimicrobial mechanism, especially in the vagina of healthy women [78]. Pridmore et al [79] described an in vitro role for H₂O₂ in the anti-Salmonella activity of L. johnsonii NCC533.

1.1.8.1.1f Lactic acid
Lactic acid can be considered to be a key antimicrobial compound produced by lactobacilli [78]. Besides exerting its activity through lowering the pH and through its undissociated form, lactic acid is also known to function as a permeabilizer of the Gram-negative bacterial outer membrane [80], allowing other compounds to act synergistically with lactic acid. In addition, organic acids such as lactic acid can capture elements essential for growth, such as iron, by their chelating properties [81]. The strong antimicrobial activity of L. rhamnosus GG against S. enterica serovar Typhimurium was shown to be due to the accumulation of lactic acid [82]. The exact mode of action underlying this observed antimicrobial effect of lactic acid has not yet been completely clarified, although it is clear that both Salmonella growth and the expression of virulence factors are affected by lactic acid [83].

1.1.8.1.1g Other low molecular mass antimicrobials
Antifungal cyclic dipetides, phenyllactic acid, 4-hydroxyphenyllactic acid and 3-hydroxy fatty acids are other low molecular mass compounds with antimicrobial activity against Gram-positive and Gram-negative bacteria, moulds and yeasts [84-86]. Niku-Paavola et al. discovered new types of antimicrobial compounds produced by Lactobacillus plantarum (benzoic acid, methylhydantoin and mevalonolactone) that are active against fungi and some Gram-negative bacteria [87].

1.1.8.1.2 Bacteriocins
Lactobacilli are reported to secrete antimicrobial peptides called bacteriocins. Bacteriocins are usually active against closely related bacteria that are likely to reside in the same ecological niche. Most Lactobacillus bacteriocins are small, heat-stable proteins with a high isoelectric point (class II bacteriocins) that act generally by inducing membrane permeabilization and the subsequent leakage of molecules from target bacteria [88]. Bacteriocin production is controlled in many strains in a population density-dependent manner using a secreted peptide pheromone for quorum sensing (QS). The sensing of its own growth, which is likely to be comparable to that of related species, is suggested to enable the producing organism to switch on bacteriocin production at times when competition for nutrients is likely to become more severe [88].
Table 1.2: Classification, characteristics and examples of bacteriocins [89-92]

<table>
<thead>
<tr>
<th>Classification</th>
<th>Major characteristics</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lantibiotics/lanthionine-containing bacteriocins</td>
<td>&lt;5 kDa</td>
<td>type A: nisin, lactocin S, lacticin 481 type B: mersacidin</td>
</tr>
<tr>
<td>Class II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-lanthionine-containing bacteriocins</td>
<td>5-10 kDa, heat stable</td>
<td>pediocin PA1, sakacin A, sakacin P, curvacin A, lactococcin G, lactococcin M, lactacin F, plantaricin A, acidocin B, reuterin 6</td>
</tr>
<tr>
<td>Class III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bacteriolysins</td>
<td>10-30 kDa, heat labile</td>
<td>helveticin J, helveticin V-1829</td>
</tr>
<tr>
<td>Class IV</td>
<td></td>
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<tr>
<td></td>
<td>require non-proteinaceous moieties like carbohydrate or lipid for their activity</td>
<td>plantaricin S, leuconocin S, actocin 27, pediocin SJ1</td>
</tr>
</tbody>
</table>

1.1.8.1.2a Mode of bacteriocin action

Varying modes of action have been described for bacteriocins, such as enzyme activity modulation, inhibition of outgrowth of spores and formation of pores in cell membrane. Bacteriocins produced by LAB can be of broad or narrow spectrum, but generally, the activity is directed against low G+C Gram-positive organisms [89]. The antibacterial spectrum includes spoilage organisms and foodborne pathogens such as Listeria monocytogenes and Staphylococcus aureus. Most bacteriocins interact with anionic lipids that are abundantly present in the membranes, and consequently initiate the formation of pores in the membranes of susceptible cells [93, 94]. However, generalised membrane disruption models cannot adequately describe the mode of action of bacteriocins. Rather, specific targets seem to be involved in pore formation and other activities. For the nisin and epidermin family of lantibiotics, the membrane-bound cell wall precursor lipid II has been identified as target [95]. Most of class II bacteriocins dissipate the proton motive force (PMF) of the target cell \textit{via} pore formation [96]. The class II bacteriocin activity depends on a mannose permease of the phosphotransferase system (PTS) as a specific target. They are also known to induce dissipation of
the PMF by forming cation- or anion-specific pores; though specific targets have not yet been identified [89].

1.1.8.1.2b Resistance and immunity to bacteriocins

A bacteriocin producing bacteria would have developed protection mechanisms against its own bacteriocin. Two distinct systems of bacteriocin immunity in the producing cell have been identified. Protection can be mediated by dedicated immunity protein and/or a specialised ABC-transporter system involving two or three subunits that probably pump the bacteriocin through the producer membrane. These two immunity systems can work synergistically to protect the producing cells from their own bacteriocin [97]. In the case of lantibiotic immunity, e.g. protein LanI, which is most likely localised at the cytoplasmic membrane, probably confers immunity to the producer cell by preventing pore formation by the bacteriocin. Related ABC-transporter system LanEFG acts by excreting bacteriocins that were inserted into the membrane back to the extracellular microenvironment and thus keeping bacteriocin concentration in the membrane under a critical level [90, 94]. Regulation of bacteriocin production and immunity is most frequently mediated through two-component signal-transduction systems, often as part of the quorum-sensing mechanism [98].

1.1.8.2 Competitive exclusion

Probiotics are able to use the same attachment site as the pathogen on epithelial cells, so that the pathogen is in competition for binding to the host mucosal interface and thereby could be inhibited from invading the mucosal layer. This anti-pathogenic mechanism is known as competitive exclusion and generally requires that the probiotic lactobacilli are administered in a preventive setup, as the displacement of a pathogen by a Lactobacillus strain is usually not observed. The various specific adhesins probably contribute to this mechanism of probiotic action, although non-specific mechanisms based on steric hindrance are also possible [99].

One putative competitive exclusion factor is the mannose specific adhesin Msa of L. plantarum [100]. Mack et al. demonstrated that a spontaneous and non-characterized adh mutant of the probiotic strain L. plantarum 299v, showed 10-times-less adherence to HT-29 epithelial cells and was not able to inhibit the adherence of enteropathogenic E. coli, in contrast to the wild type [101]. Surface layer extracts from L. helveticus R0052 were shown to inhibit the adhesion of E. coli O157:H7 to epithelial cells [102]. This process seems to be mediated partly by the high hydrophobicity of the S layers, and it is not yet known whether it involves interactions with specific
receptors. An additional mechanism to decrease the pathogenic load during infections is co-aggregation, which is thought to facilitate the clearance of pathogens during mucus flushing. Bergonzelli et al. described a role for the cell surface-located GroEL of L. johnsonii NCC 533 in the specific aggregation of Helicobacter pylori [103].

1.1.8.3 Beneficial interactions with gut epithelium
The first and the most important target cells of probiotic action are the intestinal epithelial cells. Discussed below are Lactobacillus molecules that can beneficially influence the optimal functioning of the gut epithelium, related to its nutritive and its barrier function.

1.1.8.3.1 Metabolic interactions
Metabolic interactions between lactobacilli and the host could alter the primarily nutritive function of the epithelium, but these interactions are difficult to describe. Butyrate is a particularly important source of energy for the colonic mucosal cells, and it was suggested to be necessary for the maintenance of the colonic epithelium [104, 105]. Some of these observed effects are due to the capacity of lactobacilli to metabolize bile acids, the primary function of which is to emulsify fats. This holistic approach is of particular interest in further investigating the potential of probiotic lactobacilli in modulating mainly metabolic disease risks for, e.g., diabetes [106], obesity [107], and colon cancer [108].

Other metabolic effects of lactobacilli on the host are less easy to describe. Some lactobacilli are able to catalyze the isomerization of the double bond at the C-9 position in linoleic acid to form conjugated linoleic acid, which is hypothesized to be involved in additional metabolic effects of lactobacilli [109]. Also, although lactobacilli are traditionally known as being auxotrophic for B vitamins, some strains of lactobacilli such as L. reuteri JCM1112 can synthesize the B vitamins folate (vitamin B9) and/or vitamin B12, which could be interesting in cases of vitamin deficiency [110, 111].

1.1.8.3.2 Preservation of epithelial barrier function
There are alterations in intestinal epithelial cell barrier function reported in a variety of intestine-related disorders including enteric infections, inflammatory bowel disease (IBD), and food allergy [112]. Probiotic lactobacilli are suggested to strengthen the epithelial barrier by various mechanisms such as the induction of mucin secretion [101], enhancement of tight-junction functioning [113-115], upregulation of cytoprotective heat shock proteins [171, 172] and prevention of apoptosis of
epithelial cells [116]. In some cases, parts of the signaling pathways have been identified, but the *Lactobacillus* effector molecules and their corresponding receptors mediating these effects need to be studied in detail.

**1.1.8.3.2a Cell surface factors**

Direct cell contact between lactobacilli and epithelial cells is needed for certain beneficial effects on the epithelium, and some studies have identified important cell surface factors of lactobacilli to be involved in this process. For instance, LTA molecules from *L. johnsonii* La1 and *L. acidophilus* La10 inhibited *E. coli*- and lipopolysaccharide (LPS)-induced interleukin-8 (IL-8) release by HT-29 epithelial cells, and the lipid moiety of the LTA molecules proved to be important [117]. Mack *et al.* showed that the induction of mucin expression by probiotic lactobacilli was dependent on direct cell contact between lactobacilli and epithelial cells [101]. Here, they used a spontaneous *adh* mutant of the probiotic strain *L. plantarum* 299v, resulting in 10-times-less adherence to HT-29 epithelial cells. This *adh* mutant had lost the ability to induce mucin expression and was not able to inhibit the adherence of enteropathogenic *E. coli*, in contrast to the wild type. Other studies have shown that some host pathways are modulated by direct contact with lactobacilli. For instance, *L. acidophilus* ATCC 4356 was reported to mediate anti-inflammatory and antiapoptotic effects in epithelial cells dependent on direct cell contact by the activation of mitogen-activated protein kinases (MAPK) and Akt, the prevention of IκB degradation, and the prevention of inactivation of the epidermal growth factor (EGF) receptor [116, 118, 119]. Yan and Polk showed that *L. rhamnosus* GG exerts a specific antiapoptotic effect through the inhibition of tumor necrosis factor alpha (TNF-α)-stimulated activation of the pro - apoptotic MAPK and p38, for which direct contact is needed [120], in addition to the activation of Akt, which is induced by soluble proteins. These effects seemed to be mediated by direct cell contact, but the probiotic effectors are yet unknown.

**1.1.8.3.2b Secreted proteins**

Other beneficial effects of lactobacilli on epithelial cells do not require direct cell contact. For instance, two secreted proteins of *L. rhamnosus* GG, designated p40 and p75, were identified to promote *in vitro* intestinal epithelial homeostasis through specific signaling pathways [116, 120]. Similar proteins were identified in the supernatant of *L. casei* ATCC 334 and ATCC 393 but not in the supernatant of *L. acidophilus* ATCC 4356 [116]. These proteins stimulated the activation of Akt, promoted epithelial cell growth, and inhibited TNF-α-induced epithelial cell apoptosis. In another
model system, p40 and p75 of L. rhamnosus GG appeared to protect the intestinal epithelial tight junctions and attenuate the H₂O₂-induced epithelial barrier disruption in Caco-2 cells. The probiotic mixture (VSL#3), which includes L. casei, L. plantarum, L. acidophilus, and L. delbrueckii subsp. bulgaricus, was also reported to stabilize tight junctions and induce mucins in intestinal epithelial cells by large but unidentified proteinaceous soluble factors (>50 kDa) [114].

1.1.8.3.2c Soluble peptides
Low-molecular-weight, heat- and acid stable peptides of L. rhamnosus GG were shown to activate the MAPK and p38 to induce cytoprotective heat shock proteins in intestinal epithelial cells [121]. Similarly, unknown peptide-soluble factors of the probiotic mixture VSL#3 were shown to inhibit the degradation of the NF-κB inhibitor IκB and induce heat shock proteins through specific proteasome inhibition [122].

1.1.8.3.2d Unmethylated CpG DNA
In the presence of pro-inflammatory stimuli, bacterial DNA of the VSL#3 mixture was shown to inhibit IL-8 secretion, reduce p38 MAPK activation, delay NF-κB activation, stabilize levels of IκB, and inhibit proteasome function [123].

1.1.8.3.2e Multiple probiotic factors and multiple signaling pathways
Multiple signaling pathways are known to mediate various cell surface as well as secreted factors of lactobacilli to exert a protective effect on intestinal epithelial cells. Some of these effects are related to immune responses. However, the exact effector-receptor interactions and downstream signaling events still need to be characterized in most cases. Also, many of these in vitro studies need to be complemented with in vivo data. L. rhamnosus GG affected the expression of genes involved mainly in the immune response and inflammation (transforming growth factor β [TGF- β] and TNF family members, cytokines, nitric oxide synthase 1, and α-defensin), apoptosis, cell growth, cell differentiation (cyclins, caspasas, and oncogenes), cell-cell signaling (ICAMs and integrins), cell adhesion (cadherins), and signal transduction [124]. This is in agreement with some of the in vitro effects of L. rhamnosus GG on epithelial cells.

