Chapter 1

Review of literature

"It is the mark of an educated mind to be able to entertain a thought without accepting it"

-Aristotle

Chapter 1

Review of Literature

1.1 Introduction

The journey of a human with microbes begin with the birth of an individual. Indeed, the number of microbial cells harboured by an adult human being is ten times more than the total number of human cells. The sterile gastrointestinal tract (GIT) of a born infant is colonized by microbes from the feeding of maternal milk. The human GIT comprises the highest density of the microbial population with approximately 1000-1150 different bacterial species, mostly including Gram-positive bacteria (Frank et al., 2007; J. Qin et al., 2010). However, the exact size and diversity of the gut microbiota are still unknown. The composition of gut microbiota depends upon the physiological conditions of GIT and is highly variable throughout the gut. Since bacteria have different adherence abilities with the mucosal surface, those with very firm adherence to the mucosa may tend to miss out during the faecal sample examination for gut microbial diversity. Also, the diversity studies based on the 16S-rDNA sequencing have revealed the presence of unculturable bacteria in high numbers (Eckburg et al., 2005). The microbiota composition in humans is majorly affected by various factors including age, socio-economic conditions, diet and consumption of antibiotics (Quigley, 2010). The gut microbiota of an infant is highly variable and unstable over a period of time. Several reports suggest the prevalence of bifidobacteria in the GIT of 17-20 week infants who are on breast-feed (Hopkins et al., 2005). In contrast, the formula-fed infants have higher abundance of aerobic bacteria and lower abundance of bifidobacteria in the GI tract compared to breast-fed infants (Balmer and Wharton, 1989; Hopkins et al., 2005).

However, by the end of the first year of life, the GI microbiota composition is almost similar to that found in the adult human gut with the dominant group of microbiota being conserved between all individuals (Spor et al., 2011).

The development of a human is dependent on both the genes they inherent and the interaction with the microbes. Both the host and the microbiota adapt to each other to maintain the mutualism and get benefited. The gut microbiota provides the host with essential nutrients, defend against colonization of opportunistic pathogens as well as foreign pathogens and thus protect the host from various infections, metabolize indigestible compounds and help in the development and maturation of the immune system. On the contrary, the microbes get nutrition and a stable environment in the gut. The imbalanced homeostasis leads to overgrowth of different subdominant opportunistic pathogens causing illness. Furthermore, the depletion of commensal microbiota causes unusual health condition since the host is deprived of the various benefits provided by this microbiota rather than the overpopulation of pathobionts (Martín R. et al., 2013). This explains the application of gut microbiota as a breakthrough for the prophylactic treatment of opportunistic infection, along with a contribution towards protection against many intestinal diseases. The bacteria providing specific health benefits upon consumption either as a food component or supplement are called probiotics.

1.2 History of probiotics:

Probiotics have a long history. The concept of functional food as medicines was first conceived by Hippocrates, the Greek philosopher and father of medicine, who once wrote:' Let food be thy medicine, and let medicine be thy food'. Over 2000 years ago, the first consumption of bacterial drinks by humans was recorded. Originated from the

Greek word, "Probiotic" translated as 'for life'. The scientific basis of the beneficial effect of probiotics was first put out by Elie Metchnikoff in 1908 (Metchnikoff E., 1908). While working at the Pasteur Institute in Paris, Metchnikoff hypothesized that the consumption of 'Soured milk' could reverse the adverse effects on the human health caused by the microflora of lower gut. This concept had originated from his knowledge of the Bulgarian farmers, who lived a ripe old age and ingested large amounts of soured milk. The sour milk or fermented milk is now called yogurt. The original strain he was working initially with was called the 'Bulgarian bacillus', which was later named as *Lactobacillus bulgaricus.* In his book published in 1907, entitled '*Essais Optimistes*', he mentioned the beneficial effects of fermented milk based on his and other people's findings. This concept was further supported by Louden Douglas in his book '*The bacillus of Long Life'*, published in 1911, where he restated the connection between consumption of fermented milk and longevity (Fuller, 1992). An independent discovery of a French paediatrician, Henry Tissier, regarding the role of bifidobacteria in breast-fed infants also played a key role in establishing the concept that specific bacteria take part in maintaining health. He observed a low number of peculiar, Y shaped bacteria in the stool of children suffering from diarrhoea compared to the healthy children and further exhibited the benefits of gut flora modulation in infants with intestinal infection (Tissier, 1906). At that point of time, people were still doubting the application of bacterial therapy and also questioned the survival of bacteria present in the yogurt in intestinal transit, reaching gut and conveying benefits to the host (Kulp and Rettger, 1924). In 1920s, Rettger and his colleagues used *L. acidophillus*, isolated from human intestine, as the dietary supplement since they observed no survival of 'Bulgarian bacillus' in the human gut (Rettger and Cheplin, 1921). So interest was diverted from 'Bulgarian bacillus' to the application of intestinal isolates. Later on, Regger and his

colleagues used *L. acidophillus* in the clinical trials to treat the patients with chronic constipation (Rettger, Levy, Weinstein, & Weiss, 1935). In 1917, during the world war, there was an outbreak of shigellosis and no remedies were available for treatment. At that time, a German Professor Alfred Nissle used *Escherichia coli*, isolated from feces of a healthy soldier, to treat patients suffering from salmonellosis and shigellosis. The strain was later known as *E.coli* Nissle 1917 (Nissle, 1918). In the early 1930s, in Japan, Shirota and his group focused on the isolation of intestinal strains which could survive in the gut. He wanted to distribute milk fermented using such strains in his clinic. The strain he used was *L. acidophilus* Shirota (later named *L. casei* Shirota) which was marketed later by Yakult Honsha Company as a fermented product (C. R. Soccol et al., 2013). In 1965, the term 'Probiotics' was first time used to represent the substances secreted by one microorganism that stimulates the growth of another (Lilly and Stillwell, 1965). Later, in 1974, Parker used the term 'Probiotics' to define "the organisms and substances which contribute to intestinal microbial balance". In 1989, Fuller defined probiotics as 'live microbial supplements which beneficially affects the host animal by improving its microbial balance'. Salminen et al., (1998) described probiotics as 'foods containing live bacteria which are beneficial to health'. Health beneficial functions of intestinal microflora only became clear at the end of the twentieth century (Guarner and Malagelada, 2003). Various health benefits exhibited by intestinal flora are described in table 1.1. Most commonly used yogurt starter culture, *Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus* has limited health benefits and moreover, they are not natural inhabitant of intestine. Therefore, in preparation of yogurt, incorporation of the probiotic strains such *as L. acidophilus, L. casei* and *Bifidobacterium* with well documented health benefits, is necessary to consider it as a probiotic product (Shah, 2007).

Table 1. 1 Health beneficial functions of intestinal microflora.

Metabolic	Trophic	Protective
• Production of vitamins • energy saving by providing short chain fatty acids • fermentation of non-digestible food residues • absorption of ions.	• development and homeostasis of the immune system • control of intestinal epithelial cell proliferation	• antagonistic effect on pathogens to prevent infections • protection against epithelial barrier dysfunction

1.3 Selection of Probiotics

Screening for potential probiotic strains includes various *in vitro* tests as practised by researchers in the field of probiotics. Determination of its taxonomic classification is the very first step in the selection process. It provides the information regarding origin, habitat, and physiology of the strain, which have important consequences on the selection of the novel strain (Morelli, 20

07). Due to an uncertainty of the origin of microorganisms, specificity of probiotic action is given more important over the source of probiotic bacteria (FAO/WHO, 2002). However, although many bacteria meet the basic criteria to be known as a probiotic, an ideal probiotic strain remains to be identified for any given indication. Moreover, a single probiotic bacterial strain will never be suited to all indications; selection of strains for disease-specific indications will be required (Shanahan, 2003). Key criteria for selection of a probiotic candidate for commercial application are described below in figure 1.1. The major and cardinal steps for selecting a novel probiotic bacteria are indicated in figure 1.2.

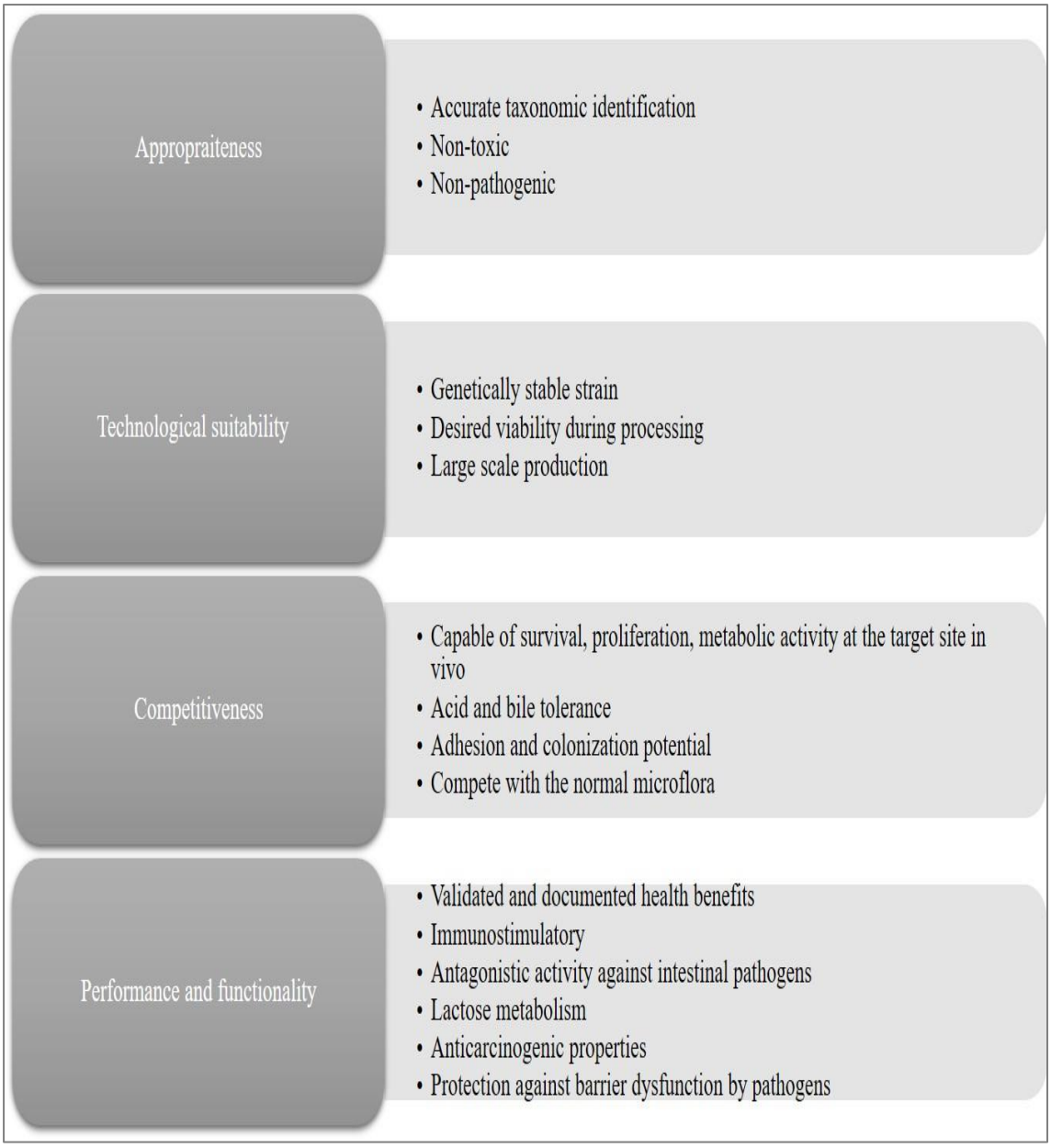

Figure 1. 1 Key and desirable criteria for selection of probiotic candidate.
(adopted from Klaenhammer and Kullen, 1999).

