Chapter 1

Introduction

1. Introduction

1.1. Probiotics

Lilly and Stillwell for the first time described the substances secreted by a type of microorganism to stimulate the growth of another: probiotic in contrast with antibiotic (Lilly & Stillwell, 1965). The term "probiotic" used subsequently for an organism which enhances human health and contributes towards intestinal microbial balance. The widely accepted definition of probiotics according to World Health Organization (WHO), is "Live organisms which, when administered in adequate amounts, confers a health benefit to the host". Probiotic strains are considered non-pathogenic, and its use in humans is considered safe.

The main health benefits of probiotics are the competitive exclusion of pathogens, colonisation of gut and strengthening of the epithelial barrier, stimulation of immune system, maintenance of gut peristalsis, inflammation control, anti-infection and antagonistic activities (Hill *et al.*, 2014). This health-promoting effects, however, varies amongst different probiotic strains and no particular strain possess all the functionalities (van Baarlen *et al.*, 2013). Microorganisms routinely used as probiotics are *Lactobacilli* and *Bifidobacteria* species along with few strains from the *Saccharomyces, Bacillus, Escherichia* and *Streptomyces* species.

1.2. The genus *Lactobacillus*

Lactobacilli are Gram-positive, anaerobic, non-sporogenous, fastidious, and strictly fermentative bacteria with low G+C content whose principal end product of sugar fermentation is lactic acid (Axelsson, 1998; Orla-Jensen, 1919). *Lactobacillus* genus comprises of more than 150 species consisting of a variety of organisms and belongs to Lactobacillales order, phylum Firmicutes and class Bacilli. *Lactobacillus* belongs to a heterologous group of bacteria known as Lactic Acid Bacteria (LAB). LAB are widely distributed in nature and share the ability to utilise nutritionally rich environments and subsequently converting the sugars into lactic acid through simple metabolic pathways (de Vos & Hugenholtz, 2004). Different species of *Lactobacillus* can be grown in a temperature range of 30 to 60°C, and can grow in a pH range between 3 and 8. However, optimal growth conditions are usually 30 to 40°C and pH of 5.5 to 6.2.

Lactobacilli are naturally persistence at mucosal surfaces, principally the

gastrointestinal tract, the vagina and the oral cavity. Based on the characteristics of metabolic products, *Lactobacilli* can divide into two main groups under the standard fermentation condition: homo-fermentative (producing more than 85% of lactic acid) and hetero-fermentative (producing lactic acid, CO2, ethanol and/or acetic acid) (Hammes, 1995; Pot B., 1994). *Lactobacillus* has requirement of complex nutrients such as carbohydrates, amino acids, peptides, salts, fatty acids and vitamins which reflects their habitats which are rich in carbohydrates containing substrates like dairy products, meat, fermented products, pickles, plants and grain related source (Tannock, 2004).

1.2.1. *Lactobacillus as probiotics*

Interest in LAB as health-promoting bacteria was raised early last century when Elie Metchnikoff suggested that the gut bacterial community was a source of toxic substances to the human host as they were capable of degrading proteins and releasing ammonia, amines, indoles, etc., which in turn were toxic to the human host. Since the lactic acid bacteria naturally fermented the milk and prevented the growth of non-acid tolerant bacteria, thus they were favoured as fermentative bacteria used to enrich the gut microbiota populations. Metchnikoff also proposed that the proteolytic ("putrefactive") bacteria of the healthy intestinal flora are damaging to human health and that adaptation of the intestinal flora by the consumption of LAB may contribute to the prolonging of life (Metchnikoff, 1907). Since then, the use of probiotics to enhance intestinal health has recommended for many years and as a consequence several probiotic strains of *Lactobacillus* spp. are currently popular as their consumption results in a health benefit to the host (Saxelin *et al.*, 2005). *Lactobacilli* strain identified as probiotics are *Lactobacillus acidophilus, Lactobacillus helveticus, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus paracasei, Lactobacillus casei, Lactobacillus reuteri, Lactobacillus delbrueckii, Lactobacillus sakei* and *Lactobacillus fermentum* (Lebeer *et al.*, 2008). The ever growing market and well-being for consumers have strongly encouraged molecular research into the biology and impending health beneficial effects of *Lactobacilli*.

1.2.2. Existence of *Lactobacillus* in human gastrointestinal tract

The human gastrointestinal microbiota exists in equilibrium between commensal and pathogenicity, nurtured by living and ingested microorganisms (Hooper & Gordon, 2001; Sonnenburg *et al.*, 2004). A metagenomics analysis of human gut microbiome revealed that the gastrointestinal tract (GIT) upholds a microbial population of about 10^{13}

- 10^{14} organisms, whose collective genome comprises no less than 100 times as many as genes of the human genome (Gill *et al.*, 2006). The colonisation of commensal microbes in human host starts as soon as the birth of a child and imparts lifelong benefits to the human host. In humans, the populations of microbes vary widely in different parts of GI tract (Figure 1.1) (Lepage *et al.*, 2013). The gut microbiota acts as a natural barrier against pathogens, foreign materials, and aids in the digestion of complex food components, stimulates hosts immune system and produces certain vitamins, enzymes and essential nutritional elements. The complex dietary requirement of huge gut microbiota residing in the human host gets accomplished by complex carbohydrates, proteins, fats from food and host secretions like mucus (Walter, 2008). The microbial population per gram of the GI tract varies from 10^2-10^4 cells in stomach and duodenum, 10^4-10^8 cells in small intestine and 10^{10}-10^{12} cells in the large intestine and colon (Gerritsen *et al.*, 2011).

1.2.3. Effect of *Lactobacillus* on human health

Through thousands years of evolution, there is a symbiotic relationship within the multiple species of bacteria in the human gut. However, some of those microorganisms colonise GIT and due to their pathogenic potential, result in different intestinal disorders (Kosiewicz *et al.*, 2011). Though the probiotics protect the gut against colonisation of pathogenic microbes by the direct mechanism of limiting nutrients or indirect mechanism of modulation of host immune system still some of the pathogens, have evolved to enhance their replication and affect healthy microbial community leading to growing risk of pathogen infections (Kamada *et al.*, 2013). There is always a tug of war between probiotic and pathogenic bacteria in human GIT wherein disruption in the healthy microbiota community leads to pathogenic infection and intestinal diseases (Figure 1.2).