1.1.8.4 Immunomodulatory interactions
Intestinal epithelium cells (IECs) actively participate in immune reactions, in addition to their nutritive function and their role as a physical barrier that separates luminal contents from the internal environment. Together with IECs, dendritic cells (DCs) and macrophages continuously sense the
environment and coordinate various defenses for the protection of mucosal tissues. Innate defenses include the production of antimicrobial compounds (defensins and nitric oxide, etc.) and the secretion of chemokines such as IL-8 that recruit neutrophils, i.e., phagocytes that are capable of ingesting microorganisms or particles. Many adaptive immune responses against commensal, probiotic, and pathogenic bacteria are mediated in mucosal lymphoid follicles that are distributed throughout the GIT and are referred to as the gut-associated lymphoid tissue (GALT) [125]. DCs are the most important antigen-presenting cells in the mucosa. Especially, the modulation of DC function and induction of regulatory T cells are increasingly gaining attention as important mechanisms of probiotic action [126]. Live *L. rhamnosus* GG induces NF-κB with subsequent STAT1- and STAT3 DNA-binding activity in human macrophages [192]. Roessler *et al.* showed that probiotic lactobacilli can even increase the phagocytic activity of monocytes and granulocytes in healthy subjects [127]. Some interactions by lactobacilli and macrophages seem to be, mediated by the mannose receptor (CD206) [128]. Many studies that investigate the effect of lactobacilli on DCs have been carried out, as DCs play a key role in mucosal immunity. It was shown that lactobacilli can modulate DC function by differentially inducing their maturation and the expression of MHC class II, co-stimulatory, adhesion, and activation molecules for antigen presentation to T cells or regulatory cytokines such as IL-10. Christensen *et al.* showed that mouse bone marrow DCs were differentially stimulated by various *Lactobacillus* species [129]. Mohamadzadeh *et al.* demonstrated that *L. gasseri* ATCC 19992, *L. johnsonii* ATCC 33200, and *L. reuteri* ATCC 23272 were able to induce the secretion of IL-12 and not of IL-10 and hence skew T-cell polarization toward Th1 and Tc1 CD8⁺ cytotoxic T cells, although differences among the three strains in this capacity were observed [130]. Karlsson *et al.* showed that monocytes produced higher levels of IL-12 and TNF-α in response to *L. plantarum* than in response to *E. coli* [131]. Certain studies have also focused on the induction of regulatory T (Treg) cells by lactobacilli in detail. The probiotic mixture VSL#3 was reported to ameliorate Th1-mediated colitis by inducing TGF-β-bearing regulatory cells [132]. Interactions between DCs and *L. rhamnosus* were also shown to induce hyporesponsive T-helper cells [133]. Smits and coworkers reported that certain lactobacilli induced T cells to produce IL-10 when cultured with DCs and that such T cells exert mild to moderate suppressive effects on peripheral CD4⁺T cells [134].
Overall, the results suggest that various \textit{Lactobacillus} strains can direct immunological responses toward pro- or anti-inflammatory responses, depending on the specific bacterial strains applied, the specific immune cells used, and the specific experimental setup.

\subsection*{1.1.9 THE INTESTINAL EPITHELIAL-CELL BARRIER}

The intestinal epithelium exhibits numerous physical adaptations to separate the host connective tissue from the external environment. Intercellular tight junctions that prevent para-cellular traffic are coupled with actin-rich microvillar extensions that create a brush border on the apical surface of the epithelium that impedes microbial attachment and invasion. This physical barrier is reinforced by numerous biochemical adaptations such as a glycocalyx formed by the secretion and apical attachment of a heavily glycosylated mucin-rich layer by goblet cells. Together, these form a viscous and relatively impermeable sheet on the apical surface of the epithelium. IECs also secrete a broad range of antimicrobial peptides, including defensins, cathelicidins and calprotectins. Most molecules in this diverse group of peptides are rich in hydrophobic and basic residues that confer broad-spectrum antimicrobial properties through the formation of pores in the bacterial cell wall. These adaptations are consistent with the view that IECs, in addition to promoting digestion and absorption of nutrients, perform essential barrier functions that obstruct the entry of commensal and pathogenic bacteria into the underlying lamina propria \cite{125} (Figure 1.4)
Figure 1.4: The intestinal epithelial-cell surface. Simple columnar epithelial cells exhibit physical and biochemical adaptations to maintain barrier integrity including actin-rich microvillar extensions (A), epithelial-cell tight junctions (B), apically attached and secreted mucins that form a glycocalyx (C) and the production of various antimicrobial peptides (D). Specialized intestinal epithelial cells known as M (microfold) cells overlie Peyer’s patches and lymphoid follicles to facilitate luminal sampling. M cells exhibit reduced mucin secretion and have modified apical and basolateral surfaces (E) to promote uptake and transport of luminal contents to professional antigen-presenting cells that inhabit the subepithelial dome (SED) of the Peyer’s patches and lymphoid follicles (F). Specialized dendritic cell (DC) subsets can also extend dendrites between the tight junctions of intestinal epithelial cells to sample luminal contents (G) [125]
1.1.10 PROBIOTICS IN TREATMENT AND PREVENTION OF INFECTIONS

The normal human microflora is important as a barrier against colonization by exogenous pathogenic microorganisms and potentially pathogenic bacteria. Probiotic microorganisms are thought to counteract disturbances and thereby reduce the risk of colonization by pathogenic bacteria. Over the years, many in vitro studies, animal experiments as well as clinical trials have been conducted to test the efficacy of different probiotic strains in prevention and treatment of various infectious diseases including, gastrointestinal infections, urinary infections, dental caries, skin infections etc.

Considerations while using probiotics for treatment are that, unlike small molecules that are stable entities, probiotics are dynamic microorganisms and will change gene expression patterns when exposed to different environmental conditions. Probiosis is a strain-specific phenomenon. The physiology of the probiotic strain is also an important consideration. Being live microorganisms, the proteins and secondary metabolites that are being produced will change depending on growth phase.

1.1.10.1 Gastrointestinal infections

Gastrointestinal infections are a major cause of morbidity and mortality worldwide. Studies conducted in 2006 found that, globally, severe diarrhea and dehydration are responsible each year for the death of 1,575,000 children under the age of five. This represents 15% of the 10.5 million deaths per year of children in this age group [135]. Enteric pathogens include viruses (rotaviruses, noroviruses) and bacteria such as different strains of pathogenic E. coli, toxigenic Clostridium difficile, Campylobacter jejuni, and Vibrio cholerae. These pathogens produce different types of toxins that can cause severe or life-threatening dehydration and diarrhea. Moreover, infectious agents such as enteropathogenic E. coli (EPEC) may cause persistent, chronic diarrhea in children which may increase the risk of dehydration and long-term morbidities.

1.1.10.1a Gastroenteritis

Probiotic bacteria have been used as therapy for a range of digestive diseases, including antibiotic-associated colitis, Helicobacter pylori gastritis, and traveler’s diarrhea [136]. Probiotic formulations used include single strains or combinations of strains. L. reuteri is indigenous to the human gastrointestinal tract, and has never been shown to cause disease. In human trials, probiotic treatment with L. reuteri in small children with rotaviral gastroenteritis reduced the duration of disease and facilitated patient recovery, whereas in another study, it prevented diarrhea in infants [137, 138].
Despite the promising data from clinical trials, the primary molecular mechanisms underlying the antipathogenic properties of *L. reuteri* remain unknown.

Probiotics may be effective for the prevention or treatment of infectious gastroenteritis. In the perspective of disease prevention, studies with different probiotic strains have shown that these bacteria may reduce the incidence of acute diarrhea by 15–75% depending on the study [300, 314-317]. Although the relative impacts on disease incidence vary depending on the specific probiotic strain and patient population, consistent benefits for disease prevention have been demonstrated in multiple clinical studies. Studies that examined potential benefits of probiotics for preventing antimicrobial-associated diarrhea have yielded mixed results [139-142].

Probiotics are also used in treatment regimens for infectious gastroenteritis. Several meta-analyses of various clinical trials with different probiotics resulted in reduction of disease course [143, 144]. In other terms, meta-analyses of probiotics used in clinical trials of gastroenteritis noted significant reductions of incidence of diarrhea lasting longer than 3 days. [145].

**1.1.10.1b Clostridium difficile and antibiotic-associated diarrhea**

Approximately 15–25% of cases of antimicrobial-associated diarrhea are caused by *C. difficile*. *C. difficile*-associated diarrhea (CDAD) occurs primarily in patients who have undergone antibiotic therapy in a health care setting, indicating that alterations in the intestinal microbiota are important for the initiation of CDAD. CDAD is currently treated by the use of antimicrobial agents that are effective against *C. difficile*, most often vancomycin or metronidazole. Because these drugs are broad-spectrum antibiotics, they are likely to play a role in recurrent disease by suppressing the normal intestinal microbiota. Using antimicrobial compounds that target *C. difficile* while allowing restoration of resident organisms would be one possible mechanism to prevent recurrent CDAD.

Besides, the emergence of metronidazole-resistant strains of *C. difficile* has diminished the efficacy of metronidazole, and vancomycin- and metronidazole-induced cecitis reinforces the need for new therapies for the treatment and prevention of CDAD. Antimicrobial agents are not generally recommended as prevention strategies because of the problems of antibiotic resistance and antimicrobial associated disease.

Probiotics are being used in treatment of antibiotic associated as well as other gastritis to reduce the duration of infection.
1.1.10.1c *Enterohemorrhagic E. coli*

**Enterohemorrhagic E. coli** (EHEC) infections cause sporadic outbreaks of hemorrhagic colitis throughout the world. Most infections result in the development of bloody diarrhea but a subset (~5–10%) of EHEC patients (mostly children) will develop the life-threatening condition hemolytic uremic syndrome (HUS) [146]. EHEC also produces attaching and effacing lesions on host epithelial cells and reduces intestinal epithelial barrier function.

EHEC strains are characterized by the expression of Shiga toxin (Stx) genes, and thus they can be labeled as Shiga-toxin-producing *E. coli* (STEC). At present, only supportive therapy for EHEC infection is available since antibiotic therapy may increase the risk of developing HUS, and therefore, novel therapies must be developed.

1.1.10.1d *Rotavirus*

Enteric viruses including noroviruses and rotavirus represent major causes of gastroenteritis, especially in young children. Rotavirus infection results in acute gastroenteritis with accompanying dehydration and vomiting mainly in children 3–24 months of age. Human rotavirus primarily infects intestinal epithelial cells of the distal small intestine, resulting in enterotoxin-mediated damage to intestinal barrier function. Studies indicate that probiotics may reduce the duration and ameliorate disease due to rotavirus infection. Probiotics promoted intestinal immunoglobulin production and appeared to reduce the severity of intestinal lesions due to rotavirus infection in a mouse model. These findings and related investigations suggest that probiotics may diminish the severity and duration of gastrointestinal infections by mechanisms independent of direct pathogen antagonism. Probiotics may also promote healing and homeostasis by modulating cytokine production and facilitating intestinal barrier function.

1.1.10.2 *Staphylococcus aureus*

**Staphylococcus aureus** is Gram-positive cocci which occur singly, in pairs, and irregular clusters. It is a nonmotile, non-spore forming, catalase and coagulase positive organism. *S. aureus* is both a commensal, which colonizes the nares, axillae, vagina, pharynx, or damaged skin surfaces, and an extremely versatile pathogen. It causes a variety of suppurative (pus-forming) infections in humans starting from superficial skin lesions such as boils, styes and furuncles to more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis. *S. aureus* is a major cause of hospital
acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* can cause food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release of superantigens into the blood stream.

Hospital strains of *S. aureus* are usually resistant to a variety of different antibiotics. A few strains are resistant to all clinically useful antibiotics except vancomycin, and vancomycin-resistant strains are increasingly-reported[147, 148]. The term MRSA refers to Methicillin resistant *Staphylococcus aureus*. Methicillin resistance is widespread and most methicillin-resistant strains are also multiple drug-resistant.

Certain strains of *S. aureus* can produce subpopulations which are phenotypically very different from the parent strain. These naturally occurring subpopulations grow slowly, leading to colonies that are defined as being one-tenth the size of ‘normal’ *S. aureus*, hence the name ‘small colony variants’ (SCVs). Prominent features of these SCVs include decreased pigmentation and haemolysis, increased resistance to aminoglycosides and an unstable colony phenotype. The biochemical basis of this phenotypic abnormality is an auxotrophism for either menadione, haemin or thymidine [149-152] Any of these defects results in reduced tricarboxylic acid cycle metabolism and consequent reduced electron transport, yielding the typical SCV phenotype. The supplementation of the key compounds (to which SCVs are auxotroph) to the growth medium revert SCVs to normal-size colonies. Mutations of genes involved in the biosynthesis of menadione, haemin or thymidine have been identified in some SCVs [153-156]. Clinically, SCVs have been associated with relapsing or persistent infections, in particular in osteomyelitis, some foreign body-associated infections and respiratory infections in cystic fibrosis patients.