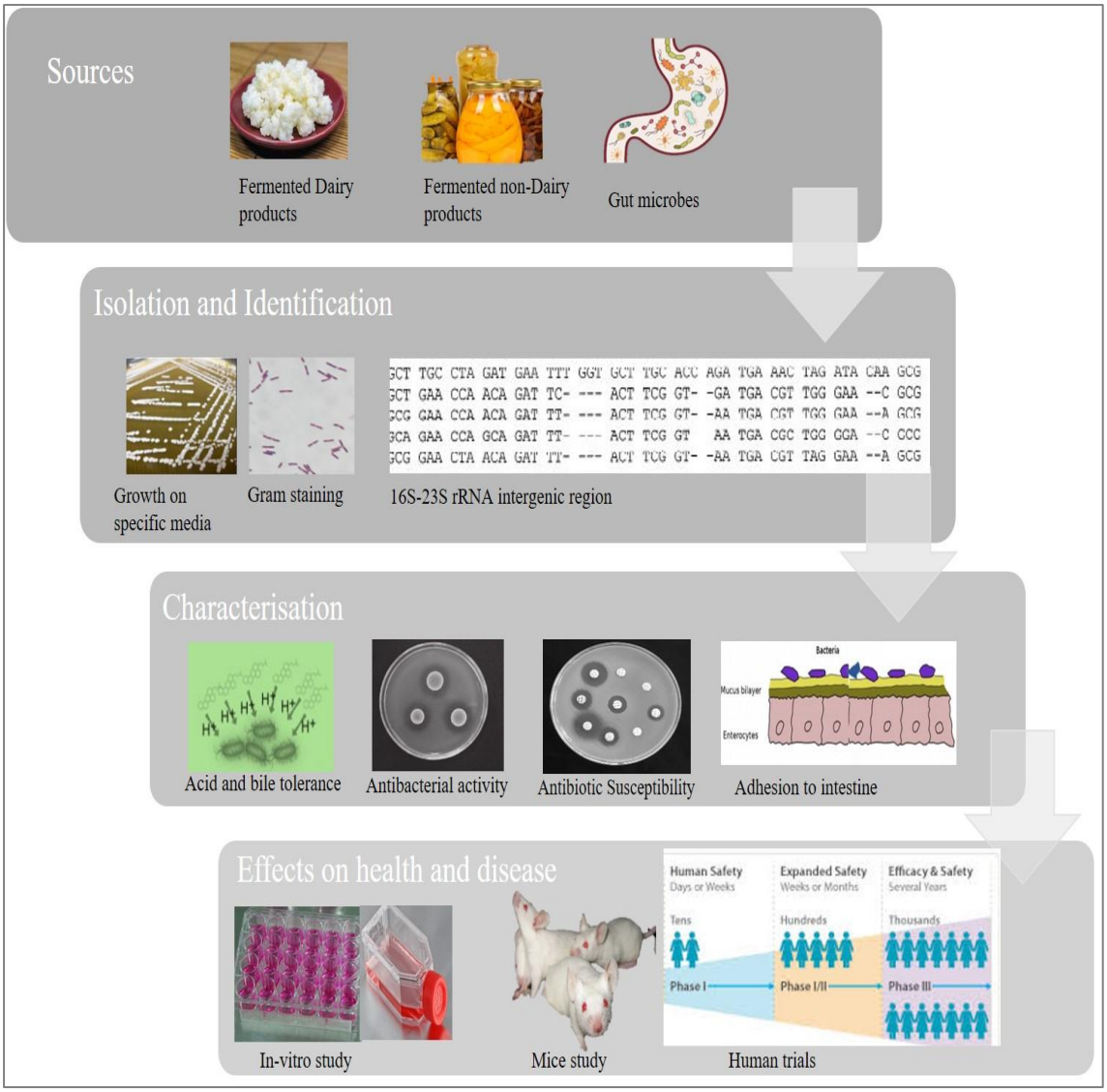

Figure 1. 2 Schematic representation of basic steps involved in the isolation of novel probiotics.

1.4 Probiotic Microorganisms

There are several groups of bacteria used as potential probiotic; most of them belong to the genera *Lactobacillus, Bifidobacterium,* and *Streptococcus*. Other than these, enterococci and yeasts have also been used as probiotics. Table 1.2 describes the most commonly used species as potential probiotic. Although belonging to the same genera and species, each strain has different probiotic property and mode of action. The claimed health beneficial effect of probiotic microorganisms ranges from the very

common constipation alleviation to the prevention of most life-threatening diseases such as cancer (Wollowski et al., 2001), inflammatory bowel disease (Kim S.E. et al., 2015; Sadeghzadeh et al., 2014), and cardiovascular disease (Agerholm-Larsen et al., 2000; Santosa et al., 2006).

Table 1. 2 Most commonly used probiotic microorganisms.

Lactobacillus sp.	*Bifidobacterium sp.*	*Enterococcus sp.*	*Streptococcus sp.*
L. acidophilus *L. casei* *L. delbrueckii ssp.(bulgaricus)* *L. cellobiosus* *L. curvatus* *L. fermentum* *L. lactis* *L. plantarum* *L. reuteri* *L. brevis*	*B. bifidum* *B. adolescentis* *B. animalis* *B. infantis* *B. thermophilum* *B. longum*	*Ent. faecalis* *Ent. faecium*	*S. diacetylactis* *S. salivarius* *S. intermedius* *S. cremoris*

(Collins J.K. et al., 1998; Tannock, 1999)

1.4.1 The genus *Lactobacillus*

The genus *Lactobacillus* is by far the largest of the genera included in lactic acid bacteria. It is also very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. The heterogeneity is reflected by the range of mol% G+C of the DNA of species included in the genus. This range is 32-53%, which is twice the span usually accepted for a single genus (Schleifer and Stackebrandt, 1983). *Lactobacillus* is a Gram-positive, facultative anaerobic or microaerophilic, rod-shaped, catalase negative, non-spore-forming, usually non-motile bacteria (Holzapfel and Wood, 2012). The first intestinal

Lactobacillus was isolated by Moro in 1900, who typified it as *Bacillus acidophilus* (Moro, 1900). Lactobacilli can either be homo-fermentative or hetero-fermentative depending on the metabolic pathway utilized for glucose fermentation. When homo-fermentative, they could produce more than 85% lactic acid, whereas the hetero-fermentative strains produce lactic acid, carbon dioxide, ethanol or acetic acid. According to Gomes & Malcata (1999), total 56 species of genus *Lactobacillus* has been reported. Further, on the basis of 16S rRNA sequencing, lactobacilli are divided into three subgroups (Collins M. D.et al., 1991). Depending upon the various environmental factors (oxygen availability, pH, bacterial interactions, presence of secretion etc.), the distribution of lactobacilli is different in various parts of the gastrointestinal tract (GIT). Most frequent isolated species in the human GIT includes *L. salivarius, L. plantarum, L. casei, L. acidophilus, L. fermentum* and *L. brevis* (Mikelsaar et al., 1998). Lactobacilli are generally regarded as safe (GRAS) (Salminen S. et al., 1998).

1.5 Sources, isolation and identification of lactobacilli

1.5.1 Sources

Application of various fermented dairy products for health benefits has a long history and hence it is the most common and useful source of lactobacilli isolation for potential probiotic properties (Liong, 2011). Sun et al., (2010) reported the prevalence of *L. delbrueckii* subsp *bulgaricus* amongst 148 strains isolated from a traditional naturally fermented yak milk. Lactobacilli isolated from other fermented milk products, such as Maasai milk, kefir and Koumiss, have been reported with various health benefits (Golowczyc et al., 2008; Santos et al., 2003; Vinderola et al., 2005). Cheese is a dairy product with potential for the delivery of probiotic microorganisms into the human intestine. *L. plantarum* strains have been isolated from Italian, Argentinean and

Bulgarian cheeses (Georgieva et al., 2008; Ugarte, Guglielmotti, Giraffa, Reinheimer, & Hynes, 2006; Zago et al., 2011).

The isolation of probiotics is not limited to the dairy fermented products. Non-dairy fermented products are also a good source of probiotics (Rivera-Espinoza and Gallardo-Navarro, 2010). It has been reported that functional and metabolic properties of lactobacilli isolated from meat and fruit were similar to those of human GIT isolates (Haller et al., 2001). In addition, a pickle juice isolate, *L. buchneri* P2, has been characterised with probiotic properties and ability to reduce cholesterol (Zeng et al., 2010).

Other than the dairy and fermented products, breast milk is also a potent source for the isolation of lactobacilli with probiotic potential. Initially considered as sterile, human breast milk contains lactic acid bacterial (LAB) strains genotypically different from those isolated from skin (Martín R. et al., 2009; O'Hara and Shanahan, 2006). The predominant bacteria present in the human breast milk comprises streptococci, lactobacilli, lactococci, staphylococci, micrococci, enterococci and bifidobacteria. However, its intake favours the predominance of lactobacilli and bifidobacteria in the infant intestinal microbiota (Gueimondea et al., 2012; Martín R. et al., 2003, 2004). During lactation, administration of lactobacilli isolated from breast milk is considered as an efficient alternative to the antibiotics for the treatment of infectious mastitis (Arroyo et al., 2010).

Human GIT resides with approx. 500 different bacterial species, many of them colonise the intestinal tract and exert various beneficial effects to the host. Amongst the bacteria currently being used as probiotic, many of them are the isolates of human GIT. Probiotic strain, *L. acidophilus* RY2, was isolated from faeces of a healthy infants (Lin et al.,

2009). Other than the human GIT, probiotic strains have also been isolated from the guts of pigs, rats and even other poultry animals (Petrof, 2009; Yun et al., 2009). Isolates from the intestine of freshwater and marine wish have also been reported to possess probiotic properties (Amin et al., 2017; Pérez-Sánchez et al., 2011).

1.5.2 Isolation

Isolation is a crucial step in the process of developing a novel probiotic strain. The initial step for isolation of lactobacilli involves preservation of sample in its native condition. Since lactobacilli are anaerobic or facultative anaerobic, the samples to be used for isolation should be homogenized quickly, followed by appropriate dilution and culturing in a selective media. There are various selective media available for isolation of lactobacilli. The choice of selective medium depends upon the source of the sample. Depending on the source of the sample, selective inhibitory agents can be incorporated in selective medium to avoid the growth of particular group of microbes predominating in sample. For example, we incorporated cycloheximide in selection medium for the isolation of lactobacilli from faecal samples in order to avoid the yeast overgrowth (Endo and Okada, 2007). For isolation of lactobacilli from the conventional sources, such as oral and faeces samples, Rogosa medium containing Columbia agar base supplemented with propionic acid is widely used (Rogosa, Mitchell, & Wiseman, 1951). Since, lactobacilli can tolerate low pH, growth of the other predominant bacteria present in the faeces is inhibited in the acidic environment of this medium, making it selective for lactobacilli. Isolation of lactobacilli from crop and paddy rice silage fermentation requires medium containing glucose, yeast extract and peptone (GYP) (Ennahar et al., 2003; Kawahara and Otani, 2006) whereas, soil isolates of LAB have been cultivated using GYP plus BM medium (Chen Y.-S. et al., 2005; Yanagida et al.,

2006). For isolation of LAB from air, a modified version of high nutritional medium have been used (Meroth et al., 2003).

1.5.3 Identification

Traditionally, the identification of *Lactobacillus* has been based mainly on fermentation of carbohydrates, morphology, and Gram staining, and these methods are still used. The identification of *Lactobacillus* isolates by phenotypic methods is difficult because it requires, in several cases, determination of bacterial properties beyond those of the common fermentation tests (for example, cell wall analysis and electrophoretic mobility of lactate dehydrogenase) (Kandler, 1986). In general about 17 phenotypic tests are required to identify a *Lactobacillus* isolate accurately to the species level (Hammes and Vogel, 1995). In recent years, the taxonomy has changed considerably with the increasing knowledge of genomic structure and phylogenetic relationships between *Lactobacillus* spp. (Klein et al., 1998). Newly developed research methods such as DNA-DNA homology, rRDNA-DNA homology or the guanine-plus- cytosine (G+C) content of DNA have contributed much to the advances in bacterial taxonomy, and numerous new taxa of intestinal anaerobes have been described (Eckburg P. B., 2005; Suau et al., 1999). While several schemes have been developed for the identification of indigenous anaerobic bacteria, it is still difficult to identify many of these organisms by conventional tests at the species level.

The molecular based approach for identification at species level has been a method of choice since last few decades. The conserved region of 16S rRNA is used for phylogenetic classification and the sequences are compared with that in available databases (DDBJ-DNA Data Bank of Japan, ENA European Nucleotide Archive and GenBank) for species identification (Winker and Woese, 1991; Woese, 1987). This 16S rRNA gene analysis is often coupled with other methods to identify bacterial

communities of the gut or ecological sources. The amplified 16S rRNA products are separated either by temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE) using chemical denaturation (Muyzer and Smalla, 1998). Otherwise, amplicons are often digested with restriction enzymes (Terminal restriction fragment length polymorphism (T-RFLP)) or hybridised with target specific 16S probes labelled with fluorescent molecules (fluorescence in situ hybridisation (FISH)) (Fallani et al., 2010; Langendijk et al., 1995).