Few of the clinical applications of probiotics include the prevention and treatment of gastrointestinal infections, inflammatory bowel diseases (IBD) and allergic diseases and usage as adjuvants in vaccination (Borchers *et al.*, 2009). Other roles of probiotics in human health include interaction with the immune system, production of antimicrobial substances, enrichment of the mucosal barrier function and competition with pathogens for adhesion binding sites (Turroni *et al.*, 2014; Ventura *et al.*, 2009). Apart from the GIT where the *Lactobacilli* dominates and exert most health modulating activities, few other strains of *Lactobacilli* have also shown to benefit the human host at other sites such as prevention and treatment of urogenital diseases and vaginosis in women (Beerepoot &

Geerlings, 2016). Also, they are found in the prevention of atopic illness and food hypersensitivity (Jeong *et al.*, 2015), and prevention of periodontal diseases and dental caries (Gungor *et al.*, 2015). However, it is worth mentioning that different species of *Lactobacillus* confers different

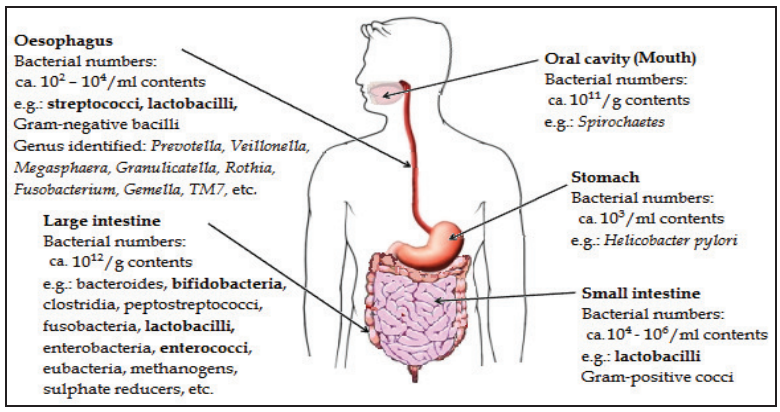

Figure 1.1: Microbiota composition Human Gastro-Intestinal tract (GIT) (Nditange Shigwedha & Jia, 2013). Source: (www.intechopen.com/source/html/42341/media/image1.png).

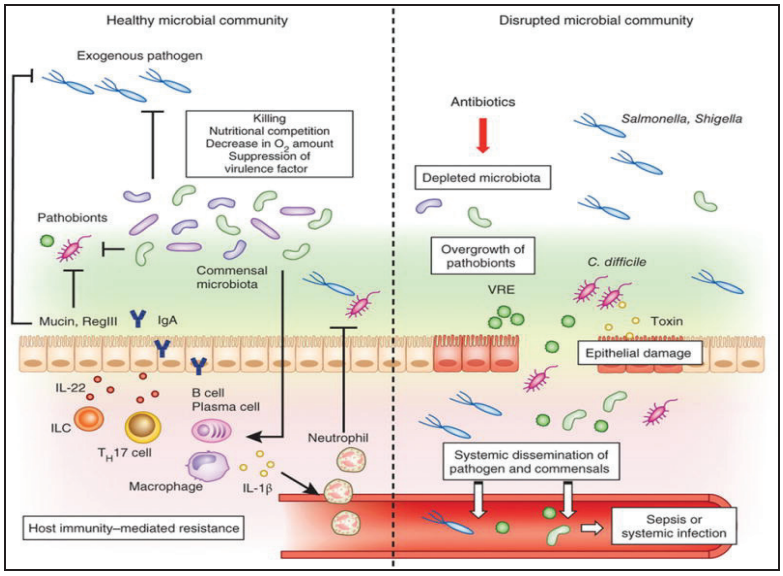

Figure 1.2: **Diagrammatic representation of healthy and disrupted commensal microbial community.** Source: (Kamada et al., 2013).

health benefits to host and also some strains of same species may not be beneficial (Sengupta et al., 2013). Table 1.1 summarise the positive effects of different strains of *Lactobacillus*.

Table 1.1: Positive benefits on human health by various strains of *Lactobacillus*.

Probiotic health effect	*Lactobacillus* species	Reference(s)
Antibiotic-associated diarrhoea & acute diarrhoea Acute gastroenteritis Ulcerative colitis	*L. casei DN114004,* *L. acidophilus* *L. acidophilus*, *L. rhamnosus, L. plantarum*, *L. casei*, *L. acidophilus*, *L. delbrueckii*	(Dietrich *et al.*, 2014; Pinto & Petrova, 2015) (Vandenplas *et al.*, 2011) (Venturi *et al.*, 1999)
Respiratory tract infections	*L. casei* shirota	(Shida *et al.*, 2015)
Colon cancer Colorectal cancer	*L. helveticus* *L. reuteri*, *L. acidophilus*, *L. rhamnosus*	(W. Li *et al.*, 2015) (Zhong *et al.*, 2014)
Urogenital disorders and bacterial vaginosis	*L. fermentum*, *L. plantarum*	(Ehrstrom *et al.*, 2010; Vicariotto *et al.*, 2014)
Cardiovascular diseases and hypercholesterolaemia	*L. plantarum* CECT *L. plantarum* 299v	(Fuentes *et al.*, 2013) (Naruszewicz *et al.*, 2002)
Antitoxicity Antioxidant Antibacterial and antiviral	*L. acidophilus*, *L. reuteri* *L. plantarum* *L. casei* shirota *L. acidophilus*	(Mechoud *et al.*, 2012) (S. Li *et al.*, 2012) (de Waard *et al.*, 2002) (Coconnier *et al.*, 2000)
Kidney diseases Liver disease Diabetes Osteoarthritis Periodontal disease and dental caries	*L. plantarum* *L. fermentum* *Lactobacillus GG* *L. casei* *L. salivarius WB21,* *L. reuteri*	(Bouhafs *et al.*, 2015) (Barone *et al.*, 2016) (Bajaj *et al.*, 2014) (So *et al.*, 2008) (Shimauchi *et al.*, 2008) (Twetman *et al.*, 2009)

1.2.4. General Mechanisms of Action of *Lactobacilli*

The modes of action by which probiotics contributes to human health fall into three broad categories (Lebeer *et al.*, 2008) (i) competitive inhibition of pathogen and maintaining microbial homoeostasis (ii) enhancement of epithelial barrier function and antimicrobial peptide production (iii) immune response modulation. The action of probiotics is graphically represented below (Figure 1.3).

Enhancement of epithelial barrier: The *Lactobacilli* are capable of modulating the epithelial barrier function by decreasing the apoptosis of cells and increasing the mucin

production. The primary purpose of this barrier is to maintain the integrity of epithelial regions as well as protect organs and organism (Bermudez-Brito *et al.*, 2012). The epithelial defence barrier consists of mucous layer, secretory IgA, antimicrobial peptides and epithelial junction adhesion complex. As soon as this barrier is disrupted the food and bacterial antigen along with toxins reaches submucosa and induces inflammatory responses and ultimately intestinal diseases (Ohland & Macnaughton, 2010). Probiotics are believed to induce the secretion of mucous which improves the barrier function and the limits the pathogens. *Lactobacillus* species are known to increase mucin production in human intestinal cell lines (Mattar *et al.*, 2002).

Production of Antimicrobial compounds: Probiotics either induces the host epithelial cells or directly releases antimicrobial peptides which intervene with the harmful pathogens. They suppress the colonisation of the pathogen through the release of antimicrobial factors like defensins, bacteriocins, H_2O_2, nitric oxides, lactic acids, acetic acids & short chain fatty acids (Lebeer *et al.*, 2008).

Competitive adherence and exclusion of pathogens: Probiotic organism mediates competitive exclusion by cell-cell interaction which ultimately results in competition for adhesion sites in the intestine and available nutrients. One such competitive exclusion example is of pathogen type I fimbriated *E. coli* which utilise oligosaccharide receptor sites in the GIT (Le Bouguenec, 2005). The study of mannose specific adhesion (*Msa*) of *L. plantarum* 299v and its deletion and overproduction effectively excludes pathogens with type I fimbriae. It is because *L. plantarum* 299v uses the same attachment site so that there is a competition for adherence in the host mucosal surface (Gross *et al.*, 2010; Pretzer *et al.*, 2005).