1.1.10.3 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative bacterium. Almost all strains are motile by means of a single polar flagellum. The bacterium is ubiquitous in soil and water, and on surfaces in contact with soil or water. *P. aeruginosa* has very simple nutritional requirements. It is tolerant to a wide variety of physical conditions, including temperature and resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. These natural properties of the bacterium undoubtedly contribute to its ecological success as an opportunistic pathogen. It also explains the ubiquitous nature of the organism and its prominence as a nosocomial pathogen. In fact, *P. aeruginosa* is the epitome of an opportunistic pathogen of humans.
It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed. *P. aeruginosa* infection is a serious problem in patients hospitalized with cancer, cystic fibrosis, and burns.

*P. aeruginosa* strains produce two types of soluble pigments, the fluorescent pigment pyoverdin and the blue pigment pyocyanin. The latter is produced abundantly in media of low-iron content and functions in iron metabolism in the bacterium. Pyocyanin (from "pyocyanus") refers to "blue pus", which is a characteristic of suppurative infections caused by *P. aeruginosa*.

*P. aeruginosa* is well known for its resistance to antibiotics and is, therefore, a particularly dangerous and dreaded pathogen. Also, its tendency to colonize surfaces in a biofilm form makes the cells impermeable to therapeutic concentrations of antibiotics. Only a few antibiotics are effective against *P. aeruginosa*, including fluoroquinolones, gentamicin and imipenem, and even these antibiotics are not effective against all strains.

*Pseudomonas* infection may be seen as composed of three distinct stages: (1) bacterial attachment and colonization on a surface (2) local invasion; (3) disseminated systemic disease. Particular bacterial determinants of virulence mediate each of these stages and are ultimately responsible for the characteristic syndromes that accompany the disease.

### 1.1.11 BACTERIAL BIOFILMS

The definition of a biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.

Planktonic bacteria can adhere to surfaces and initiate biofilm formation. Once a biofilm has formed and the exopolysaccharide matrix has been secreted by the sessile cells, the resultant structure is highly viscoelastic and behaves in a rubbery manner [157].

Biofilms were perceived as unstructured accretions of bacterial cells, surrounded by the cells’ exopolysaccharide matrices. Developed biofilms are not structurally homogeneous monolayers of microbial cells on a surface. The basic building block or structural unit of the biofilm is the
microcolony, and an elucidation of basic biofilm processes, such as quorum sensing, antimicrobial resistance, and detachment, may hinge on an understanding of the physiological interactions of microcolonies within a developed biofilm. Living, fully hydrated biofilms are composed of cells (±15% by volume) and of matrix material (±85% by volume), and the cells are located in matrix-enclosed “towers” and “mushrooms”.

The biofilm structure and the physiological attributes of biofilm organisms confer an inherent resistance to antimicrobial agents, whether these antimicrobial agents are antibiotics, disinfectants, or germicides. Mechanisms responsible for resistance may be one or more of the following: (i) delayed penetration of the antimicrobial agent through the biofilm matrix, (ii) altered growth rate of biofilm organisms, and (iii) other physiological changes due to the biofilm mode of growth.

Some human infections involving biofilms include; native valve endocarditis, otitis media, chronic bacterial prostatitis, cystic fibrosis, periodontitis etc.

1.1.12 NOSOCOMIAL INFECTION

A nosocomial infection — also called “hospital acquired infection” can be defined as: An infection acquired in hospital by a patient who was admitted for a reason other than that infection [158]. An infection occurring in a patient in a hospital or other health care facility in whom the infection was not present at the time of admission. This includes infections acquired in the hospital but appearing after discharge, and also occupational infections among staff of the facility [159].

At any time, over 1.4 million people worldwide suffer from infectious complications acquired in hospital [160]. The highest frequencies of nosocomial infections were reported from hospitals in the Eastern Mediterranean and South-East Asia Regions (11.8 and 10.0% respectively), with a prevalence of 7.7 and 9.0% respectively in the European and Western Pacific Regions [161]. The most frequent nosocomial infections are infections of surgical wounds, urinary tract infections and lower respiratory tract infections. WHO study, have also shown that the highest prevalence of nosocomial infections occurs in intensive care units and in acute surgical and orthopaedic wards. Infection rates are higher among patients with increased susceptibility because of old age, underlying disease, or chemotherapy.
1.1.13 ADHERENCE FACTORS OF LACTOBACILLUS IN THE HUMAN GASTROINTESTINAL TRACT

There are various genetically and functionally characterized Lactobacillus factors responsible for mediating adhesion to different components of the human gastrointestinal tract.

Lactobacilli adhesins can be classified according to their targets in the intestinal mucosa (i.e. mucus components, extracellular matrices), according to their localization in the bacterial surface (i.e. surface layer proteins), and/or according to the way they are anchored to the bacterial surface (i.e. sortase-dependent proteins).

1.1.13.1 Mucus-binding proteins

The epithelial cells of the intestine are covered by a protective layer of mucus, consisting of glycolipids and a complex mixture of large and highly glycosylated proteins or mucins as the main components. Bacteria adhering to mucus might have a short residence time. The mucus layer has the potential function of protecting the host against undesirable bacterial colonization. Mucus may also provide a habitat for commensal bacteria, such as lactobacilli [78]. Lactobacilli adhesion to mucus has been proposed to be mediated by proteins [100, 162], although unidentified saccharide moieties and lipoteichoic acid have also been reported to be involved [163, 164]. The lactobacilli mucus adhesins identified and functionally characterized until now are the extracellular mucus-binding protein (Mub) of Lactobacillus reuteri 1063 [162], the lectin-like mannose specific adhesin (Msa) of Lactobacillus plantarum WCFS1 [100], and the Mub of Lactobacillus acidophilus NCFM [165].

1.1.13.2 Sortase-dependent proteins

In Gram positive bacteria, a subgroup of these surface proteins contain the C-terminal motif LPxTG recognized by the enzyme sortase (SrtA), which cleaves between the T and G residues, and covalently links the threonine carboxyl group to amino groups provided by the cell wall cross bridges of peptidoglycan precursors. The product of the sortase reaction, a surface protein linked to peptidoglycan, is then incorporated into the envelope and displayed on the microbial surface [166]. Therefore, these surface proteins are commonly called sortase-dependent proteins.

The extracellular proteins of L. plantarum WCFS1 were predicted using a variety of bioinformatics methods, including domain analysis and phylogenetic profiling [167]. From this study, 12 proteins were identified as putative adhesion factors, 10 of which contained an LPxTG sortase motif. The
domain composition of the identified proteins predicted their role in the adherence to collagen, fibronectin, chitin or mucus.

1.1.13.3 Surface layer proteins
Lactobacilli surface layer proteins (S-layers) are generally monomolecular crystalline arrays exhibiting a morphologically similar, oblique lattice structure and representing 10-15% of the total protein of the bacterial cell wall [38, 164]. The biological functions of most of these proteins remain to be validated [38], except for four Lactobacillus S-layer proteins i.e. CbsA of Lactobacillus crispatus JCM 5810 [164], Slp of Lactobacillus helveticus R0052 [102], SlpA of Lactobacillus brevis ATCC 8287 [168] and SlpA of L. acidophilus NCFM [165]. These proteins have been reported to mediate adhesion to intestinal epithelial cells, extracellular matrices and/or to LTA of other bacterial species [38, 102, 165, 168]. Additionally, some of these proteins were proven effective in preventing the adhesion of pathogenic bacteria to epithelial cells [102, 168, 169].

1.1.13.4 Proteins mediating adhesion to extracellular matrices
The extracellular matrix (ECM) is a complex structural entity surrounding epithelial cells, referred to as the connective tissue. The ECM is composed of various proteins including, laminin, collagen and fibronectin. In case of damaged mucosa, ECMs can be exposed allowing undesirable microbial colonization and infection [170]. However, because of the ability of some lactobacilli to adhere to these matrices, they can compete with pathogens for the same receptors and occupy their potential binding sites in the gut [171, 172]. One of the reported ECM adhesins is the collagen-binding protein (CnBP) of L. reuteri NCIB 11951. Other examples of lactobacilli binding to collagen include S-layer proteins of L. crispatus (CbsA) [173] and L. brevis ATCC 8287 (SlpA) [174]. Another type of Lactobacillus ECM adhesins are fibronectin binding proteins. These proteins have been identified previously in L. acidophilus NCFM (FbpA) [165] and L. brevis ATCC 8287 (SlpA) [174].

1.1.13.5 Peculiar lactobacillus proteins mediating adhesion
Two unexpected Lactobacillus adhesins have been reported in Lactobacillus johnsonii La1 NCC 533. The first corresponds to the elongation factor Tu (EF-Tu), a guanosine nucleotide-binding protein involved in protein synthesis in the cytoplasm. Interestingly, the EF-Tu of L. johnsonii La1 has been shown to be localized at the bacterial surface, although no domains or motifs have been found in the protein to explain this observation [175]. Outside the cytoplasm, EF-Tu fulfils an
alternative function. In *L. johnsonii* La1, the surface-associated EF-Tu has been suggested to mediate the adhesion to human intestinal cells and mucins.

Another usually cytoplasmic-localized protein that has been found to be associated with the surface of *L. johnsonii* La1 is the heat shock protein GroEL, sometimes referred to as a chaperone of the Hsp60 class [156]. GroEL as a cytoplasmic protein is a mediator of protein folding. However, localized at the surface, it has been suggested to be involved in La1 adhesion to the GIT because recombinant GroEL has been reported to bind to human intestinal epithelial cells and mucus [103].

### 1.1.13.6 NON PROTEIN-MEDIATED ADHESION

Surface-associated factors such as LTA and exopolysaccharides have also been reported to affect *Lactobacillus* adhesion to the GIT. In *L. johnsonii* NCC 533, this molecule has been reported to mediate the adhesion to Caco-2 cells. In addition to LTA, the production of exopolysaccharides by lactobacilli has been reported to influence the adhesion to the intestinal mucosa. In *L. acidophilus* CRL639, adhesion to ECM components has been attributed to the production of different types of exopolysaccharides [172].

**AIM**

Probiotics is a relatively new concept in India. The effectiveness of a probiotic organism is considered to be population specific, due to variation in gut microflora, food habits and specific host-microbial interactions. The commercial probiotic cultures currently being used in India are of foreign origin. Inherent differences in gut flora of Indian population are known to occur, hence it is imperative to carry out efficacy studies in Indian population prior to their use in India. Hence in this study, we study an *L. fermentum* strain isolated from normal human gut for its *in vitro* ability to adhere to mammalian cells and also to inhibit growth of pathogenic bacteria.
1.2. MATERIALS AND METHODS

1.2.1 COLLECTION OF COLONOSCOPIC BIOPSY SAMPLES

Reagents:

(a) Normal Saline

- Sodium chloride 8.5 g
- Milli-Q H₂O 100 ml

Dispense 1 ml in vials and autoclave at 15 lbs (121 °C) for 15 min.

Procedure:

- Normal human colonic mucosal biopsies were obtained at the time of colonoscopy (Dept of Gastroenterology, AIMS, Kochi) in vials containing 1 ml sterile normal saline.
- Sample was transported to the lab under sterile conditions.
- The tissue was placed on sterile petri plate weighed on an electronic balance. The approximate weight of tissue was 25mg.

1.2.2 ISOLATION OF LACTOBACILLI FROM BIOPSY SAMPLES

Reagents:

(a) de Mann Rogosa Sharpe (MRS) broth / agar

- Bacteriological peptone 10 g
- Beef extract 8 g
- Yeast extract 4 g
- Dextrose / Glucose 20 g
- Tween 80 1 ml
- KH₂PO₄ 2 g
- CH₃COONa 5 g
- Triammonium citrate 2 g
- MgSO₄ 0.2 g
- MnSO₄ 0.05 g
- Bacteriological Agar * 15 g
- Milli-Q H₂O 1000 ml

* Omit if MRS broth is being prepared
Procedure:
• The biopsy tissue was homogenized in saline to dislodge the adherent bacteria.
• 450 μl of the homogenate was added to 4.5 ml of sterile saline.
• Three serial dilutions of the same were carried out.
• All dilutions were plated on to MRS agar.
• The plates were incubated at 37 °C for 48 h.
• White, opaque pinpoint colonies of 2-3 mm diameter were identified and picked from the lowest dilution plates where individual colonies were visible.
• Colonies were then suspended in MRS broth and further purified by re-plating on MRS agar.

1.2.3 PRELIMINARY IDENTIFICATION OF LACTOBACILLI

1.2.3.1 Gram Reaction
This is a basic microbiological test to distinguish between two basic groups of bacteria – namely Gram positive and Gram negative. It is based on the differences in their cell wall structures.

Reagents:
(a) Crystal Violet

Crystal violet powder 10 g
Absolute alcohol 100 ml
Milli-Q H₂O 900 ml

Crystal violet powder was added to alcohol in a mortar. It was allowed to dissolve by mixing well. This was then filtered and 900 ml distilled water was added.

Working solution: Stock 10 ml
Milli-Q H₂O 90 ml

(b) Grams Iodine

Iodine crystals 1g
Potassium iodide 2 g
Milli-Q H₂O 300 ml

Working solution: Stock 10 ml
Milli-Q H₂O 90 ml
(c) Decolourizer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>50 ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

(d) Carbol fuschin

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuschin</td>
<td>10 g</td>
</tr>
<tr>
<td>Phenol (crystalline)</td>
<td>50 g</td>
</tr>
<tr>
<td>Alcohol (95% absolute)</td>
<td>100 ml</td>
</tr>
<tr>
<td>Milli-Q H₂O</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Basic fuschin was added to phenol and kept in a 1 litre flask in a water bath at 60 °C. When the stain powder and phenol are completely liquefied, the alcohol was added and mixed thoroughly. Distilled water was added and filtered.

