CHAPTER 2

In vitro evaluation on

Adhesion abilities of selected Sporeformers
2.1 Introduction

Probiotic bacteria have an important role in keeping the intestinal balance and protection against gastrointestinal pathogens. Sporeformers with probiotic properties can be used in the therapy of intestinal disorders of various origin. Microflora of the gastrointestinal tract plays a crucial role in the anatomical, physiological and immunological development of the host. Spore forming bacteria do not belong to the commensal microbiota present in the human GIT. However, several health food supplements and therapeutic products that contain one or more *Bacillus* strains/species have been consumed by humans since the second half of the twentieth century. Collado *et al.*, 2008 found that adherence of bacteria to the intestinal epithelium of host is known to be a prerequisite for colonization and infection of the gastrointestinal tract by many pathogens. Adhesion to gastrointestinal tract is a necessary probiotic feature for effective probiosis. Criteria for the selection of probiotics include lack of pathogenicity, tolerance to gastrointestinal conditions (acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens. Adhesion is a very important physical trait of probiotic bacteria which helps in the attachment and colonization in the host intestine. Probiotic bacteria can prevent the adhesion and invasion of gastrointestinal pathogens. Gastrointestinal pathogens have the ability to disturb intestinal microbial balance and capable for subsequent pathogenesis. It is generally agreed that LAB must adhere to intestinal mucus or epithelial cells in order to persist in the gut. The ability of probiotics to adhere on mucosal surfaces prevents their rapid removal by gut contraction and subsequent peristaltic flow of digesta,
and could also confer a competitive advantage. Adhesion is regarded a prerequisite for colonization in intestinal tract, antagonistic activity against enteropathogens, modulation of the immune system and for increased healing of the damaged gastric mucosa (Rinkinen et al., 2000). The ability to colonize is often considered as a main selection criterion for probiotics, that is, the efficient adherence to intestinal epithelial cells to reduce or prevent colonization of pathogens (Vine et al., 2004b; Kesarcodi-Watson et al., 2008).

Bacterial adhesion is a complex process initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesins (usually proteins) and complementary receptors (Perez et al., 1998). In order to manifest beneficial effects, probiotic bacteria need to achieve an adequate mass through aggregation. Autoaggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cells and the ability to coaggregate with other bacteria such as pathogens may form a barrier that prevents colonization by pathogenic microorganisms (Boris et al., 1997; Del Re et al., 1998). Adherence of bacterial cells is usually related to cell surface characteristics (Bibiloni et al., 2001; Canzi et al., 2005). Physicochemical characteristics of the cell surface such as hydrophobicity may affect autoaggregation and adhesion of bacteria to different surfaces (Wadstrom et al., 1987; Perez et al., 1998; Del Re et al., 1998). Biofilm formation helps in flourishing the probiotic in intestinal niche and produce antagonistic substances against pathogens.

A large body of research has been conducted to screen probiotic bacteria for their ability to attach to intestinal cells (Goktepe et al., 2006). In vitro experimentation shows that some strains of Lactobacillus adhere to intestinal tissue cultured cells in a species-dependent way (Fuller, 1975). The survivability and colonization in the digestive tract are considered critical
factors to ensure optimal functionality and expression of health promoting physiological functions by probiotics. Enteric fever and cholera are the common epidemics which causes health problems in developing countries. Use of antibiotics in the treatment of gastrointestinal infections lead to the emergence of antibiotic resistant strains of gastrointestinal pathogens. Development of probiotic against gastrointestinal infections will prevent and cure the diseases to some extent. In the present chapter II, adhesion and cell surface properties of selected five wild species of spore formers having basic probiotic properties were compared with the enteric pathogens *Salmonella typhi*, *Salmonella paratyphi* A and *Vibrio cholera*.

2.2 Review of Literature

Several studies consider adhesion to mucus, as one of the important criteria for selecting probiotic bacteria (Olsson *et al*., 1992; Chabrillón *et al*., 2006). One of the prerequisites of a good probiotic is adhesion to mucus and epithelial cells (Savage *et al*., 1992). Adhesion to the mucosa is, however, influenced by the flow rate, competition for nutrients, adhesion sites and specific physicochemical properties of the probiotic (Wadstro¨m *et al*., 1987; Schillinger *et al*., 2005).