Modulation of host immune system: Probiotics with their immune-modulatory effects can interact with epithelial and dendritic cells along with monocytes, lymphocytes or macrophages. *Lactobacilli* have the capacity to bind to pattern recognition receptors (PRR) which are expressed on the immune cells which in turn recognises microbe-associated molecular patterns (MAMPs) and subsequently induce signals for production of cytokines, chemokines and other immune effectors (Wells, 2011).

Interference in the quorum sensing: Quorum sensing is a well-known mechanism by which bacteria communicates with each other in a niche using chemical signalling

molecules called autoinducers (Miller & Bassler, 2001). This mechanism facilitates the successful colonisation and infection in their host. Earlier it was demonstrated that *L. acidophilus* inhibits quorum sensing signalling by secreting a molecule which directly interacts with *E. coli O157* gene thus reducing colonisation (Medellin-Pena *et al.*, 2007).

1.3. Adhesion mechanisms of *Lactobacillus* in humans

The prerequisite step in bacterial colonisation is adhesion to host tissues as it promotes persistence time for colonisation which in turn modulates microbe-host interaction (Beachey, 1981). This persistence in GIT also promotes gut residence time; microbe-host cross talks through immunomodulation provides an epithelial barrier and antagonistic effects against pathogens (Servin, 2004). Thus, main criteria for selection of probiotic strains is adhesion. The microbe-host adhesion process is mediated through the electrostatic interactions, passive forces, steric forces, hydrophobic interactions, lipoteichoic acids and specific adhesins/lectins (Bermudez-Brito *et al.*, 2012). The role of surface proteins in adhesion has been proposed in several earlier studies (Rojas *et al.*, 2002; Tallon *et al.*, 2007). Although, while non-proteinaceous interactions also mediate adhesion (Granato *et al.*, 1999). In comparison to the current understanding of the adhesion mechanisms of human pathogenic bacteria, information on the surface molecules mediating *Lactobacillus* adhesion to the epithelial cells, mucus layer and/or extracellular matrices and their respective receptors is very preliminary.

The cell wall of *Lactobacilli* is composed of the lipid bilayer of the plasma membrane. The bacterial cell wall consists of a thick multilayer of peptidoglycan (PG) which consists of teichoic acids (WTA- Wall teichoic acids and LTA- Lipoteichoic acids), exopolysaccharides (EPS) and filamentous pili which are important factors of adhesion. Along with that the proteins embedded and anchored to the cell wall act as proteinaceous factors of adhesion (Figure 1.4). Few *Lactobacilli* species displays an additional para-crystalline layer of proteins around PG, known as the S-layer.

Different strains of *Lactobacillus* exhibits different adhesion properties due to the diversity in cell surface architecture and variation in the cell surface repertoire of proteins in response to the environment (Jacobsen *et al.*, 1999; Jonsson *et al.*, 2001; Taranto *et al.*, 2003). Several distinct bacterial cell surface components like adhesins, polysaccharides and surface proteins are responsible for microbe adherence and interaction with the host (Velez *et al.*, 2007). These cell surface components either act

individually or collectively in host-microbe interactions (Buck *et al.*, 2005; Granato *et al.*, 1999).

1.4. Other molecules mediated adhesion

Certain cell-wall associated factors have been earlier reported to be involved in adhesion of *Lactobacilli* to human host (Granato *et al.*, 1999). These include a thick multilayer of peptidoglycan (PG) which consists of teichoic acids (WTA- Wall teichoic acids and LTA- Lipoteichoic acids), exopolysaccharides (EPS) and filamentous pili.

1.4.1. Peptidoglycan layer

In bacteria, peptidoglycan (PG) is the largest component of cell wall found outside the cytoplasm of the cell whose primary function is to preserve cell integrity and protect bacteria cell wall from internal turgor pressure. PG also maintains the cell shape and act as a junction for covalent anchoring of other cell wall polymers, wall teichoic acids (WTA), Lipoteichoic acids (LTA), wall polysaccharides (WPS) and surface-anchored proteins (Delcour et al., 1999). It is linear polysaccharide chains of β-1, 4-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues cross-linked by two pentapeptide side chains. During the biosynthesis, assembly and incorporation of PG subunits, modifications in GlcNAc and MurNAc structures occurs which affects the interaction between *Lactobacilli* and host. One such example is the O-acetylation of the cell wall MurNAc residues in *L. casei* (Billot-Klein et al., 1997). Certain modifications affect the bacterial physiology like an increase in sensitivity to autolysis, resistance to lysozyme and helping to escape innate immunity, the hydrophobicity of cell envelope which finally affects the recognition by host receptors and bacterial adhesion to host (van Loosdrecht et al., 1987; Vollmer, 2008).

1.4.2. Teichoic acids

Teichoic acids (TA), a second major component of cell walls of *Lactobacilli* are anionic polymers made of repeating units of glycerol- or ribitol phosphate, covalently linked to peptidoglycan as WTA or attached to the cytoplasmic membrane through via lipid anchors as LTA (Neuhaus & Baddiley, 2003). The LTA also contributes to the anionic property to cell wall which in turn modulates adhesiveness of the cell wall (van Loosdrecht *et al.*, 1987). Earlier reports suggest that LTA is involved in biofilm formation of *Lactobacillus* strain on mouse gastric epithelium (Sherman & Savage, 1986). LTA binds a variety of molecules like eukaryotic target cells, membrane

phospholipids, CD14 and Toll-like receptors and studies in *L. johnsonii* NCC533 show that LTA is present on the cell surface and participates in the adhesion to Caco-2 cells (Granato *et al.*, 1999).

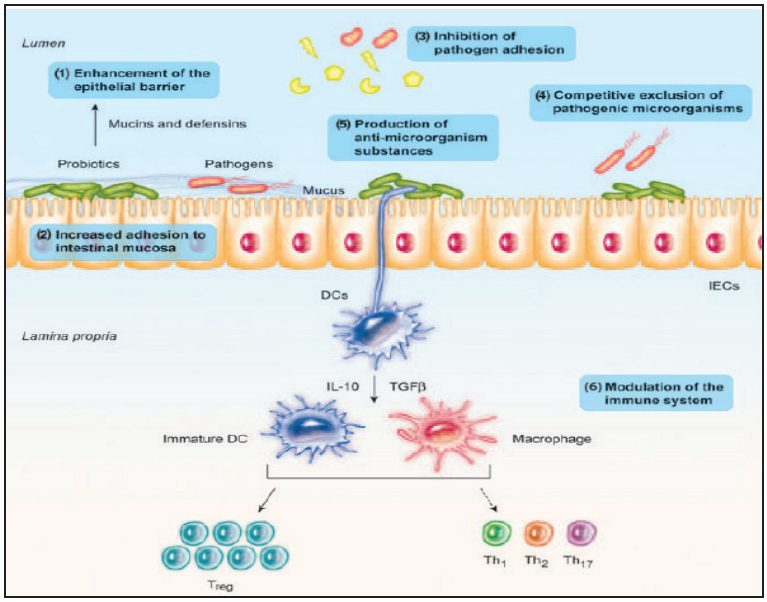

Figure 1.3: Major mode of actions of probiotics. Source: (Bermudez-Brito et al., 2012).