<table>
<thead>
<tr>
<th>Working solution:</th>
<th>Stock</th>
<th>Milli-Q H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ml</td>
<td>90 ml</td>
</tr>
</tbody>
</table>

**Procedure:**

- An isolated colony was picked using sterile tooth pick from the plate of culture.
- A smear was prepared on a clean glass slide.
- This was air dried and heat fixed.
- Stained with Crystal violet for 1 min and then washed off.
- Fixed smear in Grams iodine for 1 min and then washed off with running tap water.
- Decolourizer was added and washed off immediately.
- Smear was counter stained with carbol fuschin for 30 seconds and washed off with running tap water.
- The slide was dried and observed under microscope.

1.2.3.2 Motility test

This is to check the motility of a bacterial strain in liquid culture.

**Reagents:**

- (a) Overnight liquid culture of the bacterial strains.
Procedure:

- A drop of liquid culture was placed on a glass slide.
- A coverslip was placed over it so as to form a thin film of liquid.
- Observe under microscope to see the motility of the organism.

1.2.3.3 Growth at different temperatures (5, 15 & 45 °C)

Reagents:

(a) Overnight liquid culture of the bacterial strains.

Procedure:

- Bacterial strains adjusted to a cell density of 10⁶ cfu / ml were inoculated to four different tubes of 2 ml MRS broth.
- Tubes were incubated overnight in a shaking incubator at various temperatures of 5 °C, 15 °C, 37 °C & 45 °C respectively.
- The turbidity of the growth of the strains at varying temperatures were compared with the control tube that was incubated at 37°C by measuring in a colorimeter at 610 nm.

1.2.3.4 Catalase test

The catalase enzyme functions to convert H₂O₂ into water and nascent oxygen.

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} \text{H}_2\text{O} + [\text{O}]^\cdot
\]

Reagents:

(a) 3% H₂O₂

Procedure:

- A drop of H₂O₂ was placed on a clean, dry glass slide.
- Using sterile toothpick, an isolated colony of the organism to be tested was placed on it.
- A positive reaction showed active bubbling due to the evolution of nascent oxygen.

1.2.3.5 Nitrate reduction test

Certain bacteria contain nitrate reductase enzyme that reduces nitrate to nitrite (in the presence of suitable electron donor) to form a product which can be tested colorimetrically.
Reagents:
(a) Nitrate broth
   KNO₃  0.2 g
   Peptone  5.0 g
   Milli-Q H₂O  1000 ml
(b) Test Reagent:
   Solution A: 8.0 g Sulphanilic acid + 1000 ml of 5M CH₃COOH
   Solution B: 5.0 g α naphthylamine + 1000 ml of 5M CH₃COOH
   Mix equal volumes of A & B
Procedure:
• A drop of the test reagent was added to an overnight liquid culture of the bacterial strain.
• On addition of both A & B reagents, a positive reaction showed a red colour, while negative reaction was colourless.

1.2.3.6 Voges – Proskauer test
Carbohydrate fermentation in bacteria in some organisms can take a different pathway and lead to the production of acetoin which is tested using colour reactions.
Reagents:
(a) Glucose Phosphate broth
   Glucose phosphate broth  5 g
   Peptone  5 g
   Milli-Q H₂O  1000 ml
(b) Test Reagent
   Solution A: 40% KOH
   Solution B: 1% α naphthol in ethanol
   Mix equal volumes of A & B
Procedure:
• A drop of the test reagent was added to an overnight liquid culture of the bacterial strain.
• On addition of both A & B reagents, a positive reaction was indicated by pink colour in 2-5 min, which turns to crimson in 30 min.
1.2.3.7 Bile esculin hydrolysis

Organisms positive for esculin hydrolysis hydrolyze the esculin to esculetin and dextrose. The esculin reacts with the ferric citrate to form a dark brown or black complex.

**Reagents:**

(a) Bile esculin agar slant

**Procedure:**

- The bacterial strain was inoculated on to the bile esculin agar slant and incubated overnight at 37 °C.
- A positive reaction was indicated by formation of brownish black colour.

1.2.3.8 Arginine decarboxylation

Some bacteria can decarboxylate amino acids to their corresponding amines with the liberation of CO₂. Production of decarboxylase is induced by low pH and their action raises the pH to neutrality or above.

**Reagents:**

(a) Arginine broth

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5 g</td>
</tr>
<tr>
<td>Meat extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Bromophenol purple</td>
<td>5 ml</td>
</tr>
<tr>
<td>Cresol red</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Milli-Q H₂O</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Add 1% Arginine hydrochloride to the above solution

**Procedure:**

- Bacterial strains were inoculated into the arginine broth.
- On reading the result after incubation at 37 °C in shaking incubator for 48 h, a positive test was purple in colour, while negative remained yellow.
1.2.3.9 Sugar fermentation tests
Bacteria produce acidic products when they ferment carbohydrates. The carbohydrate utilization tests are designed to detect the fermentation pattern of the bacterial strain that is characteristic of a species or strain.

Reagents:
(a) MRS broth containing indicator phenol red
(b) Commercially available sugar discs (Hi media), for the following sugars (25 mg / ml)
- Salicin
- Arabinose
- Maltose
- Galactose
- Lactose
- Mannose
- Melibiose
- Raffinose
- Sorbitol
- Threhalose

Procedure:
- The sugar discs were placed in the MRS broth with phenol red.
- The broth was inoculated with the bacterial strain and incubated at 37 °C overnight in shaking incubator.
- A positive result was indicated by the formation of a yellow colour, while negative tubes remained pink.

1.2.4 TESTS FOR PROBIOTIC PROPERTIES
1.2.4.1 Acid tolerance
Reagents:
(a) MRS broth with pH adjusted to 2.0 with HCl[176]
Procedure:
• 100 µl was taken from an overnight culture whose concentration has been adjusted to $10^6$ bacterial cells were inoculated to 5 ml of MRS broth of pH 2 in a 150mm x 20 mm test tube.
• A control of normal MRS was also inoculated simultaneously.
• Incubation was carried out for 2 h in shaking incubator at 37 °C.
• The turbidity was measured as absorbance at 610 nm in a colorimeter.
• The reading of the control was taken as 100% and the percentage tolerance of the isolates were assessed.
• Triplicate readings were taken and the mean was calculated.

1.2.4.2 Bile tolerance

Reagents:
(a) MRS broth containing 0.3% bile salt

Procedure:
• 100 µl was taken from an overnight culture whose concentration has been adjusted to $10^6$ bacterial cells were inoculated to 5 ml of MRS broth with 0.3% bile salt in a 150mm x 20 mm test tube.
• A control of normal MRS was also inoculated simultaneously.
• Incubation was carried out for 24 h in shaking incubator at 37 °C.
• The turbidity was measured as absorbance at 610 nm in a colorimeter.
• The reading of the control was taken as 100% and the percentage tolerance of the isolates were assessed.
• Triplicate readings were taken and the mean was calculated.

1.2.5. 16S rRNA IDENTIFICATION OF LACTOBACILLI TO THE SPECIES LEVEL

Carried out at Vimta Labs, Hyderabad, India.

1.2.6 CULTURE OF BACTERIA OTHER THAN LAB

1.2.6.1 Luria-Bertani Agar / Broth

Tryptone 10 g
Chapter 1…Materials & Methods

Yeast extract 5 g
NaCl 10 g
Bacteriological Agar* 15 g
Milli-Q H₂O 1000 ml
pH 7.0
*Omit if broth is being prepared.

1.2.6.2 Nutrient Agar / Broth

Nureient broth base 13 g
Bacteriological Agar* 15 g
Milli-Q H₂O 1000 ml
*Omit if broth is being prepared.

1.2.7. LIST OF BACTERIAL STRAINS USED

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Name of Bacteria</th>
<th>Source/ Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>ATCC 25922</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>S. aureus SA113</td>
<td>[177]</td>
</tr>
<tr>
<td>2</td>
<td>S. aureus SA113 (pCtuf-gfp)</td>
<td>[178]</td>
</tr>
<tr>
<td>3</td>
<td>P. aeruginosa PA 01</td>
<td>[179]</td>
</tr>
<tr>
<td>4</td>
<td>SAΔmenD</td>
<td>[180]</td>
</tr>
<tr>
<td>5</td>
<td>SAΔhemB</td>
<td>[180]</td>
</tr>
<tr>
<td>6</td>
<td>Salmonella Paratyphi A</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>7</td>
<td>Shigella sonnei</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>8</td>
<td>Pseudomonas aeruginosa</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>9</td>
<td>Proteus mirabilis</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>10</td>
<td>Enterococcus fecalis</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>11</td>
<td>Vibrio spp.</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>12</td>
<td>Klebsiella pneumoniae</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>13</td>
<td>Methicillin Resistant S. aureus</td>
<td>10 Clinical isolates</td>
</tr>
</tbody>
</table>
1.2.8 LIST OF CELL LINES USED

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Name of Cell line</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HT 29</td>
<td>Human colon adenocarcinoma</td>
</tr>
<tr>
<td>2</td>
<td>HCT 15</td>
<td>Human colon adenocarcinoma</td>
</tr>
<tr>
<td>3</td>
<td>INT 407</td>
<td>Human intestinal epithelium (embryonic)</td>
</tr>
<tr>
<td>4</td>
<td>HEK 293</td>
<td>Human embryonic kidney epithelium</td>
</tr>
</tbody>
</table>

Cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics penicillin (50 U / ml) and streptomycin (50 μg / ml).

1.2.9 CO-CULTIVATION OF LAB AND ENTEROPATHOGENS

Procedure 1:
• 10^6 bacteria per ml concentrations each of the pathogens and *L. fermentum* were taken in test tubes containing 2.5 ml of MRS broth and 2.5 ml of LB broth and incubated in a shaking incubator at 37°C. This media has been shown to be suitable for growth of both pathogen as well as *L. fermentum* by Drago *et al* [181].
• Smears were made on microscopic glass slides from the broth at different time intervals.
• These smears were heat fixed and subsequently subjected to Gram staining.
• Bacterial cells were viewed at 100x magnification with oil immersion objective on a light microscope.
• Monocultures of all bacteria were used as controls.

Procedure 2:
• The interference of Lactobacilli with the growth of pathogenic strains was evaluated by coincubating *E. coli*, *S. Paratyphi A*, and *S. sonnei* individually with *L. fermentum* strain.
• Tubes containing 2.5 ml of MRS broth and 2.5 ml of LB broth were inoculated with 10^5 cfu / ml of both the *L. fermentum* and the respective pathogenic strain.
• The tubes were incubated at 37 °C under continuous agitation.
• At 8 to 10 h intervals, all media were refreshed to limit changes in growth due to pH variation or nutrient consumption; to achieve this, cultures were centrifuged for 15 min at 5000 × g and pellets were resuspended in fresh medium.
• Twenty four hours and 48 h after inoculation, bacterial cells were collected by centrifugation (15 min at 5000 × g) and suspended in phosphate-buffered saline (PBS) by vortexing.
• Cells were serially diluted in PBS and plated on MRS agar to evaluate the Lactobacillus growth and on LB agar to evaluate the growth of E. coli, S. Paratyphi A, and S. sonnei, respectively.
• The MRS agar plates were incubated for 48 h at 37 °C, while LB agar plates were incubated for 24 h at 37 °C.
• Pure cultures of each strain were subjected to the same treatments and used as controls.

1.2.10 EVALUATION OF GROWTH INHIBITION OF E. COLI IN THE PRESENCE OF L. FERMENTUM AND ITS CULTURE SUPERNATANT

Reagents:
Culture supernatants (CS) of L. fermentum
(a) Acidic CS (pH 3.5): Overnight cultures of L. fermentum were centrifuged to pellet down the cells and the cell free supernatant was collected and filtered using 0.22 μm syringe filters. This was stored at -20º C till use.
(b) Neutral CS (pH 7.0): The pH of the acidic culture supernatant was adjusted to 7.0 using 1N NaOH.
(c) Heat treated CS: The culture supernatant was subjected to a temperature of 90º C for 10 minutes in a dry bath.

Procedure1: Growth curve
• E. coli were grown in 2 ml of MRS broth containing 5 μg / ml of each of L. fermentum CS (acidic as well as neutral) and heat treated CS.
• The growth curve was plotted by measuring the optical density of the culture at 610 nm at different time intervals using spectrophotometer.
• Control was E. coli grown in MRS broth without any CS.

Procedure2: Agar plate diffusion assay
• The log phase culture of E. coli was adjusted to a final cell density of 10^6 cfu / ml and spread onto a Nutrient agar plate.
Wells of 5 mm diameter were made into the plates and 50 μl of *L. fermentum* overnight culture, filter sterilized CS, neutralised CS as well as heat treated CS were added into the wells.

- The plates were incubated overnight at 37 °C.
- The zones of inhibition formed around the wells were measured and photographed.

### 1.2.11 STUDY OF GROWTH INHIBITION OF OTHER ENTERIC PATHOGENS IN THE PRESENCE OF *L. FERMENTUM* AND ITS CULTURE SUPERNATANT

**Procedure:**
- The log phase culture of *E. coli, Salmonella Paratyphi A, S. sonnei, P. aeruginosa, P. mirabilis, E. s. fecalis, Vibrio spp., K. pneumoniae* were adjusted to a final cell density of $10^6$ cfu / ml and spread onto a nutrient agar plates.
- Wells of 5 mm diameter were made into the plates and 50 μl of *L. fermentum* overnight culture, filter sterilized CS, neutralised CS as well as heat treated CS were added into the wells.
- The plates were incubated overnight at 37 °C.
- The zones of inhibition formed around the wells were measured and photographed.