Adhesion to epithelial cells is facilitated by cell surface carbohydrates, proteins (including S-layer proteins) hemagglutins and lipoteichoic acids (Bernet *et al*., 1994; Greene and Klaenhammer 1994; Andreu *et al*., 1995; Alderberth *et al*., 1996; Granato *et al*., 1999; Satoh *et al*., 1999; Roos and Jonsson 2002; Vidal *et al*., 2002; Frece *et al*., 2005). A relationship between autoaggregation and adhesion ability in *Bifidobacterium bifidum* (Perez *et al*., 1998) and *B. suis* (Del Re *et al*., 1998) has been reported and a correlation between hydrophobicity and adhesion ability has been observed in some *lactobacilli* (Wadstrom *et al*., 1987).
Many authors have reported that the coaggregation abilities of *Lactobacillus* species might enable it to form a barrier that prevents colonization by pathogenic bacteria (Boris *et al*., 1997; Schauder and Bassler *et al*., 2001). Autoaggregation ability test together with cell-surface hydrophobicity and coaggregation abilities with pathogen strains could be used for preliminary screening identifying potentially adherent bacteria with properties suitable for commercial purposes (Vlkova *et al*., 2008). *Roseobacter* showed high adhesion capability and formation of biofilm under growth conditions, which facilitates its production of an antibacterial compound (Bruhn *et al*., 2005). Therefore, it is speculated that the biofilm mode of growth could facilitate antagonism. Following section provides a brief review of selection of potential probiotic strains on different criteria such as adhesion, autoaggregation, coaggregation with pathogens, cell-surface hydrophobicity, and biofilm formation.

2.2.1 Adhesion on intestinal mucus

Probiotics make up part of the resident microflora and contribute to the health or well-being of their host (Gatesoupe, 1999). To remain within their host, they must either attach to the intestinal tract or grow fast enough to prevent them from being flushed out by the movement of food through the digestive tract. Adhesion is a complex trait that could be a multistep process in which both non-specific (e.g., hydrophobicity) mechanisms and a specific ligand receptor play a role. Servin and Coconnier (2003) showed that the *Lactobacilli* adhesion process included passive forces, electrostatic interaction, hydrophobic and steric forces. It has been reported that lipoteichoic acids are one of the factors responsible for adhesion of *Lactobacillus johnsonii* La I (Granato *et al*., 1999). In addition, adhesion-promoting proteins, which are present on the cell surface of *Lactobacillus*...
*fermentum* and *Lactobacillus brevis* have been isolated and characterized (Rojas *et al.*, 2002).

Adhesion of probiotic bacteria to the intestinal mucosa has been shown to enhance their antagonistic activity against pathogens (Coconnier *et al.*, 1993). In humans, the antibody titres in serum treated with probiotic bacteria were directly correlated with the adherence ability of that strain (Juntunen *et al.*, 2001) suggesting that probiotic attachment enhanced the health of its host. In a healthy gut, attachment may allow the probiotic to exert its beneficial effects while in a diseased gut it may reduce the possibility of pathogen translocation when the host’s defence mechanisms are impaired (Apostolou *et al.*, 2001). Adhesion studies of bacteria to mucosal surfaces has been tested widely (Balcázar *et al.*, 2007b; Collado *et al.*, 2007; Vine *et al.*, 2004b; Nikoskelainen *et al.*, 2001).

By attaching to the intestinal mucosa, probiotics can extend their time within the gut thereby influencing the gastrointestinal microflora of their host. Urdaci *et al.*, studied adhesion capabilities of some *Bacillus* strains from commercial probiotic preparations on three different model intestinal surfaces (mucin, Matrigel and Caco-2 cells). He concluded that, adhesion of spores was higher than that of vegetative cells to the three matrices, and overall strain *Bacillus cereus* CH displayed the best adhesion. Many *in vitro* models have been used to study the adhesion of probiotic cells to epithelial cells. Of these, the colon adenocarcinoma cells Caco-2 and HT-29 are the most popular choice (Tuomola and Salminen 1998; Ouwehand *et al.*, 1999; Gopal *et al.*, 2001; Sambuy *et al.*, 2005).