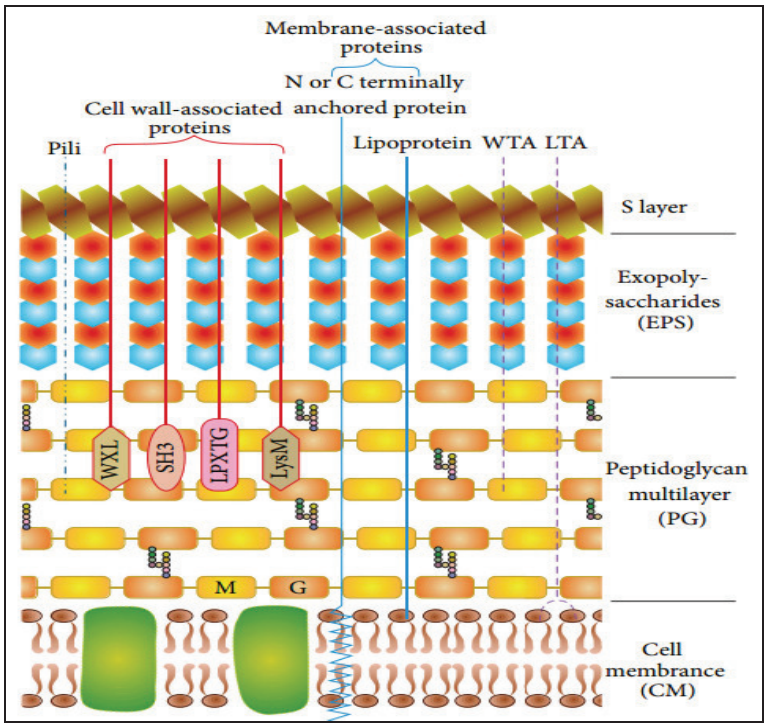

Figure 1.4: Cell surface of *Lactobacilli* with a schematic representation of cell-wall components and membrane-embedded proteins. Source: (Sengupta et al., 2013).

1.4.3. Exopolysaccharides

Exopolysaccharides (EPS) are long-chain polysaccharides composed of branched, repeating units of sugars or sugar derivatives loosely attached to the cell surface or secreted into the gut environment (Ruas-Madiedo & de los Reyes-Gavilan, 2005). They contribute to cell-surface physiochemical properties and blocking of other cell surface adhesins binding (Lorca *et al.*, 2002) and *L. rhamnosus* (Ruas-Madiedo *et al.*, 2006). Recent evidence suggests that EPS can have an influence on bacterial aggregation, biofilm formation, adhesion, and survival (Lebeer *et al.*, 2009). In *L. acidophilus* CRL639, adhesion to ECM components has been attributed to the production of different types of exopolysaccharides (Lorca *et al.*, 2002). Bacterial polysaccharides further divided into three classes: capsular polysaccharides (CPS), which is a thick outermost layer around the cell wall, cell wall polysaccharides (WPS) which are either covalently or loosely bound to the cell wall and extracellular polysaccharides (EPS) which get secreted into the medium. In *Lactobacilli*, the term EPS is preferred and relates to extracellular polysaccharides that are attached to the cell wall or gets secreted into the surroundings (Delcour *et al.*, 1999).

1.4.4. Pili and flagella

Pili are polymeric components of multi-subunit protein which are mainly characterised in *L. rhamnosus* GG (Kankainen *et al.*, 2009). The role of pili in immunomodulation, bacterial invasion and adhesion, biofilm formation and aggregation is well studied although receptors in the host which recognise pili are still unknown (Danne & Dramsi, 2012; Lebeer *et al.*, 2012). The flagellum consists of protein polymers known as flagellin, which is believed to act as a ligand and mediate signalling pathway activation and immunomodulation of host (Tallant *et al.*, 2004). Till date, twelve species of *Lactobacilli* have been recognised as motile species having flagella (Neville *et al.*, 2012).

1.5. Protein-mediated adhesion

As discussed earlier the cell wall of *Lactobacilli* play a crucial role in mediating host-microbe interaction as apart from its primary purpose, it also confers physical support for surface-associated proteins and provides attachment sites for certain exported proteins (Sanchez *et al.*, 2008). The protein which mediates the adhesion process from *Lactobacilli* cell surface further divided into the secreted proteins that detach from the bacterial cell into the external medium and surface-associated proteins. The surface

associated proteins divide into two broad categories (a) Covalently anchored and (b) Non-covalently anchored (Kleerebezem *et al.*, 2010) (Figure 1.5).

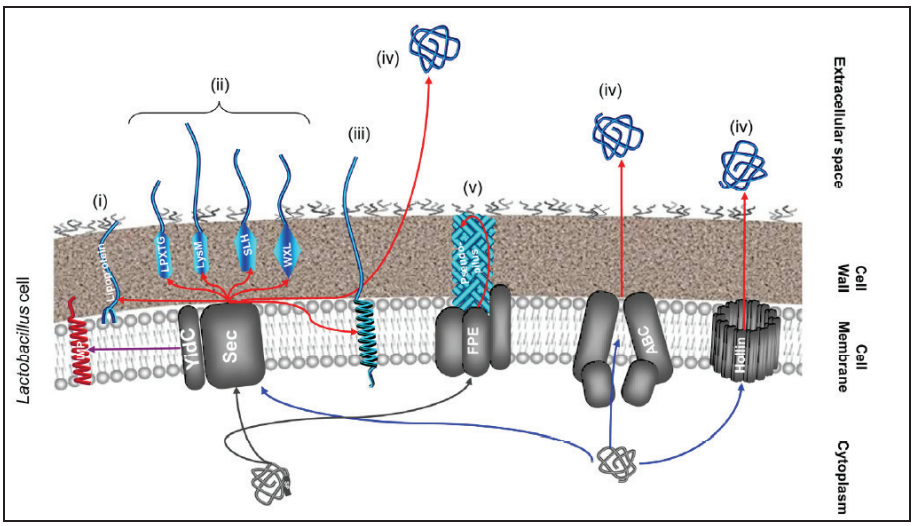

Figure 1.5: Schematic representation of the secretion systems and the surface associated proteins in *Lactobacillus*: (i) the proteins anchored to lipids in the CM (lipoproteins); (ii) attached to the CW either covalently (LPxTG proteins) or non-covalently (e.g. LysM, SLH or WXL domains/motifs); (iii) anchored to the CM via the N- or C- terminal TM helix; (iv) secretory proteins released via Sec, holin or ABC transporters; (v) being part of the competence pseudo-pili (assembled via FPE). Black arrows indicate the route of proteins to the CM and carrying the N-terminal SP, whereas blue arrow marks the route for proteins lacking such an SP. Red arrow indicates secreted proteins. Abbreviation: CM -Cell Membrane; CW -Cell Wall; TM -Transmembrane; SP -Signal Peptide. Source: (Kleerebezem *et al.*, 2010).