### 1.2.12 pH, HEAT AND PROTEASE SENSITIVITY OF *L. FERMENTUM* CULTURE SUPERNATANT

**Reagents:**
(a) CS with varying pH: The pH of the culture supernatant was adjusted to 3, 5, 7 and 9 using 1N NaOH / HCl.

(b) CS with varying temperature treatments: The culture supernatant was subjected to a temperature of 40 °C, 60 °C, 80 °C, and 100 °C for 15 min each.

(c) CS with protease treatment in varying concentrations: The neutralized (pH7) culture supernatant was treated with varying concentrations of trypsin viz. 0.2, 0.4, 0.6, 0.8 and 1.0 mg / ml at 37 °C, overnight.

**Procedure:**
- The log phase culture of *E. coli* was adjusted to a final cell density of $10^6$ cfu / ml
- It was then spread onto the nutrient agar plates.
- Wells of 5 mm diameter were made into the plates.
- 50 μl of L. fermentum CS of varying pH, varying temperatures, and CS with trypsin at varying concentrations were added into the wells.
- The plates were incubated overnight at 37 °C.
- The zones of inhibition formed around the wells were measured and photographed.

1.2.13 ADHESION ASSAY

Procedure:
- Adhesion assay was carried out with HT 29 and HCT 15 (human colon adenocarcinoma) cells.
- Cell lines were seeded (5 x 10^4 cells / well) into 96-well tissue culture microtitre plates.
- The plates were incubated for 48 h at 37 °C; 5% CO₂.
- Viable bacterial cell suspension or surface proteins (or trypsin treated proteins or bovine serum albumin) in antibiotic free RPMI medium were added to the wells.
- Incubation of the bacterial cells or surface proteins with colon cells were carried out for 4 h. The media was aspirated and discarded.
- Distilled water was added (100μl for 10 min at room temperature) to lyse the colon cells and detach the bacterial cells that adhered.
- The viable bacterial cell count was obtained by serially diluting and plating in duplicates (5 μl) in MRS or Nutrient agar.
- Plates were incubated at 37 °C for 24h.
- Viable colony count was performed and the log value of cfu / ml was used as a measure of adhesion.

Serial dilution:

<table>
<thead>
<tr>
<th>Vial</th>
<th>Vial 1</th>
<th>Vial 2</th>
<th>Vial 3</th>
<th>Vial 4</th>
<th>Vial 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contents</td>
<td>100 μl from 96 well plate + 900 μl saline</td>
<td>100 μl from earlier dilution + 900 μl saline</td>
<td>100 μl from earlier dilution + 900 μl saline</td>
<td>100 μl from earlier dilution + 900 μl saline</td>
<td>100 μl from earlier dilution + 900 μl saline</td>
</tr>
<tr>
<td>Dilution</td>
<td>10⁻²</td>
<td>10⁻³</td>
<td>10⁻⁴</td>
<td>10⁻⁵</td>
<td>10⁻⁶</td>
</tr>
</tbody>
</table>
Colony count:
The number of colonies of bacteria in the lowest dilution that was plated was counted. This was multiplied by a factor of 200 to get the number of colony forming units per ml (cfu / ml).

1.2.14 ISOLATION OF NON-COVALENTLY LINKED CELL SURFACE ASSOCIATED PROTEINS (SAPS) FROM *L. FERMENTUM*

Reagents:
(a) 4M NaCl
   - Sodium chloride 23.4 g
   - Milli-Q H₂O 100 ml
(b) Phosphate buffered Saline (PBS)
   - 137 mM NaCl 8.0 g
   - 2.7 mM KCl 0.2 g
   - 10 mM Na₂HPO₄ 1.7 g
   - 2 mM KH₂PO₄ 0.27 g
   - Milli-Q H₂O 1000 ml
   - pH 7.4
(c) 10 kDa cut off Centricon centrifugal filter units.

Procedure:
• *L. fermentum* cells were grown overnight in 500 ml MRS broth.
• Bacterial cells were harvested by centrifugation, washed twice with PBS (pH 7.4).
• It was then suspended in 20 ml of 4 M NaCl and incubated at 4°C for 2 h in a rotary shaker.
• Cells were pelleted down by centrifugation.
• The supernatant was dialysed against PBS.
• Protein was concentrated using 10 kilo Dalton molecular weight cut off Centricon Centrifugal Filter Units.

1.2.15 PROTEIN ESTIMATION OF SAPs – BRADFORD’S ASSAY

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins and it is estimated colorimetrically.
Reagents:
(a) Protein Standard
   Bovine serum albumin 4 mg
   Milli-Q H₂O 1 ml
(b) Bradford reagent
   Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol; add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman 1 paper just before use.

Procedure:
   • The protein standards were prepared using BSA in milli-Q water.
   • 10 μl of the protein standards of concentration 0.75 μg, 1 μg, 1.5 μg, 2 μg and 3 μg were taken in 5 tubes.
   • Blank was set using 10 μl milli-Q water.
   • 300 μl of Bradford reagent was added to all the tubes and incubated for 5 min at room temperature.
   • 10 μl test sample was added to 300 μl of Bradford reagent.
   • The absorbance was then measured at 595 nm using Nanodrop spectrophotometer.

1.2.16 TRYSIN TREATMENT OF SAPs
Reagents:
   Trypsin Stock solution 1 mg/ ml

Procedure:
   Trypsin was added to the SAPs at a concentration of 100 μg/ml protein and incubated at 37 ℃ for 16 h.
1.2.17 SODIUM DO DECEYL SULPHATE – POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

(a) Separating Gel
- 30% Acrylamide: 2 ml
- 750 mM Tris: 3 ml
- 10% APS (pH 8.8): 60 µl
- 10% SDS: 60 µl
- TEMED: 4 µl
- Milli-Q H₂O: 900 µl

(b) Stacking Gel
- 30% Acrylamide: 340 µl
- 1M Tris (pH 6.8): 250 µl
- 10% APS (pH 8.8): 20 µl
- 10% SDS: 20 µl
- TEMED: 2 µl
- Milli-Q H₂O: 1360 µl

(c) Running Buffer (10X)
- 250 mM Trisbase: 30.28 g
- 1920 mM Glycine: 144 g
- 10% SDS: 10 g
- Milli-Q H₂O: 1000 ml
- pH: 8.3
- Dilute 10 x buffer for 1x buffer

(d) Gel loading buffer (4X)
- 1M Tris-HCl, pH 6.8: 4 ml
- SDS: 2 g
- 2% Bromophenol blue: 4 ml
- Glycerol: 8 ml
- Beta-mercaptoethanol: 0.6 ml
1.2.18 STAINING OF ACRYLAMIDE GELS

Reagents:

(a) Coomassie Blue Stain
   - Coomassie brilliant blue R-250 0.1%
   - Milli-Q H₂O 40%
   - Methanol 40%
   - Glacial acetic acid 10%

(b) Destaining solution
   - Milli-Q H₂O 40%
   - Methanol 40%
   - Glacial acetic acid 10%

Procedure:
- The glass plates of the gel casting apparatus were cleaned with alcohol, dried and assembled.
- The resolving gel constituents were mixed and casted.
- It was then overlaid with isopropanol and kept undisturbed till the gel sets.
- After the gel was set, the overlay buffer was poured off.
- Then the stacking gel was prepared and poured over the separating gel.
- Comb was then inserted into the stacking gel carefully without causing air bubbles, and allowed to set.
- After the gel was completely set, the comb was removed and transferred into the electrode assembly filled with the gel running buffer.
- Buffer was filled in completely to submerge the wells and then the molecular weight markers and the samples were loaded.
- The samples were mixed with gel loading dye prior to loading into the wells.
- Power supply was connected and the gel was run till the tracking dye front reached the bottom.
- The gel was removed from the glass plates and placed into the Coomassie Stain.
- After staining for 3-5 h, the gel was destained till bands were clear.
• The gel was then stored in water.

1.2.19 DOSE DEPENDENT ADHESION INHIBITION OF E. COLI
Increasing concentrations (0, 1.5, 3, 6, 12 and 24 µg/ml) of SAPs or Bovine serum albumin (BSA) were added to HT 29 cells and adhesion assay was performed.

1.2.20 CYTOTOXIC EFFECT OF ENTEROPATHOGENS IN PRESENCE OF L. FERMENTUM ON HT-29 CELLS

Procedure:
• Confluent HT 29 cells in 60 x 15 mm tissue culture plates were infected with 10^8 cells / ml of E. coli and L. fermentum cells (MOI = 1000).
• After incubation at 37 °C; 5% CO₂, cell lines were washed thrice using PBS and fresh medium added.
• The monolayer cells were then photographed using Nikon microscope.

1.2.21 GROWTH INHIBITION OF S. AUREUS & P. AERUGINOSA WHEN GROWN IN CO-CULTURE WITH L. FERMENTUM

Procedure 1:
• 10^6 bacteria per ml concentrations of the pathogens and L. fermentum were taken in the same MRS broth tube and incubated in a shaking incubator at 37 °C.
• Smears were made on microscopic glass slides from the broth after 24 h.
• The slides were heat fixed and subsequently subjected to Gram staining in the case of P. aeruginosa.
• The wet mount smears were used in the case of S. aureus.
• Bacterial cells were viewed at 100x magnification with oil immersion objective on a light microscope.
• Monocultures of all bacteria were used as controls.

Procedure 2:
• The interference of Lactobacilli with the growth of pathogenic strains was evaluated by coincubating S. aureus and P. aeruginosa individually with L. fermentum.
• Tubes containing 2.5 ml of MRS broth and 2.5 ml of LB broth were inoculated with $10^5$ cfu / ml of both the *L. fermentum* and the respective pathogenic strain (*S. aureus* or *P. aeruginosa*).

• The tubes were incubated at 37 °C under continuous agitation.

• At 8 to 10 h intervals, all media were refreshed to limit changes in growth due to pH variation or nutrient consumption; to achieve this, cultures were centrifuged for 15 min at 5000 × g and pellets were re-suspended in fresh medium.

• 24 h and 48 h after inoculation, bacterial cells were collected by centrifugation (15 min at 5000 × g) and suspended in phosphate-buffered saline (PBS) by vortexing.

• Cells were serially diluted in PBS and plated on MRS agar to evaluate the *Lactobacillus* growth and on LB agar to evaluate the growth of *S. aureus* and *P. aeruginosa*, respectively.

• The MRS agar plates were incubated for 48 h at 37 °C, while LB agar plates were incubated for 24 h at 37 °C.

• Pure cultures of each strain were subjected to the same treatments and used as controls.

1.2.22 DOSE DEPENDENT INHIBITION OF PYOCYANIN PRODUCTION OF *P. AERUGINOSA* BY CULTURE SUPERNATANT OF *L. FERMENTUM*  

Procedure:

• 100 μl of log phase cultures of *P. aeruginosa* in MRS broth was adjusted to a cell density of $10^6$ cfu / ml and added to 96 well microtitre plates.

• To this was added 2.5 and 5 μg / ml filter sterilized culture supernatant of *L. fermentum*.

• Control was fresh filtered MRS broth.

• The microtitre plates were incubated at 37 °C for 48-72 h.

• The pyocyanin production was visualised and photographed.

1.2.23 DOSE DEPENDENT *S. AUREUS AND P. AERUGINOSA* BIOFILM INHIBITION AND DISPERSION BY CS OF *L. FERMENTUM*  

Procedure:

• For biofilm assay *S. aureus* and *P. aeruginosa* was grown in filter sterilized MRS broth in 96 well flat bottom plates and also on glass slides.
• Filter sterilized CS of *L. fermentum* (2.5 and 5 µg / ml) were added to the wells.
• The plates were incubated at 37 °C for 48 h under static conditions.
• Biofilms formed in 96 well microtitre plates were stained with 0.1% crystal violet.
  For quantification of biofilm formation 100 µl of 70% ethanol was added to the wells to extract the crystal violet.
• The optical density was read at 580 nm using plate reader instrument (Biorad) and plotted graphically.
• For biofilm dispersions, fresh MRS broth containing 2.5 and 5 µg / ml *L. fermentum* CS were added to the pre-adhered bacterial cells and incubated further for 24 h.
• Wells were washed with PBS and biofilms were quantified as described above.
• Biofilm inhibition was similarly studied for small colony variants (SCVs) of *S. aureus* and also clinical Methicillin resistant *S. aureus* (MRSA) isolates and multidrug resistant (MDR) *P. aeruginosa* strains from clinical samples.

### 1.2.24 Confocal Microscopic Study of *S. aureus* Biofilm Inhibition by CS of *L. fermentum*

**Procedure:**
• Green Fluorescent Protein (GFP) expressing *S. aureus* were grown in filter sterilized MRS broth on the glass slides.
• Filter sterilized spent media of *L. fermentum* (2.5 and 5 µg / ml) were added to the culture.
• This was then incubated at 37 °C for 48 h under static conditions.
• The slides were washed in PBS and thickness of the biofilm was visualized using confocal microscope.