Adherence is strain-specific and is not defined to specific levels in any species. *Lactobacillus rhamnosus* GG, one of the best studied probiotics,
adheres at a relatively high level (9.7%) to Caco-2 cells (Elo et al., 1991; Tuomola and Salminen 1998). Strains of *L. casei, L. paracasei, L. acidophilus* and dairy strains of *L. rhamnosus*, on the other hand, adhered to Caco-2 cells at levels ranging from 3 to 14% (Forestier et al., 2001; Gopal et al., 2001; Bertazzoni-Minelli et al., 2004; Baccigalupi et al., 2005; Schillinger et al., 2005; Pennacchia et al., 2006). In general, much higher levels of adhesion (20–40%) have been recorded to mucus producing HT29 MTX cells (Schillinger et al. 2005). Adhesiveness to the human intestine is one of the most important characteristics of probiotic bacteria. Attachment of probiotic strains to the epithelial cells and intestinal mucosal is prerequisite for the intestine colonization as it influence the time of bacteria retention in the intestine and the functional activity of bacteria (Lin et al., 2006). For effective mechanism of probiotic action, the probiotic bacteria should be properly adhered to intestinal mucosa.

### 2.2.2 Hydrophobicity

Several mechanisms are involved in the adhesion of microorganisms to intestinal epithelial cells (Savage, 1992). The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue (Kiely and Olson, 2000). This property could confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract (Naidu et al., 1999). LAB commonly expresses cell surface hydrophobicity as measured by the salt aggregation test, contact angle and adhesion to xylene (Wadström et al., 1987). Collado et al. (2007) showed that autoaggregation of LAB correlates with their adhesion ability. The determination of microbial adhesion to hexadecane as a way to estimate the ability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach (Kiely and Olson, 2000). Ong et al. (1999)
showed that hydrophobic interactions were dominant in the adhesion of two genetically similar *E. coli* strains, differing only in their lipopolysaccharide composition. They also revealed that modification of the surface hydrophobicity would be effective in controlling the adhesion of cells. The hydrophobic nature of substrate had great influence on the adhesion rate of *S. cerevisiae* cells (Kang and Choi, 2005).

Bujnáková *et al.*, 2004 performed correlation between cell-surface hydrophobicity and autoaggregation activity of human and pig *Bifidobacteria*. Gómez-Zavaglia *et al.*, (2002) showed a decrease of surface hydrophobicity of *Bifidobacteria* growth in bile in correlation with the loss of adherence and autoaggregation. Collado *et al.*, (2008) determined the hydrophobicity of *Bifidobacteria* by their affinity to hydrocarbons and showed a positive correlation between aggregation ability and hydrophobicity among all *Bifidobacteria* tested. In contrast, Vlkova *et al.*, (2008) demonstrated that *Bifidobacterial* hydrophobicity did not significantly correlate with their aggregation properties (autoaggregation and coaggregation). This corresponds with the results of Gueimonde *et al.* (2005), who reported that hydrophobicity levels of *Bifidobacteria* showed considerable variability and did not correlate with the degree of their adhesion to intestinal mucus, which corresponds with autoaggregation.

Ouwehand *et al.* (1999) observed no correlation between cell surface hydrophobicity and the ability to adhere to intestinal mucus. Furthermore, all tested strains had relative low surface hydrophobicity (Ouwehand *et al.*, 1999). This result was in agreement with observations made by Savage (1992), who did not observe any correlation between surface hydrophobicity and adhesion of *Lactobacillus* strains to the murine gastric mucosa. However, Wadstrom *et al*. 1987 observed that *Lactobacillus* strains, isolated
from porcine small intestine, showing high surface hydrophobicity adhered in high numbers to porcine enterocytes. The observed differences in measured cell surface hydrophobicity can be explained by the use of different methods; partitioning in hexadecane (Savage, 1992; Ouwehand et al., 1999) and the salt aggregation method (Wadstrom et al., 1987). According to Del Re et al., 1998 hydrophobicity results obtained by one method need not be comparable with those obtained by another one, because cell surface hydrophobicity methods do not measure the intrinsic microbial cell surface hydrophobicity but rather the bacterial adhesion to certain hydrophobic substrates.

2.2.3 Microbial Aggregation

Research in microbial aggregation dates back over a century. Early researchers tried to explain microbial aggregation with summations of numerous physical inter-particle forces, homologous with the flocculation of colloidal solutions by salt (Buchanan, 1919). According to this theory, aggregation occurs when the similar electric charges on cell surfaces are decreased to amounts such that they will no longer overcome the pull of surface tension. However, since microorganisms, including bacteria, viruses, algae, and protozoa, are hydrophilic colloids, the stability of dispersion of these bio-colloids depends on both the forces between particles themselves and on the forces between particles and the solvent (i.e., water).