Although, the molecular approach based 16S rRNA is widely accepted, extremely small size of the 16S DNA (1500 bp) when compared with whole genome (30,000-40,000 bp), often fails to differentiate strains from the same species. Therefore, complementary information is often needed in certain cases to discriminate the strains of a given species. Alternatively, the spacer sequence between 16S and 23S rRNA gene shows a great deal of sequence and length variation (Leblond-Bourget et al., 1996) (Figure 1.3). This intergenic spacer region is about 200 bases in length if tRNA genes are absent (small spacer sequence) and hypervariable in case of lactobacilli (Berthier and Ehrlich, 1998; Tilsala-Timisjärvi and Alatossava, 1997). The fact that most bacterial species harbour multiple copies (alleles) of the ribosomal operon in their genome increases the possibility that a substantial amount of sequence variation exists in these spacer regions, even among strains of the same species. This diversity represents a powerful tool for the design of specific oligonucleotides for PCR-based detection protocols or to discriminate species on the basis of the band patterns obtained by PCR amplification of the spacer regions (Daffonchio et al., 1998). It is a simple way to identify the bacteria at species level. Tannock et al. (1999) showed the identification of lactobacilli at species level using intergenic region sequence analysis of isolates from human faeces, rodent gastrointestinal samples and porcine gastrointestinal contents. Additionally, it can be

used as a qualitative technique to confirm isolates as lactobacilli by simply comparing the electrophoretic mobility pattern of amplified products on agarose gel with the same of any standard lactobacilli strain.

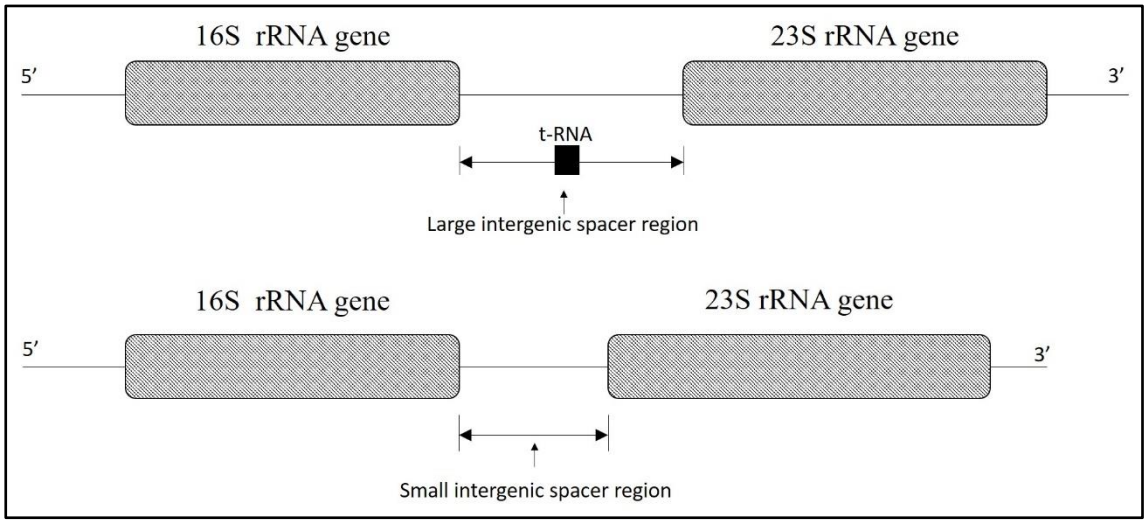

Figure 1. 3 Schematic representation of 16S-23S rRNA gene intergenic spacer region of lactobacilli

1.6 Characterization of lactobacilli as probiotics

1.6.1 Resistance to acidic environment

The acidic environment of the GIT inhibits the growth of the bacteria by increasing the energy consumption to maintain pH homeostasis (Herrero Alejandro, 1983). The resistance to this acidic stress by some bacteria is attributed to their greater range of the internal pH and thereby decreased dependency on the energy-consuming proton pumps (Booth, 1985). The other mechanisms involved in the acid tolerance in gram-positive bacteria are an alteration in the composition of cell envelope, proteins engaged in repair and degradation of damaged cell components, and increased expression of a regulator that promote minor or global response (Cotter and Hill, 2003; Yousef and Juneja, 2003).

The maintenance of cell integrity in acid stress is attributed to the different macromolecules comprising the cell membranes and cell walls of lactobacilli. A shift

in the fatty acid composition of the cell membrane of *L. casei* was observed under low pH environment (Fozo et al., 2004). The involvement of a putative esterase gene, lr1516 and gene encoding a putative phosphatidyl glycerophosphatase has been reported in some lactobacilli strains under acidic shock (Wall et al., 2007).

Several proteins involved the protection or repair of DNA and proteins also have an essential role in acid resistance. An increased expression of a gene *uvrA* which codes for subunit A of the exonuclease ATP-binding cassette (ABC) complex, involved in nucleotide excision repair, under low pH suggested a role for this system in adaptation to acidic stress in *L. helveticus* CNBL 1156 (Cappa et al., 2005). In a study using *L. acidophilus,* the authors demonstrated the production of DnaK, GrpE, DnaJ, GroEL and GroES as a response to acid adaptation (Lorca et al., 2002). In the other reports using acid-tolerant mutants *L. sanfranciscensis,* upregulation of GrpE was observed (Bini et al., 2001). Additionally, in another study, after 1 h of incubation of *L. reuteri* ATCC 23272 at pH 4, expression of GrpE and DnaK was increased (Lee K. et al., 2008).

Amachi et al., (1998) demonstrated the importance of cell membrane-bound H+-ATPase for acid tolerance of *Lactococcus lactis*. In bacteria, it is necessary to increase the H+-ATPase activity quickly and pump out H+ to maintain intracellular pH. In gram-positive bacteria, the multisubunit F_0F_1 ATPase is utilized as the main proton pump to facilitate the release of protons from the cytoplasm by proton motive force. Kullen & Klaenhammer, (2002) also reported increased levels of F_0F_1 ATPase mRNA in *L. acidophilus* when exposed to low pH. Other than F_0F_1 ATPase, the amino acid decarboxylation-antiporter systems are also one of the main proton pumps utilized by these microorganisms. In this system, an amino acid is transported into the cell and decarboxylated via consumption of a proton. An antiporter exports the product outside the cell and as a result of this process intracellular pH increases. Ueno et al., (1997)

demonstrated the utilization of glutamate decarboxylase (GAD) by *L. brevis* via this system.

1.6.2 Resistance to bile salts

The ability of probiotic bacteria to survive in the small intestine is also dependent on its bile tolerance capacity. Although intrinsic bile tolerance appears to be strain dependent, lactobacilli can progressively adapt to the presence of bile salts. Margolles et al., (2003) reported the cross-resistance to other stress factors by bile-adapted strains which suggest the existence of common mechanisms in bacteria against various stresses. Bile-specific mechanisms mediating resistance in lactobacilli include active efflux of bile salts (Bustos et al., 2011; Pfeiler and Klaenhammer, 2009), changes in the architecture/composition of cell membrane and cell wall (Gómez Zavaglia et al., 2002; Ruiz et al., 2007) and bile salt hydrolysis (Kumar et al., 2006; Lambert et al., 2008).

A common mechanism to counteract bile toxicity by bacteria is the active extrusion of the bile salts from the cytoplasm through efflux pumps (Piddock, 2006). A role of several multidrug transporters (MDRs) of the ATP-binding cassette or of the major facilitator superfamily has been suggested in mediating bile tolerance in lactobacilli strains. In *L. acidophilus* NCFM, deletion of any one of LBA1429, LBA0552, LBA1679, and LBA1446 transporters had increased the sensitivity of the mutant strains towards bile and certain antibiotics (Pfeiler and Klaenhammer, 2009). A mutation in lr1584 decreased the growth of *L. reuteri* in the presence of bile (Whitehead et al., 2008).

Other than the above described mechanisms, the activity of bile-salt hydrolyses (BSHs) is considered as an important mechanism to counteract the harmful effect of bile. BSH enzymes belong to the chologlycine hydrolase family of enzymes. BSH catalyses the deconjugation of the bile salts by hydrolysis of an amide bond, releasing glycine or

taurine. The gut microbiota further metabolises these unconjugated bile acids to secondary bile salts by the process of oxidation and dihydroxylation (De Boever and Verstraete, 1999; De Smet et al., 1995). The comparison of wild-type *L. plantarum* with the mutated BSH established a link between BSH activity and bile tolerance (Begley et al., 2005). Although the exact mechanism by which BSH confers bile salt resistance to bacteria is not completely understood, it has been proposed that since the protonated form of bile salts may exhibit toxicity through intracellular acidification in a manner similar to organic acids, BSH activity may protect bacteria through the formation of the weaker unconjugated counterparts. This could help negate intracellular pH from a drop by recapturing and exporting the co-transported proton (De Smet et al., 1995). *In vitro* study of bacterial growth at different pH revealed that the tauroconjugated bile salts were less toxic than the glycoconjugated bile salts at low pH (De Smet et al., 1995). Given the fact that BSH preferentially hydrolyses glycoconjugated bile salts and have slightly acidic pH optima, BSH activity is of great importance at the point when bacteria enter the duodenum from the stomach and are exposed to bile salts in an acidic environment.

BSHs are generally produced by intestinal bacteria such as *Lactobacillus, Bifidobacterium, Clostridium, Enterococcus and Bacteroides spp* (Bateup, McConnell, Jenkinson, & Tannock, 1995; Coleman & Hudson, 1995; Kawamoto, Horibe, & Uchida, 1989). As of now, the BSHs have been reported in Gram-positive bacteria except *Bacteroides*. Duary et al. reported six-fold increase in the BSH gene expression of *L. plantarum* following their exposure to 2 % bile which indicates inducible activity of BSH (Duary et al., 2012). In bifidobacteria, constitutive expression of BSH gene was observed in *in vitro* experiments (Sánchez et al., 2005), whereas *in vivo* experiments using *B. longum* indicated intracellular accumulation of this enzyme in the rabbit gut

which suggested role of other intestinal factors in triggering the expression of BSH gene (Yuan et al., 2008). In the genomes of lactobacilli and bifidobacteria, several copies of BSH genes are present which participate in the deconjugation of bile salt (Ren et al., 2011). In lactobacilli strains of human origin, BSH and bile salt transporters are found organized in operons (Elkins et al., 2001; Elkins and Savage, 2003).

1.6.3 Antimicrobial activity

Antimicrobial activity of probiotics has been evidenced by their inhibitory effect on the growth of potential pathogens. It has been exhibited by either competition for nutrients and adhesion, and/or production of several antimicrobial metabolites like organic acids, H_2O_2, bacteriocins etc. Production of organic acids such as lactic acid and acetic acid are the main antimicrobial compounds which account for the inhibitory effect on both Gram-positive and Gram-negative bacteria (Alakomi et al., 2000; De Keersmaecker et al., 2006; Makras et al., 2006). Once they enter the bacterial cell, organic acids dissociate inside the cytoplasm triggering a decrease in the intracellular pH which leads to the death of the pathogen. The ionized form of organic acid accumulation inside the cell results in the death of the pathogen (Russell and Diez-Gonzalez, 1997; Salminen S. et al., 2004). The cell-free supernatant of *L. casei* inhibited the growth of several human pathogens (Forestier et al., 2001). The strong antimicrobial activity against *Helicobacter pylori* by different lactobacilli strain has been attributed to the accumulation of lactic acid and other organic acids (Bhatia et al., 1989; Midolo et al., 1995). Production of H_2O_2 is another important antimicrobial molecule produced by lactobacilli which non-specifically inhibits both Gram-positive and Gram-negative organisms mainly in the normal vaginal ecosystem (Reid and Burton, 2002; Reid Gregor, 2002). The anti- *Salmonella* activity exhibited by *L. johsonii* NCC533 was due to the production of H_2O_2 (Pridmore et al., 2008).

Lactic acid bacteria are also known for the synthesis of antibacterial peptides, bacteriocins, which kill the target cells mainly by pore formation and/or inhibition of cell wall synthesis (Hassan et al., 2012). The "bacteriocins" initially included only colicin type of protein antibiotics, but now the term is used for most peptide inhibitors from both Gram-positive as well as Gram-negative bacteria. Bacteriocins are classified into four main groups on the bases of their targets, size, and mechanisms of action and immunity as described in table 1.3. (Garneau et al., 2002; Klaenhammer, 1993; Van Belkum and Stiles, 2000). Synthesis of bacteriocin by lactobacilli may confer them a competitive advantage within the complex ecological system and facilitate them to establish and increase their prevalence within the GIT by direct inhibition of pathogen growth (O'Shea et al., 2012). Recently, the studies have been focused on their application in preventing the growth of harmful bacteria in the fermentation process, preservation of various dairy products and as an anti-infective drug. The bacteriocins produced by most of the lactobacilli, including plantaricin from *L. plantarum,* lactacin from *L. acidophilus* and nisin from *L. lactis*, have antibacterial activity only against closely related bacteria (Bierbaum and Sahl, 2009). However, the bacteriocins produced by some *Lactobacillus* spp. exhibit a broad spectrum of activity against both Gram-positive and Gram-negative organisms.