### 1.2.25 Alteration of Protein Profile of Pathogens in the Presence of CS of *L. fermentum*

**Procedure:**
• *S. aureus* and *P. aeruginosa* were grown in the absence and presence of 5 µg / ml of *L. fermentum* CS.
• Cell pellet of the overnight cultures were extracted with 10% Sodium dodecyl sulphate (SDS).
• Non-covalently linked surface associated proteins thus obtained were run on SDS PAGE to study the protein profile.

1.2.26 DIMINISHED CYTOTOXIC EFFECT OF S. AUREUS AND P. AERUGINOSA IN PRESENCE OF L. FERMENTUM

Procedure:
• MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cytotoxicity of HT-29, INT-407 and HEK-293 cells.
• Cells were seeded at a density of 5 x 10^4 cells per well in 96 well tissue culture plates.
• 20 µl of culture supernatant of each pathogen and CS of pathogens grown in presence of 5 µg/ml L. fermentum CS for 72 hrs were added to the wells.
• After 2 hrs incubation, media was removed and 100 µl of MTT reagent was added to each well and upon colour development was read using plate reader (Biorad) at 570 nm.
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1.3 RESULTS AND DISCUSSION

1.3.1 ISOLATION AND IDENTIFICATION OF PROBIOTIC *LACTOBACILLUS* STRAINS FROM HUMAN COLONIC BIOPSY

1.3.1.1 Isolation of LAB from colonic mucosa
74 viable strains of LAB were isolated from colonic biopsy sample of 9 patients. The details of the isolates are given in Table 1.3.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Type of Sample</th>
<th>Total LAB count (per g tissue)</th>
<th>No. of isolates</th>
<th>No of viable isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal mucosa</td>
<td>$2.26 \times 10^6$ cfu</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>Normal mucosa</td>
<td>$2.7 \times 10^4$ cfu</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Normal mucosa</td>
<td>$2.08 \times 10^6$ cfu</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Normal mucosa</td>
<td>$2.26 \times 10^6$ cfu</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Normal mucosa</td>
<td>$4.2 \times 10^4$ cfu</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>Normal mucosa</td>
<td>$2.19 \times 10^6$ cfu</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>Normal mucosa</td>
<td>$3.0 \times 10^3$ cfu</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>Normal mucosa</td>
<td>$3.2 \times 10^4$ cfu</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>Normal mucosa</td>
<td>$2 \times 10^4$ cfu</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>$80$</td>
<td>$74$</td>
<td></td>
</tr>
</tbody>
</table>

1.3.1.2 Identification of best probiotic strain

From among all 74 strains, the strain with the best acid and bile tolerance was chosen for further characterization and studies.
1.3.1.3 Biochemical identification of the strain

The various biochemical tests carried out to identify the strain to species level are given in Table 1.4. From the results of the biochemical tests, it was concluded that the strain was *Lactobacillus fermentum* (*L. fermentum*)

**Table 1.4: Results of the biochemical tests carried out for identification of the probiotic strain.**

<table>
<thead>
<tr>
<th>Biochemical Tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Reaction</td>
<td>Gram Positive</td>
</tr>
<tr>
<td>Morphology</td>
<td>Bacilli</td>
</tr>
<tr>
<td>Bile Tolerance</td>
<td>98%</td>
</tr>
<tr>
<td>Acid Tolerance</td>
<td>82%</td>
</tr>
<tr>
<td>Motility</td>
<td>Non Motile</td>
</tr>
<tr>
<td>Catalase</td>
<td>-</td>
</tr>
<tr>
<td>Gas from Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 5 °C</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 15 °C</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 45 °C</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate Production</td>
<td>-</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>-</td>
</tr>
<tr>
<td>Esculin Hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Arginine decarboxylation</td>
<td>+</td>
</tr>
</tbody>
</table>

**Sugar fermentations**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicin</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
</tr>
</tbody>
</table>

All Lactobacilli are Gram positive, non-motile and catalase negative. *L. fermentum* is heterofermentative, thus producing gas from glucose fermentation. Nitrate reduction and
Chapter 1...Results & Discussion

Voges proskauer (VP) were negative, while arginine decarboxylation and esculin hydrolysis were positive. Though growth at 5 and 15 °C were negative, it was positive at 45 °C. *L. fermentum* is positive for fermentation of the following sugars; salicin, arabinose, maltose, galactose, lactose, mannose, melibiose, raffinose, sorbitol and trehalose.

1.3.1.4 Identification by molecular methods

Identification was carried out by amplifying, sequencing, and comparing sequence data of 16S rRNA region (first 500 bp) at Vimta Lab, India. The strain was identified as *L. fermentum*.

1.3.2 ANTI-MICROBIAL ACTIVITY OF *L. FERMENTUM* AGAINST ENTERIC AND FOOD BORNE PATHOGENS

1.3.2.1 Co-cultivation of LAB and enteropathogens

To find out whether *L. fermentum* is capable of inhibiting the growth of enteric pathogenic bacteria, it was grown as monoculture and as co-culture along with the respective pathogens. Aliquots of cells when stained using Gram stain and visualized under microscope showed growth inhibition of all tested enteric pathogens that includes *E. coli*, *S. Paratyphi A*, and *S. sonnei*. *L. fermentum* is a Gram positive bacilli while *E. coli*, *S. Paratyphi A*, and *S. sonnei* are Gram negative organisms. This helps in easy differentiation on performing a Gram stain. It was found that *L. fermentum* induced inhibition of growth of the enteric pathogens became apparent after 24 hours in culture. While the Gram positive (purple) *L. fermentum* dominated in the co-culture, the Gram negative (pink) pathogens were less in number (Figure 1.5).
**Figure 1.5: Gram staining of enteropathogens in monoculture and in co-culture with *L. fermentum***. Cells of *E. coli*, *S. paratyphi A*, and *S. sonnei* were grown till stationary phase as monoculture or cocultivated with *L. fermentum*. Following Gram staining bacterial cells were photographed. Purple coloured cells represent Gram-positive *L. fermentum*, whereas Gram-negative *E. coli*, *S. paratyphi A*, and *S. sonnei* appear as pink coloured cells.
1.3.2.2 Antimicrobial activity of *L. fermentum*

The capability of the *L. fermentum* to inhibit the *in vitro* growth of intestinal pathogens was evaluated in co-culture experiments. In a series of experiments, *L. fermentum* was inoculated simultaneously with the pathogens. *L. fermentum* inhibited the growth of the pathogens such as *E. coli*, *S. paratyphi* A, and *S. sonnei* after 24 h incubation: a decrease in the cfu was observed for pathogens. Similar results were observed at 48 h. No significant difference in growth was observed for *L. fermentum* when grown as monoculture or in co-culture with the respective pathogens (Figure 1.6).

*Lactobacillus fermentum* isolated from the intestinal biopsy sample inhibited the growth of enteric pathogens such as *E. coli*, *S. Paratyphi*, and *S. sonnei* in coculture. The antibacterial mechanisms of the action of LAB against enteropathogens appear to involve the lowering of the pH due to the production of lactic acid and antibacterial molecules including nonbacteriocin molecules. *Lactobacillus acidophilus*, *L. bulgaricus*, and *L. casei* have been shown to secrete antimicrobial short-chain fatty acids that are active only at low pH environment (pH 3.0 to 5.0) and were heat stable, as well as resistant to various proteases including proteinase K, trypsin, and carboxypeptidase A.
Figure 1.6: Inhibition of in vitro growth of *E. coli*, *S. Paratyphi A*, and *S. sonnei* by *L. fermentum*. A & B represent the mono culture of *L. fermentum* and pathogen respectively, while, C & D represent the growth of *L. fermentum* and pathogen respectively in co-culture. The coloured bars represent the growth in cfu/ml after 24 h of culture and the white bars after 48 h.
1.3.2.3 Growth inhibition of *E. coli* in the presence of *L. fermentum* and its culture supernatant

Growth inhibition zones were observed when agar plate diffusion assays were performed using *L. fermentum* culture of pH 3.5 and neutralized culture of pH 7.0, as well as with filter sterilized culture supernatant (CS) of pH 3.5 and 7.0. To find out whether the growth inhibition of these enteropathogens was not solely due to the secreted lactic acids (low pH) in the CS, growth inhibition assays were also performed using neutralized CS of pH 7.0. The heat stability of the antimicrobial compound was also tested by using CS that has been subjected to at 90 °C for 10 min to denature the proteins. No growth inhibitions of *E. coli* was observed, when CS was heated. These results indicate that *L. fermentum* secretes antimicrobial protein(s) or peptide(s), which are heat sensitive and have growth inhibitory properties (Figure 1.7A). Similar results were observed in growth curve in broth culture where *E. coli* was grown in MRS broth in the presence of various CSs of *L. fermentum* and the OD was measured at varying time intervals. While the control (MRS broth alone) and in the heat treated CS did not inhibit the growth of *E. coli*, there was significant decrease in the OD with respect to control, when *E. coli* was grown in the presence of acidic *L. fermentum* CS indicating growth inhibition. The neutralized CS also showed inhibition to a certain extent but not as evident as acidic CS (Figure 1.7B).
**Figure 1.7: Inhibition of *E. coli* in the presence of *L. fermentum* culture and culture supernatant**

(A) Agar plate diffusion assay showing the growth inhibition of *E. coli* in the presence of acidic as well as neutralised culture of *L. fermentum* (CuI\textsubscript{acidic} and CuI\textsubscript{neutral}) and also the acidic and neutralized culture supernatant of *L. fermentum* (CS\textsubscript{acidic} and CS\textsubscript{neutral}). The heat treated CS (CS\textsubscript{heat}) had no inhibitory effect. (B) Growth curve of *E. coli* grown in the presence of culture supernatant of *L. fermentum* (acidic, neutralized as well as heat treated).
1.3.2.4 Growth inhibition of other enteric pathogens in the presence of *L. fermentum* and its culture supernatant

Growth inhibition zones were observed when agar plate diffusion assays were performed using *L. fermentum* culture of pH 3.5 (Figure 1.8, well 2) and neutralized culture of pH 7.0 (Figure 1.8, well 3), as well as with filter sterilized CS of pH 3.5 (Figure 1.8, well 6). To find out whether the growth inhibition of these enteropathogens was not solely due to the secreted lactic acids (low pH) in the CS, growth inhibition assays were also performed using neutralized CS of pH 7.0 (Figure 1.8, well 5). Growth inhibition zones of *E. coli*, *S. paratyphi A*, *S. sonnei*, *P. mirabilis*, *P. aeruginosa*, *Vibrio* sp., *K. pneumoniae*, and *E. faecalis* were observed when neutralized culture (Figure 1.8, well 3) and CS (Figure 1.8, well 5) were added to the wells, but to a lesser extent. No growth inhibitions of pathogens were observed, when neutralized CS was heated at 90 °C for 10 min (Figure 1.8, well 4) to denature the proteins. These results indicate that *L. fermentum* secretes antimicrobial protein(s) or peptide(s), which are heat sensitive and have growth inhibitory properties against enteric pathogens.
Figure 1.8: Agar plate diffusion assay showing growth inhibition of various enteric pathogens in the presence of culture as well as culture supernatant of *L. fermentum*. 1: Control; 2: Culture (acidic); 3: Culture supernatant (acidic); 4: Heat treated culture supernatant; 5: Culture supernatant (neutral); 6: Culture (neutral).
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1.3.2.5 pH, heat and protease sensitivity of *L. fermentum* culture supernatant

The pH of the culture supernatant tested was varied from 3-9 (3, 5, 7 and 9) and the antibacterial activity of the culture supernatant of *L. fermentum* was tested by agar well diffusion assay against *E. coli*. All pH except pH 9 gave an inhibition zone showing that the antimicrobial compound was inactive at an alkaline pH. The zone at pH 7 was slightly smaller than the zones when pH was acidic (Figure 1.9A).

Heat sensitivity of the antimicrobial compound was tested by heating the culture supernatant of *L. fermentum* at varying temperatures (40 °C, 60 °C, 80 °C, and 100 °C for 15 min each). Agar well diffusion assay was then carried out. Zones were obtained for 40 and 60 °C while 80 °C and 100°C gave no zones indicating that the activity of the antimicrobial compound was lost on heating above 60 °C. The growth inhibition zone obtained for 60 °C was smaller than for 40°C (Figure 1.9B).

The antimicrobial compound from *L. fermentum* was found to be sensitive to trypsin. Treatment of the culture supernatant with varying concentrations of trypsin (0.2, 0.4, 0.6, 0.8 and 1.0 mg / ml) were carried out at 37 °C overnight. Agar well diffusion assay was then carried out. Results indicate that the compound is inhibited by trypsin at concentration above 0.4 mg / ml (Figure 1.9C).