1.5.1. Covalently anchored surface proteins

The covalently anchored proteins consist of those proteins which are anchored into the cell membrane and classified as N-/C- terminally anchored proteins anchored via a single hydrophobic domain into the cytoplasmic membrane, Lipoproteins which are N-terminally lipid-anchored proteins and proteins anchored to peptidoglycan via C-terminal LPxTG motif (Kleerebezem *et al.*, 2010).

1.5.1.1. N- or C- terminally anchored proteins

The proteins which get secreted via Sec translocation pathway contains N-terminal signal peptide has N, H and C regions. After the translocation, the C region exposes to the extracytoplasmic side of membrane followed by the cleavage of signal peptide releasing the mature protein in extracellular space. However, many proteins do not possess the cleavage motif and remain N-terminally anchored (Zhou *et al.*, 2008). Such N-terminal protein constitutes the largest number of membrane-anchored proteins

in *Lactobacillus* genomes and contains typical extracellular domains or unique functionalities. These proteins are involved in extracellular functions such as transport, cell-envelope metabolism, protein turnover and adhesion. If the C region of signal peptide consists of the type-I signal site, the C-terminal transmembrane domain will anchor it within the cytoplasmic membrane. *Lactobacillus* genome consists of C-terminally anchored proteins and their function is not entirely known yet (Kleerebezem *et al.*, 2010).

1.5.1.2. Lipoproteins

Lipoproteins constitute the second largest components of the membrane-anchored group in the *Lactobacillus* genomes. They are usually transported via Sec pathway and possess signal peptide containing N, H and C domains. The C region which contains the lipobox motif [L-(A/S)-(A/G)-C] helps in anchoring the mature protein to the membrane via thioether linkage (Hutchings *et al.*, 2009). Lipoproteins from *Lactobacillus* mainly constitute the substrate binding proteins of ABC transporters, but few of them are also involved in antibiotic resistance, adhesion, protein secretion and folding (Kleerebezem *et al.*, 2010). It also is known that bacterial lipoproteins bind to TLR2 thus facilitating recognition in microbe-microbe communication and eventually adhesion (Lebeer *et al.*, 2008).

1.5.1.3. LPxTG-anchored protein

Some secreted proteins contain an LPxTG motif located in the C-terminal region. This motif is followed by a C-terminal membrane anchor domain which consists of a stretch of hydrophobic residues followed with a positively charged tail (Marraffini *et al.*, 2006). The Sortase (SrtA) enzyme recognises the LPxTG motif, which cleaves between the Thr and Gly residues thus finally link the exported protein covalently to the cell wall peptidoglycan layer and subsequently displays it on the microbial surface (Marraffini *et al.*, 2006). It is well-known fact that the *Lactobacillus* adhesion to mucus involves mucus binding protein (MubP) which has mucus binding domain as well as N-terminal signal peptide and C-terminal LPxTG motif. They are also known as sortase-dependent proteins. Studies indicate that the sortase-dependent proteins of *L. reuteri* 1063 (Roos & Jonsson, 2002), the MubP of *L. acidophilus* NCFM (Buck *et al.*, 2005) and the Msa of *L. plantarum* WCFS1 (Pretzer *et al.*, 2005) are identified as mucus adhesins having a role in adherence to collagen, fibronectin and mucin. A study predicted twelve extracellular proteins from *L. plantarum* WCFS as adhesion factors, and ten of them contained the

LPxTG motif (Boekhorst *et al.*, 2006).

1.5.2. Non-covalently anchored surface proteins

The Non-covalently anchored surface proteins are bound to the bacterial cell surface via binding domains. They are also found anchored to other proteins through protein-protein interactions while few are known to re-associate to the cell wall and polymers like teichoic acids and polysaccharides through electrostatic interactions (Avall-Jaaskelainen & Palva, 2005). The genomes of *Lactobacilli* contains many such proteins with specific domains such as LysM domain, choline-binding domain or YG repeats, WxL domain, SH3 domains and S-layer homology domain which keeps them anchored to the bacterial cell surface

1.5.2.1. LysM domain

The LysM domain module is a highly conserved carbohydrate binding module found in plants, viruses, bacteria, fungi and animals. LysM domain (PF01476) recognises polysaccharides having N-acetylglucosamine (GlcNAc) residues found in peptidoglycan layer of the cell membrane (Mesnage *et al.*, 2014). Surface proteins having LysM domain are also involved in adhesion recognition of host molecules. A putative adhesion gene is characterised from *L. plantarum* BFE 5092 which coded for LysM domain (Grimm *et al.*, 2011). Also, an autolysin from *Staphylococcus aureus* and *Staphylococcus epidermidis* having LysM domain is identified as an adhesin which binds to fibrinogen and fibronectin (Heilmann *et al.*, 2005; Heilmann *et al.*, 2003).

1.5.2.2. Choline-binding domains

The choline binding domain (PF01473) mainly found in extracellular enzymes and protein are conserved tandem repeats of glycine and aromatic acids (Y and G) in a stretch of 20 amino acids. They can bind choline residues of cell wall teichoic and lipoteichoic acids (LTA), thereby anchoring the protein to the cell surface (Wren, 1991). Proteins containing these Y-G repeats are known to involve in adhesion and recognises carbohydrate as a ligand (Giffard & Jacques, 1994; Kingston *et al.*, 2002). Pneumococcal surface protein PspC - a choline-binding protein when expressed heterologously on the surface of Lactococcus exhibits adhesive properties (Asmat *et al.*, 2012). A choline binding surface protein (Cbp) from *Streptococcus pneumonae* is reported to be a multifunctional protein involved in adherence and bacterial pathogenesis (Mann *et al.*, 2006).

1.5.2.3. Surface layer (S-layer) protein

Surface-layers (S-layers) proteins form a para-crystalline assembly of proteins/glycoproteins on the cell surface of bacteria and archaea which are primarily involved in mediating adhesion to host surfaces (Sleytr *et al.*, 2014). These proteins are highly basic (pI 9-10.4), with a molecular weight in the range of 25kDa to 71kDa in *Lactobacillus* (Avall-Jaaskelainen & Palva, 2005), while some bacterial species the molecular mass ranges up to 200kDa (Sara & Sleytr, 2000). S-layer proteins are often non-covalently linked with the cell wall polysaccharides, teichoic acids and EPS, thus providing structural stability as well as adhesion factor. S-layer proteins from prokaryotes are glycoproteins except in *Lactobacilli* where most of the S-layer proteins appear to be non-glycosylated (Lebeer *et al.*, 2008). The S-layer protein domains responsible for non-covalent anchoring to the cell wall is S_layer_C (PF05124), S_layer_N (PF05123), SLAP (PF03217) and SLH (PF00395) in the Pfam database.

The primary function of S-layer proteins is to act as adhesins to epithelial cells, mucus and extracellular matrix proteins. Role of S-layer proteins of *L. acidophilus* (SlpA), *L. crispatus* (CbsA), and *L. brevis* (SlpA) in adhesion has validated experimentally, and they are also thought to act in competitive exclusion by inhibiting adhesiveness of pathogenic microorganism and thus contributing to probiotic effect (Avall-Jaaskelainen *et al.*, 2003; Chen *et al.*, 2007; Kos *et al.*, 2003). The deletion of the S-layer gene (SlpA) from *L. acidophilus* NCFM results in 84% reduction of the bacterial adherence to human intestinal epithelial cell line, with an assumption of the loss of multiple surface proteins embedded in the S-layer (Buck *et al.*, 2005). Functions of S-layer proteins in *Lactobacilli* are not species but strain specific as a different type of S-layers have various roles in adhesion, pathogen exclusion and aggregation.