Table 1. 3 Classifications of bacteriocins from lactic acid bacteria

1.	Lantibiotics	• Ribosomal produced peptides that undergo extensive post-translational modification • Usually small peptides of <5 kDa molecular weight, containing lanthionine and methyl lanthionine
2.	Non-lantobiotics	Sub-class: A • Low-molecular-weight (<10 kDa) • Heat stable • Formed exclusively by unmodified amino acids

		• Ribosomally synthesized as inactive peptides, activated during post-translational modifications
		Sub-class: B • High molecular weight (>30 kDa) • Heat-labile proteins
3.	others	• Complex bacteriocins with lipid or carbohydrate moieties • Hydrophobic • Heat stable

1.6.4 Antibiotic susceptibility

Probiotic bacteria can either be sensitive towards a particular drug or exhibit natural resistance towards it, based on the presence or absence of resistance genes in the genome. Lactobacilli are increasingly used in foods, mainly in animal feed and dairy products, and as a probiotic product due to their health-promoting attributes in the body. Depending upon the presence or absence of resistance genes in the genome, lactobacilli can either be sensitive to a particular antibiotic or exhibit natural resistance towards it. Resistance to antibiotics by lactobacilli could be both advantageous as well as disadvantageous for their potential use as a probiotic. The application of antibiotic resistant lactobacilli in patients could rapidly establish the desirable gut microflora and thus could help them to recover faster (Arnold, 2013; Courvalin, 2006; Tambekar and Bhutada, 2010). Moreover, lactobacilli with known antibiotic resistance may be beneficial in some of the preventive and therapeutic cases like prevention of ulcers, control of intestinal infections and antibiotic associated diarrhea (Ouwehand A.C., Salminen, et al., 2002). However, the potential transfer of the acquired resistance genes present in the lactobacilli genome to the other bacteria of the same species or to pathogenic commensals of the GIT is a major disadvantage. Usually, lactobacilli are

found to be more susceptible towards the antibiotics which inhibit protein synthesis (example includes chloramphenicol, tetracycline, gentamicin, streptomycin etc.) (Gueimonde et al., 2013). On the other hand, lactobacilli have an inherent property of resistance towards antibiotics that target on nucleic acid synthesis like norfloxacin, nalidixic acid, enoxacin, ciprofloxacin etc. (Ammor et al., 2007). The studies on the antibiotic sensitivity profile of lactobacilli can help to characterize them for either acquired or inherent resistance. Although resistance to the vancomycin by some *Lactobacillus* species (like *L. casei, L. rhamnosus, L. plantarum, L. brevis, L. fermentum* and *L. curvatus*) is reported to be a non-transferable inherent property, the possibility of transfer across species or genus cannot be ruled out (D'Aimmo et al., 2007). The most commonly detected acquired resistance genes in lactobacilli are *tet (M)*, which codes for tetracycline resistance, and *erm (B)*, which codes for erythromycin resistance, followed by the gene coding for chloramphenicol resistance (*cat*). However, most of the resistance genes found in *Lactobacillus* are intrinsic and being an inherent property of the bacteria, these genes are non-transferable via horizontal gene transfer (Seale and Millar, 2013).

1.7 Adhesion of lactobacilli strains

The ability of lactobacilli to adhere to the intestine is considered as an important probiotic property (Lee Y.-K. and Puong, 2002). In the intestine, the peristalsis movement tends to wash off the microbes present within it. Adherence ability allows the lactobacilli strains to persist and colonize in the intestine, particularly in the small intestine where flow rates are relatively high. Other than longer persistence and colonization, adhesion has been associated with enhanced healing of damaged mucosa (Elliott et al., 1998) and immunomodulation (Blum S. et al., 2002). Additionally, adhesion of lactobacilli to the intestinal mucosa may prevent binding of pathogenic

microbes by competing with binding site and nutrient availability (CenciČ and Langerholc, 2010). Contrary strains with extremely high adhesion ability may lead to potential pathogenesis, especially when tissue is damaged (Finlay and Falkow, 1997; Wilson et al., 2002). However, the lactobacilli are generally regarded as safe and hence rarely cause any disease (Borriello et al., 2003; Salminen M. K. et al., 2002). The adhesion ability of lactobacilli depends upon the structure, composition, and organization of the cell wall. Since adhesion studies are difficult using *in vivo* models, different *in vitro* based models are widely used. *In vitro* models to study adhesion abilities mostly human-derived adenocarcinoma cell-lines (mostly includes Caco-2 and HT-29), immobilized extracellular matrices and immobilized intestinal mucus. Various detection methods have also been developed for the quantitative measurements of adhesion which are based on quantitative culturing, radiolabelling, microscopic enumeration, FISH and immunological detection (Vélez et al., 2007).

1.7.1 Adhesion models

1.7.1.1 Tissue culture cells

Tissue culture cells are most widely applied models for assessment of adhesion ability of probiotics. Intestinal epithelial cells, particularly, Caco-2, T84 and HT-29 cells are commonly used for adhesion study (Aissi et al., 2001). The potential disadvantage in using tissue culture cells include their cancerous nature (Rousset, 1986), which may or not be different from normal intestinal epithelial cells. Caco-2 cells are derived from the large intestine of a 72-year-old man with the neoplastic tumor. It grows as a monolayer expressing brush border microvilli on the apical surface with a cylindrical polarized morphology (Sambuy et al., 2005). For adhesion assay, Caco-2 cells are cultured as a monolayer on the cell-culture treated multi-well plates. Once confluent, they express proteins characteristic for both colonocytes and enterocytes. After

confluency, proteins specificity for enterocytes increases. Such heterogeneity of Caco-2 cell-line is responsible for variation in adhesion ability of the same lactobacilli strain to make the comparison of data between different laboratories difficult. To improve the homogeneity of the population, various clonal cell-lines were obtained from the parental Caco-2 culture. C2BBe cell-line, isolated from Caco-2, is one of the examples of such cell-lines with homogeneous brush border expression (Peterson and Mooseker, 1992).

HT-29 cell-line was isolated from a large intestine adenocarcinoma tissue of a 44-year-old women of the Caucasian race. The morphology of HT-29 cells is similar to the *in vivo* epithelial cells. It does not fully differentiate in *in vitro* cultures; hence lack the brush border on the apical surface. Unlike Caco-2 cells, this cell-line produces mucin because of the presence of goblet-like cells in its cell-population. Moreover, subpopulations of HT-29 cells with higher mucin secretions have been isolated upon exposure with either 5-fluorouracil (HT-29-FU) or methotrexate (HT-29-MTX). The mucins of this secretory sub-clones are similar to the mucins of the human colon (Leteurtre et al., 2004). This provided more feasibility to researchers for studying the complicated process of bacterial adhesion to the intestine.

Other than the intestinal cell-lines, HeLa cell-line, isolated from malignant cervical cells, has also been very popular to study the bacterial adhesion because of its ease of maintenance (Fourniat et al., 1992; Mastromarino et al., 2002). Vaginal epithelial cells, buccal epithelial cells, and urinary tract epithelial cells can also serve as an alternative source of cells for adhesion-related studies. Since these cells possess normal microbiota and covered with mucus, they may serve as a better representative of *in vivo* conditions. However, these properties are dependent on the source, gender, method, and timing of collection of a sample (Arjuna N. B. Ellepola, Lakshman P., 2001).

1.7.1.2 Intestinal mucus

Immobilized intestinal mucus, isolated from resected tissue or faeces, is also a widely accepted model to study adhesion *in vitro*. Mucin is immobilized on the microtiter well plate and bacteria is allowed to adhere to this immobilized mucin. The bacterial adhesion is then measured either quantitatively or qualitatively (Lee Y.K. et al., 2000; Li et al., 2008; Tallon et al., 2007). However, the absence of underlying enterocytes is the major disadvantage of this model.

In vitro model systems do not take into account the normal microbiota that is present in the intestine, thus they do not completely mimic the actual *in vivo* conditions. Therefore, development of *in vivo* models to study the adhesion process is required. Few models which take into account the presence of normal microflora are described below.

1.7.1.3 Whole tissue

Human intestinal mucosa consists of three major parts- enterocytes, mucus and the normal microbiota has been developed to study adhesion to resected human intestinal tissue (Ouwehand A.C. et al., 2002b). This model can also be used to investigate the effect of probiotic adhesion on the specific intestinal diseases. Since the availability of the resected tissue is less, comparison with the normal intestine is difficult. This model system is used for relatively very short incubation times. Moreover, studies related to the influence of probiotics adhesion on the morphology and physiology is not possible using this system.

1.7.1.4 Organ culture

The limitation of whole tissue as a model can be overcome by using organ culture model. This model offers the possibilities to study the changes in enterocyte morphology and physiology upon bacterial adhesion. Organ culture has been used in

assessing the adhesion of pathogenic bacteria and their influence on the intestinal epithelium, both with animal and human tissue (Phillips et al., 2000). However, there are no reports available for use of this model to assess the properties for probiotics.

1.7.1.5 *In vivo* animal models

Animal models provide a more accurate representation and allied complexity of the human GIT, however, its application for adhesion study is limited by ethical restrictions. Conventional animal models present few ethical restrictions and a high degree of similarities with human GIT, but the inherent complexity factors fail to depict the interaction and limit their use in bacterial adhesion studies (Boureau, L. Hartmann, T. Karjalaine, 2000). Therefore, bacterial adhesion studies are mostly carried out with the gnobiotic and germ-free animals, although they do not exactly represent the human GIT as conventional animals. Moreover, lack of complex microflora in gnotobiotic animals simplifies the interpretation of data obtained from bacterial adhesion and bacterial host interaction studies. Furthermore, the key role of a single bacterial population on normal development, establishment and maintenance of the mucosa-associated immune system, and epithelial-cell functions can be studied using germ-free animals. (Boureau, L. Hartmann, T. Karjalaine, 2000).

1.7.2 Adhesion mechanism of lactobacilli

Cell adhesion is a complex process involving contact between the bacterial cell membrane and interacting surfaces. The molecular mechanisms of the adhesion of *Lactobacillus* spp. have not been fully studied and understood yet. Therefore, different contemporary approaches that aim to reveal the biological and genetic factors of lactic acid bacteria (LAB) adhesion are needed. *In vitro* studies assessing these properties are often not able to simulate the *in vivo* conditions completely, but they could be reliable indicators in the selection of new probiotic strains. Many scientists have reported that

multiple components such as proteins, carbohydrates (possibly glycoproteins), and divalent cations are involved in the adhesion mechanism between bacteria and epithelial cells (Bernet et al. 1993; Chauviere et al. 1992; Coconnier et al. 1992). In most cases, the adhesion of lactobacilli to the mucosa is described as a protein-mediated process, but there is evidence for the involvement of carbohydrate components of cell surfaces in this process (Ouwehand A.C. et al., 1999a). The physical and chemical characteristics of the cell surface could be assessed critically based on bacterial cell surface hydrophobicity (depends on surface components of bacteria) (Boonaert and Rouxhet, 2000) and electrical mobility/charge (rate of migration under electric field due to bacterial surface charges) (Van der Mei and Busscher, 2001). Both the hydrophobicity and the electric charge are the consequences of the chemical composition of the bacterial surfaces. Microbial adhesion is a complicated interplay of van der Waals and electrostatic forces and various other short-range interactions, hence strains adhering well to the hydrocarbons are considered to be hydrophobic and strains adhering poorly are considered hydrophilic. However, other studies indicated that there was no correlation between cell surface hydrophobicity and adhesion to intestinal mucus (Muñoz-Provencio et al., 2009; Ouwehand A.C. et al., 1999a). In these studies, highly adhesive bacteria demonstrated fairly low surface hydrophobicity. This suggested that cell surface hydrophobicity is not an accurate measure of adhesive potential.

Granato et al. (1999) have reported the involvement of Lipoteichoic acid (LTA) in the adhesion of industrial probiotic strain *L. johnsonii* La1 to intestinal epithelial cells. Inhibition of adhesion of La1 to the Caco-2 cells was observed when LTA was purified from the La1 strains and the spent culture medium containing La1 was used for the adhesion studies. Moreover, the inhibition was observed in dose-dependent manner. Genome sequencing of La1 identified the role of dlt genes involved in the LTA

synthesis and the other genes which code for potential cell-wall located molecules in adhesion process (Vaughan and Mollet, 1999).