A basic physicochemical characterization of the antimicrobial compound was tried in this study. The heat and trypsin inactivation of the compound indicate the probable role of a protein like molecule. *L. bulgaricus* and *L. casei* have been shown to secrete antimicrobial short-chain fatty acids that are active only at low pH environment (pH 3.0 to 5.0) and were heat stable, as well as resistant to various proteases including proteinase K, trypsin, and carboxypeptidase A. Previous findings have shown that some bacteriocin producing *Lactobacillus* strains, although were able to inhibit a variety of pathogenic bacteria, did not influence the growth of both *Salmonella* sp. and *V. cholerae*, when the effect of acids was excluded. Holo and others showed that *L. plantarum* secreted bacteriocin plantaricin W (P1wa and P1wβ) that inhibits a large number of Gram-positive bacteria.
Figure 1.9: Agar plate diffusion assay showing the pH, heat and protease sensitivity of *L. fermentum culture supernatant* against *E. coli*. (A) pH sensitivity – The CS is active only in acidic and neutral pH. (B) The activity of CS is destroyed when heated above 60 °C. (C) The CS activity is lost on trypsin treatment. [C=control - MRS broth in case of A and B; *L. fermentum* CS in case of C]
1.3.2.6 *L. fermentum* reduces the adherence of enteropathogens to colon epithelial cell lines

Adhesion to epithelial cells followed by invasion is an important pathogenic mechanism adopted by most enteric pathogens. To find out whether *L. fermentum* inhibits the adhesion of enteropathogens such as *E. coli*, *S. paratyphi* A, and *S. sonnei*, an equal inoculum mixture of *L. fermentum* and the respective pathogens were added to colon cancer HT-29 (Figure 1.10A) and HCT-15 cell lines individually. After 4 h, adhered bacteria were counted after serial dilutions were spread on LB agar plates, where *L. fermentum* cannot grow. Results showed that 8.2 log cfu / ml of *E. coli*, 7.8 log CFU / ml of *S. Paratyphi* A, and 7 log cfu / ml of *S. sonnei* adhered to HT-29 cells after 4 h of infection. Significant decrease (approximately 2 log cfu / ml) in the adhesion of each pathogen was observed in the presence *L. fermentum*. Similar results were observed when HCT-15 cell lines were used (Figure1.10B).

1.3.2.7 Non covalently linked Surface associated proteins (SAPs) of *L. fermentum* competitively exclude the adhesion of *E. coli*

SAPs were isolated from *L. fermentum* using 4 M NaCl, desalted, and analyzed in 10% SDS-PAGE gel (Figure 1.11A). SAPs isolated from *L. fermentum* did not inhibit the growth of *E. coli*, (Figure 1.11B) but inhibited their adhesion to HT-29 cells. Adherence of *E. coli* cells was decreased by approximately 2 log units, when HT-29 cells were pre-incubated with SAPs. The role of SAPs in exclusion of *E. coli* adhesion was confirmed using trypsin digested SAPs (Figure 1.11A-lane3 and 1.11C). No significant decrease of *E. coli* adhesion was observed when trypsin treated SAPs were used (Figure 1.11C).
Figure 1.10: Effect of *L. fermentum* on adhesion of enteropathogens. HT-29 (A) and HCT-15 (B) cells were incubated with *E. coli*, S. Paratyphi A, *S. sonnei* alone, or in presence of *L. fermentum*. A decrease in the adherence of enteric pathogens was observed when *L. fermentum* was present.
Figure 1.11: *Lactobacillus fermentum* noncovalently linked cell surface associated proteins (SAPs) exclude the adhesion of *E. coli*. (A) SDS-PAGE of SAPs from *L. fermentum*. Lane 1 = protein molecular weight marker; lane 2 = SAPs isolated using 4 M NaCl; lane 3 = SAPs digested with trypsin (100 μg / ml) for 16 h. (B) Agar plate diffusion assay showing that SAPs (40 μg / well) have no growth inhibitory effect against *E. coli*. (C) Adhesion of *E.coli* to HT 29 cells. Decrease of *E. coli* adhesion when HT-29 cells were pre-incubated with SAPs. Trypsin digested SAPs failed to inhibit the adhesion of *E. coli*. 
1.3.2.8 Dose dependent adhesion inhibition of E. coli
The SAPs mediated adhesion inhibition of E. coli to HT 29 cells showed a dose dependent pattern. There was increased inhibition in adhesion with increasing concentration of SAPs. Bovine serum albumin (BSA), used as control showed no inhibition in adhesion of E. coli to HT 29 cells (Figure 1.12). The inhibitory activity showed a plateau effect above concentration of 6 µg / ml probably suggesting the presence of surface receptors on HT 29 for the SAPs, which gets saturated, thus showing no more increase in adhesion inhibition.
SAPs from L. helveticus were known to inhibit enterohaemorrhagic E. coli adhesion to epithelial cells[102]. Previously, 29 SAPs were isolated from L. plantarum strain 299v and were identified using liquid chromatography mass spectrophotometry/mass spectrophotometry (LC-MS/MS) analysis [182] that includes phosphoglycerate kinase, triose-phosphate isomerase, enolase, glucose-6-phosphate isomerase, and glyceraldehyde 3-phosphate dehydrogenase. Kinoshita and others identified GAPDH from L. plantarum LA 318 adhered to human colonic mucin [183]. Inhibition of adhesion to epithelial cells is also one of the mechanisms to prevent invasions of enteropathogens.

1.3.2.9 Diminished cytotoxic effect of enteropathogens in the presence of L. fermentum on HT-29 cells
Enteropathogens such as E. coli cause a significant cytotoxic effect on HT-29 cells, presumably because of the production of endotoxins by this strain. Figure 1.13A shows the normal appearance of HT-29 cells (untreated control). HT-29 cells remain healthy following treatment with L. fermentum (Figure 1.13B). However, after 24 h, incubation with E. coli caused cytotoxic effect (rounding and cell detachment) on HT-29 cells (Figure 1.13C). The cytotoxic effect was negligible when HT-29 cells were preincubated with L. fermentum (Figure 1.13D) indicating that L. fermentum protects HT-29 cells from E. coli mediated cytotoxicity.
Figure 1.12: The dose dependent adhesion inhibition of *E. coli* to HT 29 cells. Control BSA confers no adhesion inhibition. HT 29 cells are treated with BSA (Control ■) and SAPs(●) of *L. fermentum*.
Figure 1.13: Inhibition of E. coli mediated cytotoxicity by L. fermentum. Confluent HT-29 cultures were incubated with either E. coli or L. fermentum or with both strains. (A) HT-29 control cells. (B) Normal appearance after 24 h of incubation with L. fermentum. (C) The cytopathic effect of E. coli in presence HT-29 cells (rounding and cell detachment). (D) Appearance of HT-29 cells in presence of E. coli when preincubated with L. fermentum for 2 h.
1.3.3 ACTIVITY OF L. FERMENTUM AGAINST NOSOCOMIAL PATHOGENS

1.3.3.1 Growth inhibition of S. aureus & P. aeruginosa when grown in co-culture with L. fermentum

To find out whether L. fermentum is capable of inhibiting the growth of S. aureus and P. aeruginosa, it was grown as monoculture and as co-culture along with the respective pathogens. Aliquots of cells where taken and viewed under microscope for S. aureus and stained using Gram stain and visualized under microscope for P. aeruginosa. Co-culturing L. fermentum led to the growth inhibition of both pathogens (Figure 1.14).

The capability of the L. fermentum to inhibit the in vitro growth of nosocomial pathogens was evaluated in co-culture experiments. In a series of experiments, L. fermentum was inoculated simultaneously with the pathogens. L. fermentum mediated growth inhibition of the pathogens – S. aureus and P. aeruginosa were apparent after 24 h incubation: a decrease of 4 log units were observed in the growth of pathogens. Similarly a decrease in 7 log units was seen after 48 h. No significant difference in growth was observed for L. fermentum when grown as monoculture or in co-culture with the respective pathogens (Figure 1.15).

1.3.3.2 Dose dependent inhibition of pyocyanin production of P. aeruginosa by culture supernatant of L. fermentum

L. fermentum CS inhibit the pyocyanin production of P. aeruginosa. Pyocyanin is a redox-active phenazine compound that kills mammalian and other bacterial cells through the generation of reactive oxygen intermediates. Inhibition of pyocyanin production probably indicates that L. fermentum can regulate the virulence factor production of P. aeruginosa (Figure. 1.16).
Figure 1.14: Microscopic picture of co-culture of *L. fermentum* (LF) with *S. aureus* (SA) and *P. aeruginosa* (PA). The pathogens growth were inhibited when grown in co-culture, while monoculture showed no growth inhibition. The top panel is *S. aureus* (wet mount) and the bottom panel is *P. aeruginosa* (Gram stained).
Figure 1.15: Inhibition of in vitro growth of S. aureus (A) and P. aeruginosa (B) by L. fermentum during co-culture. A & B represent the monoculture of L. fermentum and pathogen respectively, while, C & D represent the growth of L. fermentum and pathogen respectively in co-culture. The green bars give the growth in cfu / ml after 24 h of culture and the white bar after 48 h.
Figure 1.16: inhibition of pyocyanin production of *P. aeruginosa* by culture supernatant of *L. fermentum*. Dose dependent inhibition of pyocyanin production of *P. aeruginosa* in presence of 2.5 and 5 µg / ml CS from *L. fermentum*. Pyocyanin is a blue green pigment produced by *P. aeruginosa*.
1.3.3.3 Dose dependent *S. aureus* biofilm inhibition and dispersion by CS of *L. fermentum*

*S. aureus* biofilm-associated infections are difficult to treat with antibiotics and devices need to be replaced frequently. To determine if *L. fermentum* CS can inhibit biofilm formation, 96 well plates were inoculated with *S. aureus* and *L. fermentum* CS added to *S. aureus* cells for 48 h, after which period wells were washed with PBS to remove the non-adhered cells. Fresh growth medium containing 2.5 and 5 µg / ml of *L. fermentum* CS resulted in 40 and 50% dispersion of biofilm after 24 h (**Figure 1.17A**). The absorbance of the dye was read and percentage biofilm inhibition and / or disruption was evaluated and plotted graphically (**Figure 1.17B**).

1.3.3.4 Confocal microscopic study of *S. aureus* biofilm inhibition by CS of *L. fermentum*

To study biofilm inhibition by *L. fermentum* CS, GFP-expressing *S. aureus* biofilms were grown on glass surfaces and visualized by fluorescence microscopy. The control *S. aureus* cells were able to attach to the surface but adhesion of *S. aureus* was inhibited when grown in presence of 2.5 and 5 µg / ml of *L. fermentum* CS. An optical sectioning along the Z axis by confocal microscopy showed that the *S. aureus* cells when grown in the presence of *L. fermentum* CS forms a thin layer of biofilm across the glass surface unlike the thicker textured biofilm of the control. The average biofilm thickness was 20 µm in case of control, whereas thickness of biofilm was decreased to 14 µm and 6 µm when grown in presence of *L. fermentum* CS (**Figure 1.18**).

1.3.3.5 Biofilm inhibition in *S. aureus* small colony variants (SCVs) by CS of *L. fermentum*

SCVs are known to colonize multiple organs and they possess a survival advantage during antimicrobial therapy, compared to its parent strain. SCVs of *S. aureus* were generated earlier via mutations in *menD* or *hemB*, yielding menadione and hemin auxotrophs, respectively. Both these mutants were equally sensitive against *L. fermentum* and its CS. 96 well plate biofilm assay showed that CS of *L. fermentum* can inhibit the initial adherence of *S. aureus* to PVC microtitre plates (**Figure 1.19**).
Figure 1.17: *S. aureus* biofilm inhibition and dispersion in dose dependent manner by CS of *L. fermentum*. (A) 96 well plate showing decrease in biofilm in the presence of CS of *L. fermentum* (B) graph representing the percentage biofilm inhibited and dispersed in the presence of increasing concentrations of CS of *L. fermentum*
Figure 1.18: Effect of *L. fermentum* CS on growth inhibition and biofilm formation of *S. aureus*. Confocal fluorescence microscopic image of *S. aureus* biofilm inhibition in the presence of increasing concentrations of *L. fermentum* CS. GFP expressing *S. aureus* on glass surfaces formed thinner biofilms in the presence of *L. fermentum* CS. (Control is *S. aureus* without *L. fermentum* CS). Z axis indicates the thickness of the biofilm.
Figure 1.19: Growth and biofilm inhibition of small colony variants (SCVs) of *S. aureus* in the presence of *L. fermentum* CS. (A) Growth inhibition of *S. aureus* SCVs (ΔhemB and ΔmenD) as shown by agar plate diffusion assay. (B) Biofilm inhibition of *S. aureus* SCVs (ΔhemB and ΔmenD) in 96 well plates in presence of 2.5 μg / ml of *L. fermentum* CS.
1.3.3.6 Alteration of protein profile of *S. aureus* in the presence of CS of *L. fermentum*

To investigate whether biofilm inhibition of *S. aureus* and *P. aeruginosa* is due to altered cell wall associated protein profile, we have extracted proteins that are non-covalently associated with the cell wall of both pathogens. The proteins were separated on 10% SDS PAGE and it was seen that alteration of *S. aureus* cell wall associated proteins occurs when grown in presence of *L. fermentum* CS (**Figure 1.20**) while cell surface associated protein profile of *P. aeruginosa* was unaltered (data not shown). Since *S. aureus* is Gram positive and *P. aeruginosa* is Gram negative, their protein expression pattern might be different. Further studies are needed to give definitive conclusions in this regard.

1.3.3.7 Dose dependent biofilm inhibition of *P. aeruginosa* by *L. fermentum* CS

To study the effect of CS of *L. fermentum* on biofilm formation of *P. aeruginosa*, the pathogen was grown on both PVC (96 well microtitre plates) as well as glass (glass slides) surfaces in the presence of increasing concentrations of *L. fermentum* CS. A dose dependent decrease in biofilm formation was observed on both surfaces (**Figure 1.21 A, B**).