Apart from S-layer protein in the interaction of *Lactobacilli* with other microorganisms, evidence shows that they also influence the microbial community development as biofilms. The bacterial aggregation directly impacts the biofilms development, and the subsequent removal of S-layer reduces the aggregation in *L. acidophilus*, *L. kefir*, and *L. crispatus* which suggests their functional involvement in biofilm development process (Golowczyc *et al.*, 2007; Kos *et al.*, 2003; Wasko *et al.*, 2014). Also, evidence shows that S-layer possesses lectin-like activity by interacting with glycoproteins and polysaccharides on the surface of another microorganism (Golowczyc *et al.*, 2009).

1.6. A review of well-known adhesins from bacteria

The proteins mediating adhesion are known as adhesins which allow bacteria to attach or adhere to other cells and surfaces. It is the most important step for colonisation of the microbial community in the host which further contributes to bacterial pathogenesis.

1.6.1. Adhesins from Gram-negative bacteria

Gram-negative bacteria comprises of two main class of adhesins i.e. fimbrial and non-fimbrial adhesins out of which the fimbrial adhesins are more common in gram-negative bacteria and most of these binds to glycoproteins/ glycostructures on host cells (Figure 1.6) (Soto & Hultgren, 1999).

1.6.1.1. Fimbrial adhesins/ Pili

Pilus or fimbria which is hair-like nanofibers is most well-known proteinous adhesins of gram-negative bacteria. Several kinds of protein subunits form the pili complex along with a single protein subunit called a major pilin which is arranged into a helix to form a long rod-shaped body (Soto & Hultgren, 1999). More than one type of pili is found in bacterial species which are further divided into subcategories based on secretion and assembly processes. The chaperone-usher pili or CUP, type IV pili- general secretion pathway, the alternative chaperone-usher pathway pili or CS1 pili; and curli pili (assembled by the extracellular nucleation-precipitation pathway) (Berne *et al.*, 2015) (Figure 1.7).

The chaperone-usher pili - They are more commonly known as P pili and Type I pili which are found mainly in uropathogenic *E. coli* (UPEC). They are controlled by two different operons i.e. Fim operon encoding the type I pili which express mannose-sensitive hemagglutination and the Pap operon which encodes P- or Pap-pili which interacts with di-galactoside in P-blood group antigen (Kline *et al.*, 2009). The P pili bind through PapG adhesin to the α-D-galactopyranosyl-(1-4)-β-D-galactopyranoside of urinary tract cells and whereas Type I pili has FimH as adhesin which recognises mono-mannose and tri-mannose containing glycoprotein receptors (Hultgren *et al.*, 1991; Pizarro-Cerda & Cossart, 2006). Also, a family of Afa/Dr adhesins secreted by chaperone/usher pathway are known to bind Dr-blood group antigen, type IV collagen and integrins (Servin, 2005).

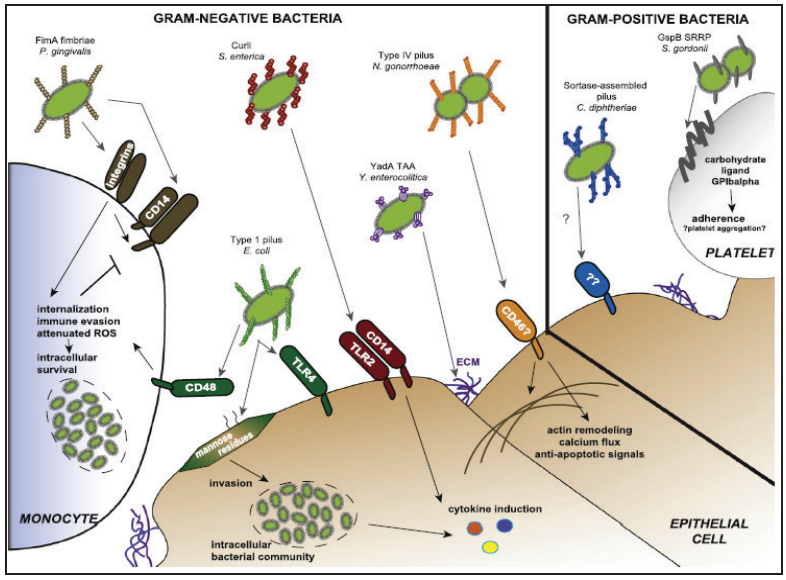

Figure 1.6: Bacterial adhesins and their host target. Source: (Kline et al., 2009).

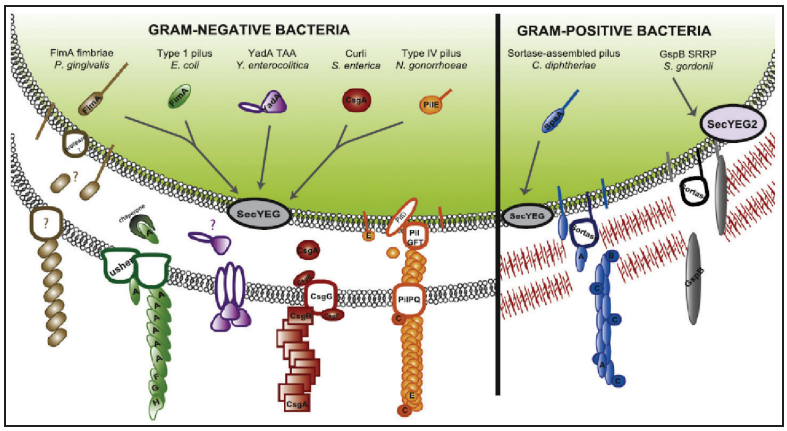

Figure 1.7: Schematic representation of Bacterial Pili or Pilus-like structure, assembly and their secretion. The bacterial cell is shown in green colour with its respective structural subunits. Source: (Kline *et al.*, 2009).

The Type IV pili - The type IV pili are composed of a homopolymer of single subunit 15-20 kDa pilin e.g. PilE and PilC in *Neisseria* spp., PilA in *Pseudomonas aeruginosa*, and TcpA in *Vibrio cholerae*. Type IV pili of *P. aeruginosa* is the primary adhesin which recognises the disaccharide β-GalNAc (1-4) βGal on epithelial cells asialo-GM1 and -GM2 gangliosides (Suh *et al.*, 2001).

The alternative chaperone-usher pathway pili (CS1 pili) - This type of pili includes several coli surface antigens (CS) of enterotoxic *E. coli* (ETEC) which are responsible for biofilm formation and intestinal epithelium colonisation (Liaqat & Sakellaris, 2012).

Another known type is the cable pili of *Burkholderia cepacia* which facilitate bacterial aggregation and its association with mucin (Ammendolia *et al.*, 2010).