Proteinaceous adhesin molecule, Mub, was also shown to be involved in the adhesion of *L. reuteri* 1063 to mucus (Roos, 1999). Mub protein is one of the largest cell-surface proteins of Gram-positive bacteria encoded by a gene of 9807 bp length. Two types of long repeat sequences possessed by this protein share some similarity with a human ocular epithelial protein (Braun et al., 1995) and an antigen from a hepatitis virus (Roos and Jonsson, 2002).

Table 1. 4 Various adhesion factors of different lactobacilli strains.

Lactobacillus strain	Adhesion	Targets for binding	References
L. acidophilus M92	S-layer protein	Porcine epithelial cells, mouse ileal epithelial cells	(Frece et al., 2005; Kos et al., 2003)
L. acidophilus NCFM	Mucus-binding protein (Mub)	Human epithelial cell-lines and mucus	(Buck et al., 2005)
	S-layer protein (SlpA)	Human epithelila cell-lines	
	Collagen-binding protein (FbPA)	Human epithelial cell-lines and fibronectin	
L. crispatus JCM 5810	S-layer protein (CbsA)	Collagen, laminin	(Sillanpää et al., 2000; Toba et al., 1995)
L. brevis ATCC 8287	S-layer protein (SlpA)	Human intestinal epithelial cell-line, Collagen, laminin, fibronectin	(Leeuw et al., 2006)
L. crispatus ST1	a-Enolase	Collagen, laminin, fibronectin, plasma components	(Antikainen and Yliopistopaino, 2007)
L. johnsonii NCC 533	Elongation factor Tu	Human epithelial cell lines and mucus	(Granato D. et al., 2004)
	Chaperonin GroEL		(Bergonzelli et al., 2006)

L. johnsonii F133	a-Enolase	Collagen, laminin, fibronectin, plasma components	(Antikainen et al., 2002)
L. plantarum WHE 92	Glyceraldehyde-3-phosphate dehydrogenase	Mucus	(Izquierdo et al., 2009)
	Elongation factor Tu		
	Chaperonin GroEL		
	Molecular chaperone DnaK		
L. plantarum WCFS1	Mannose lectin (Msa)	Mucus via Mannose Binding	(Pretzer et al., 2005)
L. plantarum 423	Glyceraldehyde-3-phosphate dehydrogenase	Human epithelial cell lines	(Ramiah et al., 2008)
	Elongation factor Tu		
	Triose-phosphate isomerase		
L. reuteri 1063	Mucus-binding protein (Mub)	Mucus	(Roos et al., 2002)
Lactobacillus gasseri SBT2055	Sortase-dependent proteins (SDPs)	Mucin, Human intestinal epithelial cell-lines, fibronectin	(Arai et al., 2016)
L. salivarius UCC 118	Surface protein (LspA)	Human intestinal epithelial cell-lines	(Van Pijkeren et al., 2006)
L. reuteri NCIB 11951	Collagen-binding protein (CnBP)	Collagen	(Roos et al., 1996)
L. reuteri ATCC PTA 6475	Mucus binding protein (cmbA)	Human intestinal epithelial cell-lines and mucus	(Jensen et al., 2014)

In some of the lactobacilli strains, cell-surface or S-layers have been involved in the adhesion process. S-layers are formed by a paracrystalline monomolecular assembly of proteins or glycoproteins on the surface of bacteria. In lactobacilli, many S-layer proteins have been reported, however, only six of them have been validated for biological functionality (Åvall-Jääskeläinen and Palva, 2005; Sára and Sleytr, 2000) which are CbsA of *L. crispatus* JCM 5810 (Antikainen et al., 2002; Sillanpää et al., 2000; Toba et al., 1995), S-layer protein of *L. acidophilus* spp. (Schneitz et al., 1993), Slp of *L. helveticus* R0052 (Johnson-Henry K.C. et al., 2007), SlpA of *L. acidophilus* NCFM (Buck et al., 2005), SlpA of *L. acidophilus* M92 (Frece et al., 2005) and SlpA of *L. brevis* ATCC 8287 (Avall-Jääskeläinen et al., 2002; Vidgrén et al., 1992). These proteins are involved in the adhesion between ECM components and intestinal epithelial cells. Moreover, some of these proteins were also involved in the antagonistic activity of lactobacilli to prevent the adhesion of pathogenic bacteria to epithelial cells (Chen X.et al., 2007; Johnson-Henry et al., 2007).

1.7.3 Factors influencing adhesion

Adhesion of probiotic microorganisms is affected by various factors mainly based on the mode of culture adopted, i.e. *in vitro* or *in vivo*. Factors related to *in vitro* conditions include bacterial concentration, incubation time, buffer composition and growth medium, whereas *in vivo* condition-related factors include normal intestinal microbiota, digestion, and the food matrix.

When lactobacilli concentration used to assess the adhesion ability is too high, it can saturate all the binding sites of the substrata used and hence a progressively smaller fractions of the lactobacilli applied will be able to adhere. While deciding bacterial concentration, secretions from gastrointestinal tract should also be considered (Marteau

et al., 1997). In most of the adhesion studies, bacteria are used in the concentration of 10^7-10^9 CFU/ml (Aissi et al., 2001; Gopal et al., 2001; Kimoto et al., 1999).

The effects of incubation time on the probiotic adhesion to intestine has not been completely investigated. In most of the studies, probiotics are allowed to adhere to substrata for 1-2 h. However, incubation time may have a major influence on the observed adhesion, some reports suggest no correlation between the adhesion and incubation time (Aissi et al., 2001; Ouwehand A.C. et al., 2002c). With the progression of incubation time, the number of bacteria sediments to the substrata increases, which leads to increase in the adhesion artificially. However, this sedimentation can be avoided by gently rocking the substratum to counteract sedimentation or by using standardized incubation time. The contact time between bacteria and the intestine is variable in different parts of the intestine, mostly ranging from 10-15 min in the upper small intestine to more than 1 h in the colon, the incubation time should be relevant to this physiological condition (Ouwehand A.C. and Salminen, 2003).

The composition of a buffer used for adhesion study may influence the adhesion assay. Effect of the presence of divalent cations such as calcium and magnesium in incubation buffer, spent culture supernatant and pH of the buffer has been investigated with respect to the adhesion assay (Bernet et al., 1993; Blum S. et al., 1999; Conway and Kjelleberg, 1989; Gusils et al., 1999). In the other reports, the presence of fatty acids also influences *in vitro* adhesion to immobilized mucin (KankaanpÃ¤Ã¤ et al., 2001).

The growth medium composition has been observed to influence the probiotic adhesion. Moreover, the ‘same’ medium obtained from different suppliers has also different adhesion. It has been observed that when lactobacilli grown in milk whey rather than MRS broth, adhesion of lactobacilli to immobilized mucin was significantly lower,

suggesting induction of different adhesive properties during growth in milk whey (Ouwehand A.C. et al., 2001). These findings suggest that appropriate growth medium should be selected.

The presence of normal intestinal microbiota has been observed to dramatically influence the adhesion of probiotics *in vivo*. Normal microbiota potentially provides protection to host by competitive exclusion of pathogens (Adlerberth, Marina Cerquetti, Isabe, 2000; Fons et al., 2000). However, the presence of such microbiota may also reduce the chances of probiotic to adhere and colonize the intestinal surfaces. The influence of faecal microbiota on the adhesion of selected probiotics have been investigated *in vitro* (Ouwehand A.C. et al., 1999b). However, thorough investigation using *in vivo* conditions is required.

1.8 Intestinal epithelial barrier

The epithelial layer is the first line of protection from external environment segregating the submucosa, gut and mucosal associated lymphoid tissues, and bloodstream from contents of the lumen. In addition to forming a protective layer, the role of the epithelium is to regulate the absorption of nutrients, and regulate secretion of lubricants, such as mucins, to hydrate the intestinal surface to mediate nutrient uptake and to compartmentalize organisms including bacteria that colonize the gut (Turner, 2009). Maintenance of this barrier is therefore extremely important. Imbalance in barrier function has been connected to diseases as IBD, irritable bowel syndrome, and celiac disease (Arumugam et al., 2011; Camilleri et al., 2012). Due to the close connection to gastrointestinal disorders, intestinal integrity may become a very relevant research field within gut ecology, gastrointestinal disease, and effects of functional foods. The word "integrity" is defined as "the state of being whole and undivided". Considering the

definition of the word integrity, intestinal integrity is here defined to deal with maintaining the intestinal barrier whole and assembled. This occurs through the mucus layer, the epithelial cells, and the connection between the intestinal epithelial cells (IEC) by tight junctions (TJ). Changes in intestinal integrity may therefore arise by changes in the mucus layer, changes in epithelial cell proliferation or cell death and/or changes in connection between adjacent cells by the TJ.

The term intestinal integrity is quite often used in connection with intestinal permeability. These two terms must be closely related, however they do not cover the same. Intestinal integrity is defined above, while intestinal permeability here is considered to reflect the passage of molecules across the IEC layer. Such a passage can be affected by the epithelial cells forming the barrier, but also interactions by TJs. However, changes in mucus layer alone may not affect the permeability. Hypothesizing that intestinal integrity is accomplished by the mucus layer, the epithelial layer, as well as TJs, while permeability does not cover the mucus layer. Based on this assumption, alterations in intestinal permeability must indicate altered intestinal integrity. One should however be aware that unaltered permeability for selected tracer molecules does not necessarily imply that intestinal integrity is not impaired, since the tracer molecule may be too large to pass across the impairment. Alterations of barrier functions are often measured by trans-epithelial electrical resistance (TEER) *in vitro* that must be a measure of barrier integrity. Additionally as it measures both the leak and the pore pathway (Shen et al., 2011), alterations in this parameter must indicate alterations in the barrier integrity.

Bacterial translocations are also used to indicate impairment of intestinal integrity (Amar et al., 2011; Bovee-Oudenhoven et al., 2003; Petersen et al., 2009). If bacteria should cross the intact epithelial layer through the TJ complex it should be through the

leak pathway. But some state that the passage between the TJ is too narrow for bacteria to cross (Turner, 2009). Therefore increased bacterial translocation is not considered here to initially occur through increased permeability at TJ. Additionally inflammation may also lead to increased permeability as specific pro-inflammatory cytokines increases permeability, this might result in complete disruption of the TJ or loss of epithelial cells, hence leading to bacterial translocation. In a recent study translocation of bacterial DNA and a labelled *E. coli* from the intestinal lumen into tissue was higher in mice fed a high fat (HF) diet than mice fed a normal diet (Amar et al., 2011), hence considered to have impaired intestinal permeability. This supports the hypothesis that impairment of intestinal integrity may lead to bacterial translocation. Conclusively bacterial translocation are here considered to result from decreased intestinal integrity, however the barrier impairment is not initially caused by transport of bacteria across the TJ.

1.8.1 Composition of tight junction proteins

Interaction between IEC is important for maintaining intestinal barrier integrity. IEC interacts through tight junctions (TJ), desmosomes, adhesion junctions, and gap junctions. (Sharma R. et al., 2010; Suzuki, 2013). TJ are mainly responsible for controlling the paracellular flux between the epithelial cells, hence the permeability of the epithelial barrier, while desmosomes and adhesion junctions are important for communication and binding between the cells (Suzuki, 2013). Therefore only the TJ proteins are considered here.

TJ is a complex of several proteins that reside in the apical section of the epithelial cell, (Suzuki, 2013). These proteins include both membrane bound and intracellular proteins that interact between adjacent cells, and also with the cytoskeleton in the cell. This helps stabilising the TJ, but also makes it possible for the cytoskeleton to regulate interactions

between IEC (Sharma R. et al., 2010; Suzuki, 2013). According to the number of transmembrane domains possessed, integral membrane proteins are classified into two major types: four-pass transmembrane proteins include claudins, occludin and tricellulin, and single-span transmembrane proteins such as junctional adhesion molecules (JAM) and Coxsackie and adenovirus-associated receptor (CAR) (Eckburg P.B. et al., 2005). The most relevant TJ proteins, for the present work, are introduced.