1.3.3.8 Diminished cytotoxic effect of *S. aureus* and *P. aeruginosa* in presence of *L. fermentum*

*S. aureus* and *P. aeruginosa* cause a significant cytotoxic effect on HT29, INT 407 and HEK 293 cells, because of the production of several toxins by these strains. **Figure 1.22A** shows the normal appearance of HEK-293 cells (untreated control). HEK-293 cells remain healthy following treatment with CS of *L. fermentum*. However, after 24 h incubation with *S. aureus* and *P. aeruginosa* CS, HEK-293 cells become cytotoxic (cell detachment and rounding). The cytotoxic effect was negligible when HEK-293 cells were incubated with *S. aureus* and *P. aeruginosa* cells grown in presence of *L. fermentum* CS, indicating that *L. fermentum* protects HEK-293 cells from *S. aureus* and *P. aeruginosa* mediated cytotoxicity.

The cytotoxicity of *S. aureus* and *P. aeruginosa* CS on HT29, INT 407 and HEK 293 was determined using MTT assay (**Figure 1.22B, C**). HT29, INT 407 and HEK 293 cells were cytotoxic when *S. aureus* and *P. aeruginosa* CS was added. Cell viability was decreased between 50 to 60 % when cells were incubated with CS of both pathogens; however cell viability was increased (80 to 95%) when pathogens were grown in presence of 5 μg / ml CS of *L. fermentum*.
Figure 1.20: Alteration of protein profile of *S. aureus* in the presence of CS of *L. fermentum* 10% SDS-PAGE of non-covalently associated cell wall bound proteins from *S. aureus* cells grown in absence (lane 1) or presence (lane 2) of 5 µg / ml *L. fermentum* CS. Molecular weight protein standard is indicated in the left
Figure 1.21: Effect of *L. fermentum* CS in biofilm formation of *P. aeruginosa*. Biofilm inhibition of *P. aeruginosa* on glass slides (A) and in microtitre plates (B) in presence of increasing concentrations (2.5 and 5 μg / ml) of CS from *L. fermentum*. 
Figure 1.22: Inhibition of cytotoxic effect of *S. aureus* and *P. aeruginosa* CS in presence of *L. fermentum*. (A) Confluent HEK-293 cells were incubated with CS of *S. aureus* and *P. aeruginosa* grown in absence and presence of 5 µg / ml CS of *L. fermentum* for 24 h. Symbols: LF: *L. fermentum*; SA: *S. aureus*; PA: *P. aeruginosa*. (B, C) Diminished cytotoxicity of *S. aureus* (B) and *P. aeruginosa* (C) CS against HT 29, INT 206 and HEK 293 cells when cultivated in the presence of 5 µg / ml CS of *L. fermentum*. 
1.3.3.9 Inhibition of growth and biofilm formation of clinical isolates of S. aureus and P. aeruginosa

Antibiotic resistance is a major problem encountered by clinicians in the successful treatment of infections. Multi-drug resistant (MDR) strains of pathogens often pose a threat to antimicrobial chemotherapy. Methicillin resistant S. aureus (MRSA) are also seen among nosocomial infections. The role of L. fermentum culture supernatant in inhibiting the biofilm formation of these pathogens (MDR P. aeruginosa and MRSA) was tested in 96 well plates. Ten clinical isolates of MDR P. aeruginosa as well as MRSA were used in the study. The antibiograms of these strains are as listed in Table 1.5a and 1.5b respectively.

The susceptibility of clinical isolates towards L. fermentum CS and biofilm forming capacity in presence of 5 μg / ml CS of L. fermentum are shown in Figure 1.23. The growth inhibition was measured as inhibition zone diameters (agar plate diffusion assay) and represented graphically. All of the P. aeruginosa MDR isolates were susceptible to L. fermentum CS and their initial adherence was impaired in presence of L. fermentum CS indicating their biofilm inhibition. However the inhibition zone diameter of the clinical isolates varied, indicating that not all the strains were equally sensitive towards CS of L. fermentum. While 7 of the MRSA were sensitive, 3 were only intermediately sensitive towards CS of L. fermentum. Similarly, 8 out of the 10 S. aureus clinical isolates failed to form biofilm in presence of L. fermentum CS, except two strains (Figure1.23).
Figure 1.23: Growth and biofilm inhibition of MRSA and MDR *P. aeruginosa* clinical isolates in the presence of *L. fermentum* culture supernatant. (A) Graphical representation of the inhibition zone diameters of clinical isolates of MRSA and MDR *P. aeruginosa* in the presence of *L. fermentum* culture supernatant. (B) Inhibition of biofilm formation by clinical isolates of MRSA and MDR *P. aeruginosa* when carried out in 96 well microtitre plates, in the presence of *L. fermentum* culture supernatant. (numbers indicate the different clinical isolates)
### Table 1.5a: Antibiogram of MDR *P. aeruginosa* clinical isolates

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<th>Cz</th>
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<th>G</th>
<th>Levo</th>
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**Ak** = Amikacin 30mcg, **Cpi** = Cefepime 30mcg, **Cpz** = Cefoperazone 75mcg, **Cz** = Ceftazidime 30mcg, **Cip** = Ciprofloxacin 5mcg, **Cotri** = Co-trimoxazole 25mcg, **G** = Gentamicin 10mcg, **Levo** = Levofloxacin 5mcg, **T** = Tetracycline 30mcg, **TC** = Ticarcillin-Clavulanic acid

R = Resistant, I = Intermediate, S = Sensitive

### Table 1.5b: Antibiogram of MRSA clinical isolates

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**Cn** = Cephoxitin 30mcg, **Cip** = Ciprofloxacin 5mcg, **Clarit** = Clarithromycin, **Oxa** = Cloxacillin / Oxacillin 1mcg, **Cotri** = Co-trimoxazole 25mcg, **E** = Erythromycin 15mcg, **G** = Gentamicin 10mcg, **P** = Pencillin G 10 units

R = Resistant, I = Intermediate, S = Sensitive
Table 1.6: Statistical table for inhibition of *in vitro* growth of *E. coli*, *S. Paratyphi A*, and *S. sonnei* by *L. fermentum*

\( n = 9 \)

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<th>Time</th>
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<td>24 h</td>
<td>Pathogen Monoculture</td>
<td>10.924</td>
<td>0.086</td>
<td>10.417</td>
</tr>
<tr>
<td></td>
<td>Pathogen Co-culture</td>
<td>5.232</td>
<td>0.743</td>
<td>4.359</td>
</tr>
<tr>
<td></td>
<td>p - value</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>48 h</td>
<td>Pathogen Monoculture</td>
<td>10.206</td>
<td>0.716</td>
<td>9.446</td>
</tr>
<tr>
<td></td>
<td>Pathogen Co-culture</td>
<td>3.648</td>
<td>0.538</td>
<td>3.528</td>
</tr>
<tr>
<td></td>
<td>p - value</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Table 1.7: Statistical table for Effect of *L. fermentum* on adhesion of enteropathogens

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Adhesion (Log cfu/ml)</th>
<th>E. coli</th>
<th>S. Paratyphi A</th>
<th>S. sonnei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>HT 29</td>
<td>Monoculture</td>
<td>8.273</td>
<td>0.228</td>
<td>7.931</td>
</tr>
<tr>
<td></td>
<td>Co-culture with <em>L. fermentum</em></td>
<td>6.114</td>
<td>0.467</td>
<td>5.906</td>
</tr>
<tr>
<td></td>
<td><strong>p - value</strong></td>
<td><strong>&lt; 0.001</strong></td>
<td><strong>&lt; 0.001</strong></td>
<td><strong>&lt; 0.001</strong></td>
</tr>
<tr>
<td>HCT 15</td>
<td>Monoculture</td>
<td>8.051</td>
<td>0.368</td>
<td>8.239</td>
</tr>
<tr>
<td></td>
<td>Co-culture with <em>L. fermentum</em></td>
<td>5.857</td>
<td>0.462</td>
<td>5.831</td>
</tr>
<tr>
<td></td>
<td><strong>p - value</strong></td>
<td><strong>&lt; 0.001</strong></td>
<td><strong>&lt; 0.001</strong></td>
<td><strong>&lt; 0.001</strong></td>
</tr>
</tbody>
</table>

Table 1.8: Statistical table for adhesion inhibition in the presence of SAPs

<table>
<thead>
<tr>
<th>Adhesion (Log cfu/ml)</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Pair 1</td>
<td>Monoculture</td>
</tr>
<tr>
<td></td>
<td>with <em>L. fermentum</em> SAP</td>
</tr>
<tr>
<td></td>
<td><strong>p - value</strong></td>
</tr>
<tr>
<td>Pair 2</td>
<td>Monoculture</td>
</tr>
<tr>
<td></td>
<td>with <em>L. fermentum</em> SAP – Trypsin treated</td>
</tr>
<tr>
<td></td>
<td><strong>p - value</strong></td>
</tr>
</tbody>
</table>
Table 1.9: Statistical table for Inhibition of *in vitro* growth of *S. aureus* and *P. aeruginosa* by *L. fermentum* during co-culture.

\( n = 9 \)

<table>
<thead>
<tr>
<th>Time</th>
<th>Growth (Log cfu/ml)</th>
<th><em>S. aureus</em></th>
<th><em>P. aeruginosa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>24 h</td>
<td><em>L. fermentum</em> Monoculture</td>
<td>11.523</td>
<td>0.222</td>
</tr>
<tr>
<td></td>
<td><em>L. fermentum</em> Co-culture</td>
<td>11.174</td>
<td>0.348</td>
</tr>
<tr>
<td></td>
<td>p - value</td>
<td><strong>0.031</strong></td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td><em>L. fermentum</em> Monoculture</td>
<td>11.300</td>
<td>0.437</td>
</tr>
<tr>
<td></td>
<td><em>L. fermentum</em> Co-culture</td>
<td>10.269</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>p - value</td>
<td><strong>&lt; 0.001</strong></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>Pathogen Monoculture</td>
<td>11.302</td>
<td>0.712</td>
</tr>
<tr>
<td></td>
<td>Pathogen Co-culture</td>
<td>6.857</td>
<td>0.464</td>
</tr>
<tr>
<td></td>
<td>p - value</td>
<td><strong>&lt; 0.001</strong></td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>Pathogen Monoculture</td>
<td>9.166</td>
<td>0.479</td>
</tr>
<tr>
<td></td>
<td>Pathogen Co-culture</td>
<td>4.259</td>
<td>0.208</td>
</tr>
<tr>
<td></td>
<td>p - value</td>
<td><strong>&lt; 0.001</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.10: Statistical table for Inhibition of cytotoxic effect of *S. aureus* and *P. aeruginosa* CS in presence of *L. fermentum*.

n = 9

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cytotoxicity (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. aureus</em></td>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>HT 29</td>
<td>CS of pathogen</td>
<td>71.691</td>
<td>3.719</td>
<td>82.998</td>
<td>1.833</td>
</tr>
<tr>
<td></td>
<td>CS of pathogen grown with <em>L. fermentum</em> CS</td>
<td>79.069</td>
<td>4.413</td>
<td>86.055</td>
<td>2.266</td>
</tr>
<tr>
<td></td>
<td><em>p - value</em></td>
<td>0.001</td>
<td></td>
<td>0.265</td>
<td></td>
</tr>
<tr>
<td>INT 407</td>
<td>CS of pathogen</td>
<td>74.103</td>
<td>3.512</td>
<td>75.185</td>
<td>0.402</td>
</tr>
<tr>
<td></td>
<td>CS of pathogen grown with <em>L. fermentum</em> CS</td>
<td>91.216</td>
<td>3.675</td>
<td>101.099</td>
<td>2.112</td>
</tr>
<tr>
<td></td>
<td><em>p - value</em></td>
<td>&lt; 0.001</td>
<td></td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>HEK 293</td>
<td>CS of pathogen</td>
<td>78.273</td>
<td>4.767</td>
<td>64.475</td>
<td>1.493</td>
</tr>
<tr>
<td></td>
<td>CS of pathogen grown with <em>L. fermentum</em> CS</td>
<td>88.176</td>
<td>4.407</td>
<td>81.535</td>
<td>1.432</td>
</tr>
<tr>
<td></td>
<td><em>p - value</em></td>
<td>0.007</td>
<td></td>
<td>&lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>
1.4 CONCLUSIONS

- *L. fermentum* isolated from human colonic mucosa possess probiotic property.

- *L. fermentum* CS inhibits the growth and adhesion of pathogens like *E. coli*, *S. Paratyphi*, *S. sonnei*, *Vibrio sp.*

- Antimicrobial activity was due to heat labile, protease sensitive compound(s).

- The surface proteins of *L. fermentum* competes out the adhesion of other pathogens.

- *L. fermentum* CS inhibits the growth of nosocomial and opportunistic pathogens like *S. aureus* and *P. aeruginosa*.

- Biofilm formation by *S. aureus* and *P. aeruginosa* were inhibited in a dose dependent manner in the presence of *L. fermentum* CS.

- *L. fermentum* CS reduces the cytotoxicity of pathogens to HT-29, INT-407 and HEK-293 cells.

- Growth as well as biofilm formation of SCVs and clinical isolates of *S. aureus* and *P. aeruginosa* were inhibited by *L. fermentum* culture supernatant.

- The antimicrobial compound(s) present in *L. fermentum* culture supernatant needs to be identified and characterized so that it may be used as a successful antimicrobial chemotherapeutic agent.