Curli Pili - Curli is thin fimbrial adhesins (6-12nm) which get assembled via the nucleation-precipitation pathway and present in wide variety of bacteria (Berne *et al.*, 2015). Best known curli fimbriae are associated with initial adherence and subsequent biofilm formation in *E. coli* producing shigatoxin (Cookson *et al.*, 2002), gastrointestinal commensal *E. coli* (Bokranz *et al.*, 2005) and enterohemorrhagic *E. coli* (EHEC) (Saldana *et al.*, 2009).

1.6.1.2. Non-fimbrial adhesins

The non-fimbrial adhesins are non-polymeric, short mono- or oligomers of a single protein structure widely found in bacteria which may be cell-associated or secreted. They are mostly involved in cell-cell attachment of host cells and extracellular matrices such as collagens, mucin, laminin, hyaluronan and proteoglycans (Berne *et al.*, 2015). Non-fimbrial adhesins are covalently or non-covalently anchored to the outer cell membrane and involved in cell-cell interactions and aggregation. Aso known to interact with components of biofilm ECM which helps to link the bacteria to the matrix and maintains the architect of biofilm (Berne *et al.*, 2015). Non-fimbrial adhesins in Gram-negative bacteria are usually of two types: the adhesins secreted through a type 1 secretion system (T1SS) and the adhesins secreted through one of the type 5 secretion system (T5SS).

A well-studied non-fimbrial adhesin secreted by T1SS is Bap (Biofilm-associated protein) family of proteins, first identified in *S. aureus* (Cucarella *et al.*, 2001) and since then numerous other such proteins involved in cell adhesion and biofilm formation has been identified (Lasa & Penades, 2006). Protein from *Pseudomonas fluorescence* LapA (Large adhesion protein A) is known to play a crucial role in biofilm formation (Hinsa *et al.*, 2003). *Salmonella enterica* genome which encodes two T1SS secreted adhesins BapA and SiiE, of which SiiE is 595kDa and binds to glycostructures on the cell surface (Gerlach *et al.*, 2007; Latasa *et al.*, 2005). Two adhesins BapA and SabA from *Helicobacter pylori* are involved in binding with Lewis B antigens and sialic acid respectively (Walz *et al.*, 2009).

The other type of non-fimbrial adhesins secreted by T5SS also termed as adhesins

secreted by autotransporter pathway (Linke *et al.*, 2006). One of the best-characterized protein is *Yersinia* adhesin YadA which is also called as a trimeric autotransporter adhesin (TAA) (Nummelin *et al.*, 2004). YadA is similar to BadA from *Bartonella henselae* which binds to ECM proteins like fibronectin and collagen (Riess *et al.*, 2004).

1.6.2. Adhesins from gram-positive bacteria

Well-studied adhesive proteins from gram-positive bacteria are MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules) which gets covalently linked to cell wall peptidoglycan and interacts with extracellular matrix proteins like fibrinogen, collagen, fibronectin, vitronectin, etc. (Patti *et al.*, 1994). Discussed below are the major categories of gram-positive adhesins are collagen binding adhesins, fibrinogen binding adhesins, fibronectin binding adhesins and gram positive pilins.

1.6.2.1. Gram-positive pilins

First, Gram-positive pili were identified in *Corynebacterium renale* (Yanagawa *et al.*, 1968) and since then pili have been discovered in many other gram-positive bacteria including *Runimococcus*, *Enterococcus*, *Actinomyces*, *Clostridium* and *Streptococcus* (Telford *et al.*, 2006). Unlike pili in gram-negative bacteria, the gram-positive bacteria pili are formed by covalent polymerization of pilin subunits in cell wall polysaccharides with the help of sortase enzymes. The role of sortase-assembled pili of Gram-positive bacteria in pathogenesis and interaction with host cells is limited (Kline *et al.*, 2009).

1.6.2.2. Collagen binding adhesins

Collagen as one of the most abundant structural proteins components of extracellular matrix proteins (ECM), participates in cell attachment, differentiation and migration. Similarly, both eukaryotes and prokaryotes express collagen-binding adhesins which recognise collagen (Vengadesan & Narayana, 2011). The first detailed structural aspect is obtained from the structure of a collagen bound eukaryotic protein complex is the I-domain of integrin α2β1 (Emsley *et al.*, 2000).

1.6.2.3. Fibronectin binding adhesin

Fibronectin (Fn) is a ~440 kDa glycoprotein found in the ECM of host and is involved mainly in cell adhesion, growth, migration, tissue repair and blood clotting.

Staphylococci and S*treptococci* have a vast repertoire of adhesin which targets Fn using the D repeats in their C-terminal region. The major adhesins SfbI of *S. pyogenes* and FnBP-A of *S. aureus* bind ECM-associated fibronectin are such examples (Joh *et al.*, 1999; Ozeri *et al.*, 2001).

1.7. Role of adhesins/lectins in *Lactobacillus* adhesion to host tissues and ECM.

The gram-positive bacteria colonizing the GIT expresses structures called adhesins embedded on cell wall or gets secreted, mediates adherence to host epithelial cells receptors, extracellular matrix proteins like fibronectin, laminin, collagen etc., mucus layer and oligosaccharides of glycoproteins (Antikainen *et al.*, 2002; de Leeuw *et al.*, 2006; Mukai *et al.*, 1992). *Lactobacillus* have more than one adhesin, and the interplay and expression of adhesins are regulated by bacteria depending on the external environmental conditions such as shown in *L. brevis* for S-layer protein (Jakava-Viljanen *et al.*, 2002). *Lactobacilli* adhesins molecules can broadly classify according to their targets in the intestinal mucosa (i.e. mucus components, extracellular matrices), to their localisation in the bacterial surface (i.e. surface layer proteins), and/or to the way they anchor to the bacterial surface (i.e. sortase-dependent proteins). The important surface and adhesin proteins are discussed further below.

1.7.1. Mucus binding proteins

The host epithelial cells of the intestine secrete a protective layer of mucus, which is mainly composed of glycolipids and a complex mixture of large and highly glycosylated proteins or mucins as the main components (Hansson, 2012). Thus, mucus binding by *Lactobacillus* has a significant role in colonisation which also provides nutrition and habitat (Kirjavainen *et al.*, 1998; Ouwehand *et al.*, 2001). *Lactobacilli* protein which primarily adheres to mucus is mucus-binding proteins (MubP). Earlier known MubP adhesins are the MubP of *L. reuteri* 1063 (Roos & Jonsson, 2002), the MubP proteins of *L.* acidophilus NCFM (Buck *et al.*, 2005) and the lectin-like mannose-specific adhesin (Msa) of *L. plantarum* WCFS1 (Pretzer *et al.*, 2005). This three mucus binding protein shares a similar domain that has also been identified abundantly in several species of LAB that inhibit the GIT, and the Mub repeat domain may be an evolutionary adaption for its survival and persistence in GI tract (Sengupta *et al.*, 2013). The protein adhering to mucus has shared features like presence of signal peptide, a C-terminal cell wall anchoring motif (LPxTG), the occurrence of repeated domains with adhesion function and few with unknown function (Navarre & Schneewind, 1999).

Evidence shows that fucose, as well as glycoprotein fetuin and asialofetuin, inhibits the binding of MubP to mucus, suggesting lectin-like interaction mechanism (Roos & Jonsson, 2002).