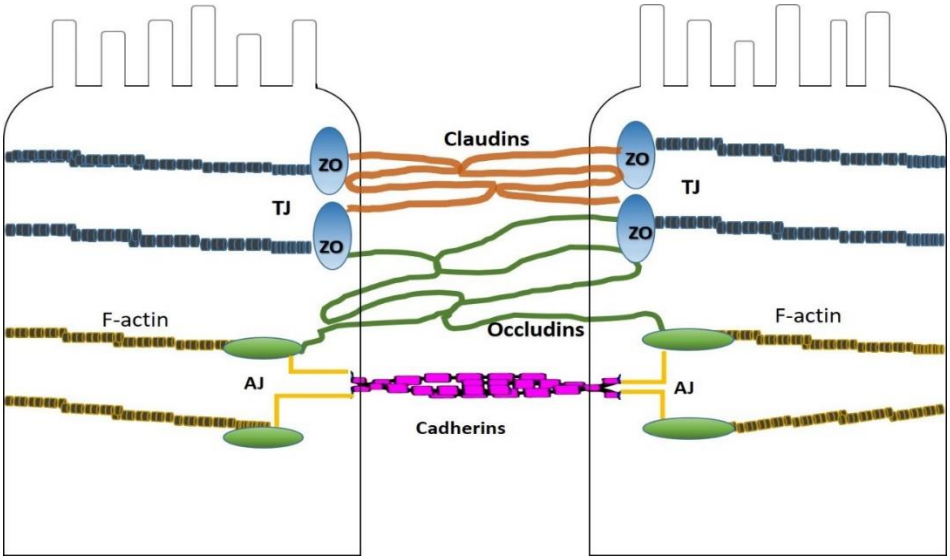

Figure 1. 4 Schematic representation of intestinal epithelial junction.

1.8.1.1 Occludin

Occludin, first identified tight junction integral membrane protein, is a tetra-spanning protein with 2 extracellular loops, and 1 intracellular loop: a short N-terminal and a long C-terminal domain project into the cytoplasm (Feldman et al., 2005; Furuse et al., 1993). It is expressed extensively at tight junctions in epithelial and endothelial cells (Furuse et al., 1993). The extracellular loops of occludin direct the insertion of occludin into tight junctions and may also be required to maintain occludin in tight junctions. In addition, the extracellular loops and the transmembrane domains of occludin are critical for the regulation of selective paracellular permeability likely mediated by intercellular occludin interactions and the regulation of the assembly of tight junction complexes

(Balda et al., 1996; McCarthy et al., 1996). The N-terminal portion of occludin plays a role in tight junction ultrastructure and in the barrier function of tight junctions (Bamforth et al., 1999; Huber et al., 2000).

Although occludin has no role in the cell viability, several studies indicate that it may be required for maintenance of tight junctions and intestinal integrity. Intestinal Caco-2 cells and mouse intestines with occludin knockdown were observed to have increased paracellular permeability to macromolecules (Al-Sadi R. et al., 2011). Similarly, mutated occludin expressed cells although formed TJs but with relatively more permeability towards macromolecules than ions (Balda et al., 1996; Schneeberger and Lynch, 2004). Depletion of occludin affected the expression of another TJ protein claudin-2 at mRNA and protein level (Al-Sadi R. et al., 2011), and occludin-deficient mice had inflammation in the gastric epithelium (Saitou et al., 2000); occludin may therefore have importance for intestinal integrity.

Regulation of occludins interactions and localisation have been linked to phosphorylation (Suzuki 2013). For example some studies have shown that phosphorylation and de-phosphorylation of occludin by protein kinase C isoforms and the phosphatases PP2A and PPI regulates the assembly and disassembly of the TJ complex, respectively (Rao, 2009).

1.8.1.2 Claudins

The claudin super family is comprised of over two dozen proteins, ranging in size from 20-24 kDa. Claudins have a very crucial role in maintaining the intestinal integrity, therefore considered as the backbone of the TJ complex (Suzuki 2013). Structurally claudins are similar to the occludins, with four transmembrane domains, two extracellular loops, and an intracellular loop with short N-terminal and a long C-terminal (Van Itallie and Anderson, 2006). Residues in the cytoplasmic C-terminus

interact with the PDZ binding domains of ZO-1 for anchorage to the cytoskeleton (Groschwitz and Hogan, 2009; Van Itallie et al., 2006).

The extracellular loops of the claudins intercalate with the adjacent cells to form a barrier or pores (Groschwitz et al., 2009; Suzuki, 2013). Specifically the first loop is considered to influence charge selectivity (Van Itallie et al., 2006). On the bases of barrier or pores forming ability, the claudins can be subdivided into two groups. The barrier-forming claudins are considered to be claudin-1, -3, -4, -5, -8, -9, -11, and -14, while the pore-forming claudins are claudin-2, -7, -12, and -15 (Suzuki 2013). The pore forming claudins may form a pore in the TJ specific to charge and size of molecules or ions that can pass through the intestinal barrier. For example the induction of claudin-2 increases the number of pores that can transport small molecules, while mannitol flux remains unaffected (Van Itallie et al., 2008).

The expression level of claudins varies throughout the GIT (John et al. 2011; Suzuki 2013). For example, in the duodenum and colon, mainly the barrier forming claudins, such as claudin-1, -3, -4, -5, and -8 are expressed while in ileum and jejunum the pore-forming claudins, such as claudin-2, -7, and -12 are expressed more (John et al. 2011). However, both pore- and barrier-forming claudins seem to be expressed in all the sections in the GIT (John et al. 2011). Claudins -1, -4, and -5, each confer decreased epithelial permeability mainly by selectively excluding sodium ions (Van Itallie et al., 2006). Conversely, claudin-2 increases paracellular permeability by allowing the passage of sodium ions (Furuse et al., 2001; Van Itallie et al., 2008).

The regulation of the claudins still needs to be elucidated. However, phosphorylation of claudins is considered to affect localization and the interaction with other proteins, hence affect the permeability of the intestine (Groschwitz et al., 2009; Suzuki, 2013). Claudins must be important for the intestinal integrity, as up-regulation of claudin-2

have in some studies been connected to Crohn's disease (CD) and ulcerative colitis (UC), while other claudins, as claudin-1 was down-regulated (John et al. 2011).

1.8.1.3 Zonula occludens

Zonula occludens (ZO) proteins are the intracellular proteins in the TJ. There are three ZO proteins; ZO-1, ZO-2, and ZO-3, where the effect of ZO-1 has been studied the most with regard to intestinal integrity. The ZO proteins have several domains all important for the regulation and maintenance of the TJ structure.

The multi-domain structure of ZO protein includes three PDZ domains, a Src homology-3 (SH3) domain, and a guanylate kinase (GUK) domain. Interestingly, many proteins in the TJ interact with ZO through these domains, for example the claudins binds to ZO-1 at the PDZ-1 domain (Itoh et al., 1999; Suzuki, 2013); JAM-1 binds to PDZ-3 of ZO-1 (Bazzoni G. et al., 2000), and occludin interacts with GUK domain of ZO-1 (Furuse et al., 1994). Since ZO proteins also are able to bind to each other and the actin skeleton (Van Itallie et al., 2008), the TJ proteins seem to interconnect with each other and the actin skeleton (Fanning A.S. et al., 2002; Gumbiner et al., 1991; Haskins et al., 1998). The ZO proteins are therefore important for the assembly of the TJ complex, but also for the interaction between the different proteins in the TJ and the actin skeleton.

Among all the ZO proteins, ZO-1 has been well characterized for its properties and biochemical function. In both, cell culture models and animal models, ZO-1 localizes to the nascent cell-cell contacts. In spite of many intensive efforts, the functional role of ZO proteins in TJ regulation have not been clarified till date with clear evidence. For example, the exogenous expression of full-length or truncated ZO-1 has only a slight effect on the distribution of other TJ proteins and the formation of TJs (Balda and Matter, 2000). According to Umeda et al. (2004), the functional redundancy in ZO

proteins was responsible for such unexpected results. To validate their hypothesis, they have generated epithelial Eph cells lacking both ZO-1 alleles and they observed that these cells were still able to form normal TJ structures and exhibited normal permeability. However, delay in the assembly of other TJ proteins such as occludin and claudins indicates some role of ZO proteins in the regulation of TJ assembly.

1.8.1.4 JAM

The Junctional Adhesion Molecule (JAM), a glycosylated transmembrane protein of 43 KDa, is a member of the Ig superfamily and found at TJs of endothelial as well as epithelial cells. Structurally, JAM has three distinct domains: 2 extracellular Ig domains, one transmembrane domain, and one intracellular C-terminal domain (Martìn-Padura et al., 1998). Through its extracellular N-terminal domains, JAM family members bind to various ligands by homophilic and heterophilic interactions (Gianfranco Bazzoni, 2003). The JAM family members with homophilic interactions play a role in the formation of TJs and the cell-cell border.

In the intestinal epithelial cells, JAM-1 and JAM-4 are expressed and involved in TJ regulation. Inhibition in TEER recovery in a transient calcium depletion assay using antibodies against JAM-1 in T84 cells indicates the role of JAM in TJ sealing (Liu Y. et al., 2000). In another study using JAM-1 knockout mice, the importance of JAM-1 in intestinal barrier function is indicated by higher permeability to dextran and myeloperoxidase activity in colon (Laukoetter et al., 2007). Furthermore, in JAM-1 knockout mice, Dextran sodium sulphate (DSS) induced the colonic injury and inflammation was more severe than in wild-type mice.

1.8.2 Signalling pathways involved in regulation of tight junction

Various physiological and pathological stimuli are involved in the regulation of the assembly, disassembly, and maintenance of TJ. Several signalling proteins are known

to control the signalling pathway associated with TJ regulation, and interactions between transmembrane proteins and the actomyocin ring. These signalling proteins include protein kinase C (PKC), mitogen-activated protein kinases (MAPK), the Rho family of small GTPases, and myosin light chain kinases (MLCK). Epithelial barrier function is also known to be regulated by phosphorylation of TJ proteins. It was observed that MDCK monolayers with high TEER exhibited less phosphorylation in ZO-1 compared to the monolayers with low TEER (Stevenson et al., 1989). Depending on the phosphorylation status of claudin, TJ permeability either increases or decreases (Findley and Koval, 2009). Moreover, assembly of occludin in TJ complex requires its phosphorylation (Wong, 1997).

Many PKC isoforms expressed by the intestinal epithelial cells are involved in the signal transduction pathways. Also different PKC isoforms have different effect on the TEER of intestinal epithelial monolayers (Song et al., 2001). Moreover, PKC is also linked to the TLR-2 pathway (Cario et al., 2004). TLR-2 is associated with microbial recognition and immune modulation, and stimulation. TLR-2 activates PKCα and PKCδ, which cause increase in TEER and redistribution of ZO-1 (Cario et al., 2004). MAPK has been associated to respond under various stress conditions and growth factors (Plotnikov et al., 2011). For example, prevention of hydrogen peroxide induced TJ disruption by epithelial growth factor involves MAPK pathway (Basuroy et al., 2006).

Phosphorylation of myosin-light chain (MLC) is also implicated in the assembly and regulation of TJ. MLCK induction in the Caco-2 monolayers led to decrease in TEER and redistribution of occludin and ZO-1 (Shen et al., 2006). Regulation of TJ structure and function also involves the Rho family of small GTPases, Rac, RhoA, and Cdc42. Rho kinases (ROCK) phosphorylate MLC and thus induce actomyosin ring contraction

(Kimura et al., 1996). Moreover, during TJ assembly in T84 monolayers, improper localization of TJ proteins was observed with inhibition of ROCK (Walsh et al., 2001).