1.7.2. Adhesins binding the extracellular matrix

The extracellular matrix (ECM) is a complex structure layer surrounding epithelial cells and composed of complex glycoproteins such as laminin, collagen, mucin, heparin and fibronectin. When the mucus layer is damaged, this layer gets exposed allowing pathogenic microbial colonisation and infection (Styriak *et al.*, 2003). Some *Lactobacilli* with an ability to adhere to these ECM proteins could potentially compete and block the adhesion of pathogens by occupying the same binding sites mostly carbohydrate binding specificities in the gut and help in maintaining the intestinal harmony (Lorca *et al.*, 2002; Neeser *et al.*, 2000).

An earlier report indicated that various strains of *L. acidophilus*, *L. helveticus*, *L. reuteri*, *L. gasseri*, *L. delbrueckii*, *L. fermentum*, *L. casei*, *L. rhamnosus*, and *L. paracasei* binds to both collagen and fibronectin, though a molecular determinant for binding was not identified (Lorca *et al.*, 2002). Well identified adhesins binding to ECM are the fibronectin-binding protein (FbpA) of *L. acidophilus* NCFM (Buck *et al.*, 2005), the collagen-binding protein (CnBP) of *L. reuteri* NCIB11951 (Aleljung *et al.*, 1994), the collagen binding S-layer proteins of *L. crispatus* (CbsA) (Antikainen *et al.*, 2002), and S-layer protein of *L. brevis* ATCC 8287 (SlpA) (Hynonen *et al.*, 2002).

1.7.3. Anchorless adhesins

Few proteins contribute to the adhesion of *Lactobacilli* to the human intestinal mucosa and ECM proteins, but they do not belong to the above-discussed family of adhesins. The majority of the anchorless adhesins are intracellular proteins and when on the cell surface, they function in binding to ECM, as an adhesin to recognise to the host cells, or as cell surface receptor. Such intracellular proteins often termed as "Moonlighting protein" (Jeffery, 1999) which binds to structural components of the host ECM like fibronectin, mucin, collagen, laminin, etc. This recognition and interactions help bacteria to physical adhere to the host which is a crucial step for colonisation for a pathogen or a commensal gut bacteria. A well-known characterised elongation factor (EF-Tu) is shown to be cell surface-associated in *L. johnsonii* NCC533 as well as unbound in the cytoplasm, and it mediates the adhesion of Caco-2 cells and mucins

(Granato *et al.*, 2004). EF-Tu is found to be localised at the cell surface although it lacks signal sequence, cell anchoring motif or transmembrane domain. Adherence of EF-Tu to cells and mucus was found to be pH dependent as seen in other mucus-binding proteins (Granato *et al.*, 2004; Roos & Jonsson, 2002).

Also, another protein from *L. johnsonii* NCC533, GroEL, was recently shown to bind undifferentiated HT29 cells and mucin. Like EF-Tu, GroEL also lacks signal motif or cell anchoring domain though detected on the surface of La1 by a whole-cell enzyme-linked immunoassay, as well as it is detectable in the spent culture medium. *E. coli* expressed recombinant La1 GroEL is shown to be capable of attaching to the mucus as well as to the HT29 cell line (Bergonzelli *et al.*, 2006).

Other prominent examples of this category of proteins include proteins such as GAPDH-glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate, aldolase, enolase, other metabolic enzymes such as succinyl-CoA synthase subunit and chaperones like DnaK identified from multiple species like *Staphylococcus*, *Streptococcus*, *Helicobacter pylori*, and *Mycobacterium tuberculosis* (Henderson & Martin, 2011).

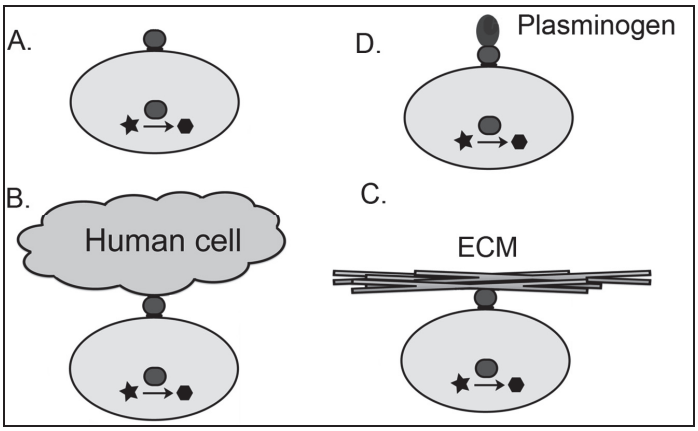

Figure 1.8: Schematic representation of anchorless adhesins: Proteins such as intracellular enzymes, chaperones, and other proteins are being found on the cell surface performing other functions: (A) an intracellular protein inside the cell converts a substrate (star) to a product (hexagon), is also found on the cell surface; (B) Few of these proteins moonlight as adhesins and binds surface protein on the host cell; (C) or as adhesins bind with the extracellular matrix (ECM), playing a role in infection and virulence; (D) few of the proteins bind to the plasminogen and converts in to a protease called plasmin which then aide to degrade and invade host tissues. Source: (Amblee & Jeffery, 2015).

1.8. Rationale behind the current study

It is reasonably well-documented that persistence of *Lactobacilli* in human host has a significant health promoting effect. The *Lactobacilli* and human host interaction are of great importance as it is a principal host-microbe interaction. The colonisation of *Lactobacilli* in human host starts right from birth and changes throughout the life span with response to the environment, food habits and health of an individual. The prerequisite step in bacterial colonisation is adhesion to host tissues as it promotes persistence time for colonisation which in turn modulates microbe-host interaction by promoting gut residence time, microbe-host cross talks through immunomodulation, providing an epithelial barrier and antagonistic effects against pathogens (Servin, 2004). Thus, primary criteria for selection of probiotic strains is adhesion.

The adhesion mechanism and factors are well documented, and numerous adhesins have been identified across the different strains of *Lactobacilli* (Velez *et al.*, 2007). Though most of the work on adhesins of *Lactobacilli* focused on its identification and microbiological point of view, the molecular and structural characterization insights are limited. Till date, the presence of adhesins in various species and strains are reported. Though the targets of this adhesins in human host are broadly characterised such as mucus and ECM, their molecular determinant is yet to explored. Though these factors and their adhesion mechanism have earlier described to a considerable extent in some pathogens, information on the surface molecules mediating *Lactobacillus* adhesion to the epithelial cells, mucus layer and/or extracellular matrices and their respective receptors is very preliminary.

The overall aim of this research project is to gain structural insight into mucus adhesins of *Lactobacillus*, to investigate their adhesive properties, and to functionally and structurally characterise them. Also, the involvement of other surface proteins, lectins and adhesins in *Lactobacilli* adhesions to GI tract are shown by previous studies, but detailed structural and mechanistic characterization is yet unexplored. In the present study, we are attempting to have a better understanding of the mechanisms of these targets through structural insights and biochemical & biophysical characterization.

1.9. Aims and objectives of the present study

In the present study, we will be investigating the genome of *Lactobacillus* species to identify new adhesins and characterise the known adhesins though structural and functional studies. The primary objectives of the current study will be

i. **Bioinformatics analysis**

ii. **Cloning, expression & purification of important candidates**

iii. **Biochemical and biophysical characterization**

iv. **Crystallisation and structure solution**