1.8.3 Cytokines mediated TJ regulation

Various pro-inflammatory and anti-inflammatory cytokines are involved in the modulation of inflammatory response in the GIT. Consistent with their role in the pathophysiology of various diseases, cytokines have also been known to regulate TJ barrier. Interferon-γ (IFN-γ), a pro-inflammatory cytokine, increases paracellular permeability in T84 cells through the redistribution and expression of TJ proteins and the rearrangement of the actin cytoskeleton (Bruewer et al., 2003, 2005). It has been demonstrated that IFN-γ induces TJ protein internalization by increasing acto-myosin contractility in a ROCK-dependent manner, thus resulting in TJ barrier disruption. Another pro-inflammatory cytokine, TNF-α, regulates the TJ barrier through various mechanisms. TNF-α induces TEER reduction and increase in the paracellular permeability of Caco-2 monolayer which is reported to be mediated by MLCK pathway (Ma et al., 2004, 2005). Moreover, TNF-α was associated with decrease in TEER through claudin-2 expression in intestinal epithelial cell-line (Mankertz et al., 2009). Thus, impairment of intestinal barrier by TNF-α involves cytoskeleton rearrangement and TJ expression. Interleukin-1β (IL-1β), a pro-inflammatory cytokine of an IL-1 family membrane, decreases TEER and increases inulin permeability in Caco-2 monolayers (Al-Sadi R. et al., 2008; Al-Sadi R. et al., 2007). Treatment of Caco-2 cells with IL-1β induces decreased expression and redistribution of occludin, resulting in such increased inulin influx and TEER reduction (Al-Sadi R. et al., 2007). Regulation of TJ barrier function by IL-1β is mediated through MLCK expression and MLC phosphorylation causing rearrangements of cytoskeleton (Al-Sadi R. et al., 2008, 2010). Another cytokine, interleukin-4 (IL-4) impairs the intestinal TJ barrier through

induction of claudin-2 expression and localization of TJs (Wisner et al., 2008). However, claudin-2 is known to be associated with pore formation for cations which indicates involvement of additional mechanisms. Interleukin-6 (IL-6) has both the pro-inflammatory and anti-inflammatory effects. It selectively increases the paracellular permeability to cations by increasing the expression of claudin-2, a pore forming TJ proteins, in intestinal Caco-2 cells (Suzuki et al., 2011). An anti-inflammatory cytokine, interleukin-10 (IL-10), is associated with protection of the intestinal barrier. In a mice model of colitis, induced by IL-10 knockout, intestinal permeability was increased with elevated expression of TNF-α, IL-1β, and IL-6 (Madsen K. L. et al., 1999). Additionally, INF-γ-induced increases in mannitol and inulin influx in T84 cells was prevented by IL-10 (Madsen K. L. et al., 1997). Interleukin-17 (IL-17), associated with the pathogenesis of various autoimmune diseases, increases the expression of two functionally different isoforms of claudins in T84 cells (Kinugasa et al., 2000). Interestingly, this increased expression of claudin-1 and claudin-2 by IL-17 induction resulted in the increased TEER and decreased mannitol permeability (Kinugasa et al., 2000). Increase in TEER and decrease in paracellular permeability of T84 cells has also been observed by transforming growth factor (TGF-β) induction in both a dose- and time-dependent manner (Howe et al., 2005). This protective effect of TGF-β on intestinal epithelial barrier is mediated by claudin-1 expression through MEK/ERK signalling pathway (Hering et al., 2011).

1.8.4 Effect of altered intestinal integrity

Impairment of intestinal integrity is connected to inflammation and gastrointestinal disorders, hence impairment of intestinal integrity can cause adverse effects. Impaired intestinal integrity or altered intestinal permeability has been connected to among other Crohn's disease (CD), ulcerative colitis (UC), Celiac disease, irritable bowel syndrome,

necrotizing enterocolitis (NEC), and obesity (Bergmann et al., 2013; Camilleri et al., 2012; Cani P.D. et al., 2006; John et al., 2011). According to Turner (2009), patients with diseases such as CD have a barrier defect before clinical disease is initiated, while enteropathogenic *E. coli* infection leads to a defective barrier then disease occur. Additionally, some pathogenic bacteria have been shown to decrease TEER indicating that increased permeability may be exploited by such pathogens, hence leading to bacterial translocation. In mice with induced NEC, intestinal permeability was also increased before the disease onset (Bergmann et al., 2013). This indicates that permeability may be increased before disease onset. Based on a literature review Turner (2009) stated that disease may not be caused by only changes in the TJ, but such changes would increase immune response in the host, hence increasing the risk of disease. This occurs potentially by an increased inflammatory state. Others support this hypothesis. The permeability for fluorescein isothiocyanate-dextran (FITC-dextran) was positively correlated to plasma LPS (Cani P.D. et al., 2009), indicating that LPS can translocate together with FITC dextran. This was suggested by the same authors in another paper, to lead to inflammation (Cani P.D. et al., 2006) and hence bacterial translocation.

The epithelial layer must however exploit the opening of the TJ complex during e.g. nutrient uptake. One may argue that increased permeability leading to increased flux of specific ions or nutrients across the epithelial layer may be beneficial. For example an increased flux of calcium may be beneficial as it would increase its uptake. It should however subsequently not result in an inflammatory stated, barrier impairment, and bacterial translocation. Changes in permeability up to a certain size or a specific duration may therefore be favourable. It is therefore relatively difficult to state if changes in intestinal permeability are beneficial or not; it depends on how much the integrity subsequently is changed. If the integrity is impaired leading to inflammation

and disease the altered permeability is adverse, however if integrity is maintained following the altered permeability, host health may not be affected.

1.8.5 Modulation of intestinal barrier function by lactobacilli

Both *in vitro* and *in vivo* studies have demonstrated that probiotics including lactobacilli improve and/or protect the intestinal integrity which may have been demonstrated under the influence of stress, infection, or cytokines. Treatment of Caco-2 cells with *Lactobacillus plantarum* MB452 (one of the probiotic strains from VSL#3) lead to increased gene expression of occludin and cingulin (Anderson et al., 2010). In another report using IL-10 knockout mice as a colitis model, administration of *L. plantarum* restored the expression of TJ proteins and improved TEER and mannitol flux in the colon (Chen H.-Q. et al., 2010). In Caco-2 cells, treatment of *L. plantarum* prevented TNF-α- and phorbol ester-induced dislocation of occludin and ZO-1, and associated reduction in TEER by supressing the NF-κβ signalling pathway (Karczewski J. et al., 2010; Ko et al., 2007). In a different report, *L. plantarum* CGMCC No. 1258 prevented enteroinvasive *E. coli* (EIEC)-induced loss of expression and redistribution of TJ proteins in Caco-2 cells when cultured simultaneously (Qin H. et al., 2009). Co-culturing of Caco-2 cells with *L. plantarum* DSM 2648 exhibited protection against enteropathogenic *E. coli* (EPEC)-induced TEER reduction. It possibly does so by reducing EPEC adherence to Caco-2 cells (Anderson et al., 2010).

Lactobacillus rhamnosus (*L. rhamnosus)* strains exhibited protection against epithelial barrier dysfunction in both *in vitro* and *in vivo* conditions. Administration of *L. rhamnosus* OLL2838 in the mouse model of DSS-induced colitis ameliorated intestinal inflammation and barrier function (Miyauchi E. et al., 2009). In the same study, the OLL2838 strain demonstrated inhibition of TNF-α induced decrease in TEER in Caco-2 cells through normalization of ZO-1 and MLCK expression. *L. rhamnosus* GG

(LGG), widely accepted probiotic strain, is reported to have protective effect on epithelial barrier dysfunction induced by infection and cytokines. Pre-treatment of Caco-2 cells with LGG inhibits impairment in barrier integrity induced by TNF-α and IFN-γ. When co-stimulated, TNF-α and IFN-γ redistributed ZO-1 and decreased TEER (Miyauchi E. et al., 2009). Similar observation was reported when T84 were treated with LGG prior to infection with EHEC (Donato et al., 2010).

Besides live lactobacilli culture, the metabolites secreted by certain lactobacilli is also known to exert beneficial effect on the TJ expression and barrier integrity. For example, soluble proteins such as p40 and p75 from LGG inhibited oxidative stress-induced barrier dysfunction (Seth et al., 2008). This effect is mediated through activation of the ERK and PKCβ/ε pathway. In DSS-induced colitis model of mice, p40 ameliorated intestinal barrier defect, indicated by dextran influx and ZO-1 distribution (Yan F. et al., 2011).

1.8.6 Methods used to study intestinal integrity *in vitro*

Primary epithelial cells *in vivo* develop a tightly packed selectively permeable membrane with measurable transepithelial resistance (also abbreviated as TEER) and VT (transepithelial potential difference); *in vitro* epithelial cells that polarise should develop these features when grown on microporous membrane. Expression of tight junction proteins is necessary for the formation of epithelial barrier, integrity and polarity (Shin et al., 2006). Tight junction permeability in cell culture is generally measured using either of two techniques: transepithelial electrical resistance (TEER) or paracellular tracer flux. In most of the *in vitro* studies related to intestinal integrity, human colon tumorigenic cell lines Caco-2, T84 and HT-29 have been used. In the present study also, intestinal epithelial cell-lines Caco-2 and HT-29 were used to study the intestinal integrity *in vitro*.

To study the TEER of an epithelial barrier, access to the culture medium bathing both the apical and basal regions of the cells is required. This is the basis behind the use of Transwell® supports. Transwells® are composed of a semi-permeable membrane made of an inert polymer (most commonly polystyrene). The membrane is supported by an impermeable plastic frame that forms a small well, which is suspended in a larger well. Both the inner and outer wells contain cell culture medium to form an apical and basolateral environment for the cell culture (see figure 1.5). Confluent cell cultures are trypsinized into single cell suspensions and seeded into the inner well of the apparatus. The choice of membrane pore size depends on the application. The smallest pore sizes (approximately 0.4 μm in diameter) are generally used for studying the passage of ions, macromolecules, and in bacterial infection systems. When using larger pore sizes, bacterial translocation across a monolayer can be quantified (Kalischuk et al., 2009). Once the cell monolayer has formed and becomes polarized, physiological measurements can be made to test barrier function. The simplest and least invasive is to measure passive ion flow with a voltmeter and electrodes. An electrode plate is placed on either side of the Transwell apparatus and TEER is measured.

Electrical resistance across a monolayer represents the sum of the paracellular resistance, which consists of the resistance of the junction and the intercellular space, and the transcellular resistance, which consists of the resistances of the apical and basolateral cell membranes (Claude, 1978). TEER is inversely proportional to the permeability of the epithelium to ions (McCormick, 2003).

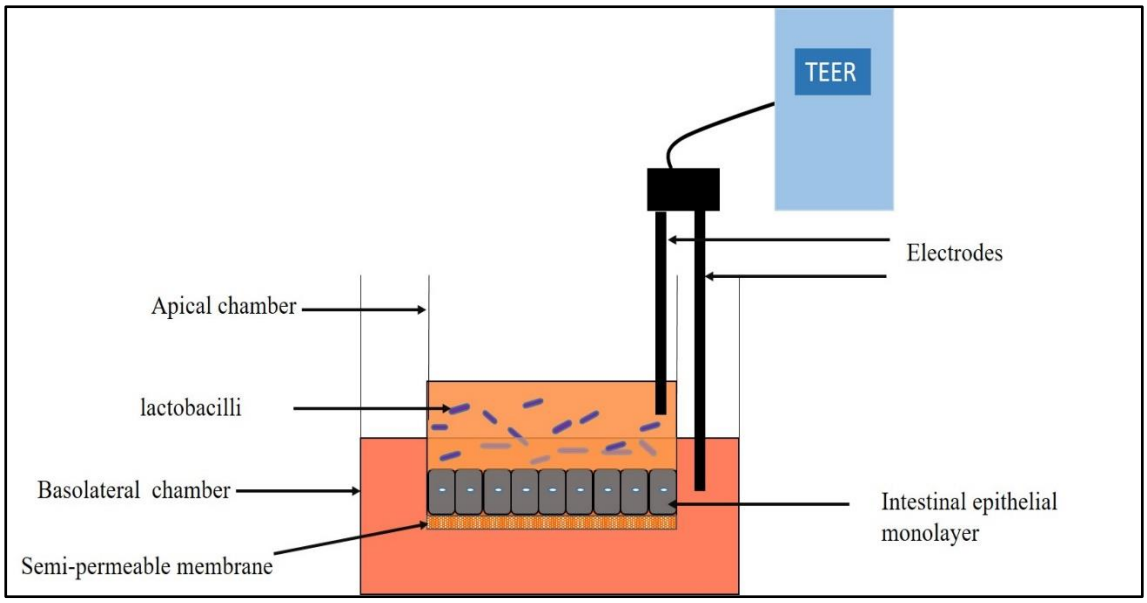

Figure 1. 5 Transwell apparatus for studying epithelial barrier function.

As a complementary measure, conjugated solutes or enzymes can be added to one face of the epithelium and allowed to diffuse into the opposing environment for a designated time period. Such molecules should be inert (that is, not consumed by the cells for energy or permanently incorporated into the cells), and care must be taken to conserve the integrity of the conjugate. There are a variety of widely used probes ranging in different sizes. The size of the probe must also be taken into account because large probes, such as horseradish peroxidase (44 kDa), generally do not diffuse across intact monolayers in a paracellular fashion through the apical junctional complex (Matsuzawa et al., 2005). In the present study intestinal permeability have been determined using FITC-inulin and FITC-labelled latex beads (2.0 μm).

1.9 Ulcerative colitis

Ulcerative colitis (UC) is a long-term condition that results in inflammation and ulcers of the colon and rectum (Ford et al., 2013). The primary symptom of active disease is abdominal pain and diarrhoea mixed with blood. Weight loss, fever, and anaemia may

also occur Often symptoms come on slowly and can range from mild to severe (Ford et al., 2013). Symptoms typically occur intermittently with periods of no symptoms between flares. UC starts from the rectum, spreads proximally and in continuity, involving a variable length of the colon. The exact cause of UC is still unknown, however the possible cause includes immune system dysfunction, genetics, changes in the normal intestinal flora, and environmental factors (Low et al., 2013). According to several reports, intestinal inflammation occurs due to the T-cell responses to luminal enteric microbes in hosts with genetic polymorphisms that regulate the mucosal barrier function, microbial killing, or immune response (Eckburg P.B. and Relman, 2007; Strober et al., 2007). UC induces production of various autoantibodies, such as anti-tropomyosin and anti-neutrophil cytoplasmic antibody (pANCA) which is mediated by Th-2-dependent immune response (Das et al., 1993; Saxon et al., 1990). The other evidence suggesting a Th-2 mediated immune response in UC includes pre-dominancy of Th-2 related immunoglobulin sub-classes such as IgG1 and IgG4 antibodies (Kett et al., 1987). In addition, it is associated with increased secretion of IL-5 (Fuss et al., 1996). Moreover, in UC increased expression of Epstein-Bar-virus-induced gene 3 (*EBI3*) was observed. *EBI3* encoded product has been tentatively identified as a Th-2 cytokine. These evidences suggest that UC is a Th-2 mediated disease (Christ et al., 1998). In the experimental model of oxazolone-induced ulcerative colitis, IL-13-producing NKT cells were reported to have a role in the inflammation (Heller et al., 2002). It is also observed that in UC, production of IL-15, IL-16 and IL-18 cytokines were higher, indicating their role in disease pathogenesis (Pagès et al., 2001).

Several studies indicated the role of genetic factors in the development of UC. The disease is however influenced by multiple genes rather than the involvement of a single gene. The potential loci involved in UC in human are linked to chromosomes 16, 12, 6,

14, 5, 19, 1, and 3 (Cho et al., 2000). Susceptibility to the intestinal inflammation is also determined by the host genetic background. For example, some inbred of mice strains with IL-10 deficiency are resistant to colitis whereas the others are susceptible (Mähler and Leiter, 2002). Some environmental factors such as diet also contribute in the pathogenesis of UC. One such study shows enhancement in the risk of developing UC with high intake of unsaturated fat and vitamin B6 (Geerling et al., 2000). In another report, high consumption of meat protein and alcoholic beverages also influenced the development and/or relapse of colitis (Andersen et al., 2012; Jowett et al., 2004).

1.9.1 Intestinal epithelial barrier function in UC

Intestinal barrier dysfunction is a main feature of inflammatory diseases including UC. Patients with UC suffer from inflammation-induced leak flux diarrhea which is caused by a passive loss of ions and water from the circulatory system into the lumen. Besides this, impaired barrier function also results in the increased uptake of noxious antigens from the intestinal lumen, hence enhances mucosal and systemic inflammatory processes. Several studies have reported the major role of epithelial TJ in barrier disruption characteristic of UC (Heller et al., 2005; Hering et al., 2012; Schmitz et al., 1999). Alterations in the epithelial TJ comprises altered TJ gene and protein expression and its subcellular distribution. (Schmitz et al., 1999). Investigations on colon biopsies from patients suffering from UC revealed that expression of sealing components such as occludin, claudin-1 and -4 were down-regulated while expression of pore-forming, claudin-2 was upregulated (Heller et al., 2005). The detailed structure of different TJ proteins and their involvement in the regulation of intestinal permeability has been described earlier in this chapter. Additionally, increase in the apoptosis associated leak flux was observed in UC (Heller et al., 2005). Moreover, Heller et al., (2005) reported that in UC, microerosions was due to the arrested restitution caused by a Th2 cytotkine

IL-13. Increased IL-13 production in the lamina propria was also accompanied by increased expression of claudin-2, a pore-forming TJ, which leads to the development of impaired epithelial barrier function and is thought to be responsible for diarrhoea (Amasheh et al., 2002). Thus, the important barrier pathomechanisms involved in UC includes changes in TJ structure or composition, and apoptotic leaks as well as gross lesions which allows penetration of various food or microorganisms derived noxious antigens into the mucosal barrier. The cytokines mediated TJ regulations involves expression regulation and effects on the redistribution of TJ proteins. TJ regulation by cytokines has been discussed above (Section 1.8.3).

1.9.2 Treatments of colitis

Several medications are available for the treatment of colitis. In the treatment of mild disease, oral sulfasalazine is given either alone or in combination with topical medications. For over years, sulfasalazine has been a major agent in the therapy of colitis. Topical medications include mesalamine suppositories, mesalamine and steroid enemas, and corticosteroid foam. Such treatment may reduce symptoms of distal colonic disease. Very rarely, corticosteroids such as prednisone is suggested as an immunosuppressive agent because of their associated risks of developing cancers, tuberculosis, and new or worsening heart failure (Axelrad et al., 2016). The other immunosuppressive agents include azathioprine and biological agents such as adalimumab and infliximab. The biological immunosuppressive agents are effective in the patients who are resistant to the conventional medications. Infliximab is a chimeric IgG1 monoclonal antibody developed against a pro-inflammatory cytokine TNF-α. The administration of infliximab is effective for induction and remission of UC (Rutgeerts et al., 2005; Sandborn W. et al., 2005). Adalimumab is a complete human IgG1 monoclonal antibody to TNF- α. This anti-TNF- α antibody is responsible for blocking

the interaction of TNF- α with its cell surface receptors such as p55 and p75, thus inhibiting the associated inflammation in patients with UC. Treatment of patients suffering from moderate-to-severe ulcerative colitis with adalimumab induced and maintained the clinical remission (Sandborn W. J.et al., 2012).

1.9.3 Lactobacilli in the management of colitis

Several data from *in vivo* studies suggest the involvement of luminal bacteria in inflammation and alteration of barrier function in UC. Various probiotic bacteria including lactobacilli are known to alter gut bacteria composition thereby improving the management of colitis. They interact with immunocompetent cells and modulate the production of pro-inflammatory cytokines. Lactobacilli are also known to have direct effect on the TJ structure of intestinal epithelial barrier. In IL-10 gene-deficient mice, VSL#3 treatment resulted in normalization of barrier function by decreasing the pro-inflammatory cytokines (Bibiloni et al., 2005; K. Madsen et al., 2001). Additionally, VSL#3 also diminished the alterations in the cellular cytoskeleton structure and expression of ZO-1 induced by *Salmonella* infection (Jepson et al., 1995, 2000). In DSS-induced colitis model of mice, pre-treatment with probiotics, either multispecies or single strain, inhibited gut leakiness by restoration of TJ structures and increased expression of ZO-1 and MLCK (Miyauchi E. et al., 2009; Ukena et al., 2007). In another study, psychological stress-induced colitis in rat caused increase in the transepithelial macromolecular flux in the intestine, which was prevented by administration of probiotics containing *Enterococcus faecalis, Lactobacillus* sp. and *Bifidobacterium* (Laudanno et al., 2008; Zareie et al., 2006). Moreover, *L. casei* was also reported to decrease the severity of colitis in mice induced by DSS. Pre-treatment of mice with *L. casei* prevented loss in body weight, colon shortening and histological damage in colonic tissue (Kokesova et al., 2006). Supplementation of probiotic compound VSL#3

in patients with mild UC exhibited decrease in the disease activity index score and induced remission of UC (Bibiloni et al., 2005).

1.9.4 Animal models used to study colitis

In the past decades, several different animal models of UC have been developed which have provided useful insights into the pathogenesis of the intestinal inflammatory response. Such models include inducible colitis models, spontaneous colitis models, genetically modified models, and adoptive transfer models (Byrne and Viney, 2006; Wirtz et al., 2007). However, none these models exactly reproduce the human UC conditions. The most widely used models are based on the chemical induced colitis models because of the simplicity in the process. Moreover, the inset, duration, and severity of inflammation are immediate and controllable with chemical-based models (Wirtz et al., 2007).

Colitis can be chemically induced by administration of 2, 4, 6-trinitro benzene sulfonic acid (TNBS), dextran sodium sulfate (DSS), or oxazolone. Induction of colitis with TNBS requires its administration along with alcohol. Alcohol is responsible for disruption of the epithelial layer, exposing the underlying lamina propia to the bacterial components (Neurath et al., 1995). Intrarectal administration of TNBS induces intestinal inflammatory response similar to the CD in humans. It induces severe transmural inflammation associated with diarrhea, weight loss, rectal prolapse, and inflammation driven by IL-12 with Th1-mediated response (Neurath et al., 1995). Moreover, different mouse strains exhibit different susceptibility for TNBS. For example, C57BI/6 and C57BI/10 mice are resistant whereas BALB/c and SJL are susceptible to TNBS. The susceptibility is observed to be related to the genetic factors such as high IL-12 response to the lipopolysaccharide locus in SJL/J mice (Bouma et al., 2002).

Intrarectal administration of oxazolone with ethanol induces acute colitis in mice. Oxazolone administration induces increase in the production of IL-4 and IL-5, a characteristic of Th2- type immune response. The typical symptoms of oxazolone-induced colitis are weight loss, ulcers, diarrhoea, and loss of epithelial cells in the large intestine (Boirivant et al., 1998). Colitis induction by oxazolone is limited to the distal colon only and the inflammation manifests as relatively superficial ulceration (Boirivant et al., 1998). The histological features and inflammatory distribution is similar to that observed in human UC (Boirivant et al., 1998).

1.9.4.1 DSS-induced colitis in mice

DSS-induced colitis model is the most commonly used chemical induced colitis model. This model has advantages over other chemical induced models such as by changing the concentration of DSS administered, one can easily create acute, chronic, or relapsing colitis. Moreover, it has been observed that DSS induced colitis provides a relevant model for the translation of mice data to human diseases. In 1985, Ohkusa et al. first used DSS for colitis induction in hamster. Animals are given DSS to drinking water for colitis induction (Ohkusa, 1985). They may develop either acute or chronic colitis depending on the concentration, the duration, and the frequency of DSS administration (De Robertis et al., 2011; Kanneganti et al., 2011). Differences observed in the susceptibility and responsiveness to the DSS is also dependent on the genetic and microbiological factors of animal. The genetic factors are strain and gender of the animals whereas the microbiological factors are the intestinal flora of the animal (Mähler and Leiter, 2002). DSS is a sulphated polysaccharide with a molecular weight ranging between 5 kDa to 14,000 kDa. Depending on the molecular weight used, the severity of colitis and carcinogenic activities differs. For example, when BALB/c mice were administered with 40kDa molecular weight DSS, the most severe form of colitis

was observed (Kitajima, Takuma, & Morimoto, 2000), whereas administration of mice with DSS of 50 kDa did not develop any lesions. Moreover, molecular weight of DSS has also effect on the location of colitis. Very severe and diffused colitis in the middle and distal third of the large bowel was developed when 40 kDa DSS was administered while mice administered with 5 kDa DSS developed relatively patchy lesions mainly in the cecum and upper colon (Kitajima, Takuma, & Morimoto, 2000). Administration of 2-5% DSS for a short period of 4 to 9 days causes acute colitis while continuous treatment of low concentration of DSS is responsible for induction of chronic colitis (Mannick et al., 2005; Rakoff-Nahoum, et al., 2004). In acute phase of DSS colitis, clinical manifestations observed are weight loss, watery diarrhoea, occult blood in stools, anaemia and eventually death. The histological changes include depletion of mucin, epithelial degeneration, and necrosis which leads to disappearance of epithelial cells (Perše and Cerar, 2012). Advanced phase of DSS-induced colitis is associated with neutrophil infiltration of lamina propria and submucosa crypt abscesses, cryptitis, and inflammation in mucus and submucosa (Melgar et al., 2005). The toxicity of DSS to colonic epithelial cells results in epithelial barrier dysfunction. Administration of mice with DSS results in increased colonic mucosa permeability which allows formation of large molecules suggest DSS. Changes which start appearing even after 1 day of DSS treatment include loss of tight junction complex protein ZO-1 (Ichikawa-Tomikawa et al., 2011; Laprise, 2011; Poritz et al., 2007) and increased expression of pro-inflammatory cytokines (Yan Y. et al., 2009). In colon, significant increase in the Evan's blue permeability is observed after 3 days of DSS administration. By this time, histological changes start appearing in the colonic mucosa such as loss of basal crypts and infiltration of inflammatory cells. Impairment of epithelial barrier function associated with acute DSS colitis includes loss and redistribution of occludin, claudins

and ZO-1 (Mennigen et al., 2009; Poritz et al., 2007). DSS-induced breakdown of mucosal epithelial barrier function allows the entry of luminal antigens and microorganisms into the mucosa resulting in overwhelming inflammatory response.

Inflammatory mediators involved in the pathogenesis of DSS induced colitis includes cytokines, nitric oxide, reactive oxygen species, and complement system activation product, which are similar to the pathogenesis of human colitis. After first day of the treatment, increase in the expression of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, IL-12, and IL-10 was also observed which increases progressively during DSS treatment (Yan Y. et al., 2009).