Surface charge reduction alone cannot explain the phenomenon of microbial aggregation. The forces between particles and solvent can prevent these bio-colloids from settling. One example is that microorganisms can sustain stable suspension at very close to the isoelectric point (Pavoni, Tenney and Echelberger, 1972). It is obvious that other mechanisms are
responsible for microbial aggregation. Later on, EPSs excreted by microorganisms was found to play an important role in microbial aggregation. Pavoni et al., (1972) found that a direct correlation exists between EPSs accumulation and microbial aggregation. Bacterial auto-aggregation and co-aggregation are two important interactions leading to floc formation in natural and engineered systems. In these systems, bacteria form flocs either by aggregating with genetically identical cells (auto-aggregation) or with genetically distinct cells (co-aggregation). Since they are species dependent, the process of floc formation by means of auto-aggregation and co-aggregation is more predictable if enough information is known about the interaction relationships between bacterial cells.

2.2.4 Autoaggregation

Several works have investigated the composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells (Perez et al., 1998; Del Re et al., 1998). In most cases, aggregation ability is related to cell adherence properties (Boris et al., 1997; Del Re et al., 1998). Del Re et al., 1998 performed adhesion and autoaggregation tests with 13 strains of B. longum and demonstrated that these two properties are strongly related. Collado et al., 2007 showed that autoaggregation of Lactic acid bacteria (LAB) correlates with their adhesion ability. In general, the probiotic strains showed higher autoaggregation abilities than pathogen strains (Collado et al., 2008). Clostridium butyricum in broth showed a strong autoaggregating phenotype which still existed after washing and suspending of the cells in PBS (Pan et al., 2008). Studies indicated that there is a relationship between autoaggregation and adhesiveness of L. acidophilus M92 that are mediated by proteinaceous components on the cell surface (Kos et al., 2003).
2.2.5 Coaggregation

Co-aggregation was first reported by Gibbons and Nygaard (Gibbons and Nygaard, 1970) to occur between strains isolated from an oral biofilm. It is defined as the recognition between surface molecules of genetically distinct bacterial cells so that a mixed-cell aggregate is formed (Kolenbrander., 1988). The process of recognition occurs immediately and co-aggregates are usually visible after several seconds of cell mixing. Different from the flocculation/coagulation process in physical/chemical water treatment, co-aggregation is a highly specific interaction during which only certain types of cells (Rickard et al., 2002) serve as partners. The process is little affected by pH and the extent of mixing (Cisar, Brennan and Sandberg, 1979; Kolenbrander, 1988).

Co-aggregation occurs widely among oral bacteria. The non-co-aggregating bacteria cannot be integrated in the multi-generic aggregates formed in cell suspensions and are eventually washed out along with swollen saliva (Kolenbrander and Andersen, 1986). The process of oral biofilm formation can be hypothesized as: oral biofilm accumulates mass through a combination of the sequential attachment of different cell types and the growth of micro-colonies of attached bacteria. Due to widespread co-aggregation, oral biofilm formation can be regarded as a combination of independent co-aggregation between oral bacterial cells. Aggregation of a new cell type is dependent upon the presence of the previously added cell type no matter how complex the aggregate is in composition (Kolenbrander., 1988).

Although it was once believed that co-aggregation was unique for oral bacteria (Kolenbrander., 1988), recent studies indicated that co-
aggregation occurs among strains isolated from other environments (Drago et al., 1997; Kmet and Lucchini, 1997; Reid et al., 1990; A. H. Rickard et al., 2003). The mechanism of co-aggregation among freshwater bacteria is similar to that of oral bacteria in many aspects, except that, in a freshwater biofilm, the expression of bacterial co-aggregation is dependent on their growth phases (Rickard et al., 2000). Co-aggregation occurs only during the stationary phase; exponential cells are not able to co-aggregate. Furthermore, co-aggregating pairs acquire and lose their co-aggregation ability at various times during stationary phase.

Co-aggregation has also been reported to occur between Lactobacilli and Escherichia coli strains isolated from the human urogenital tract (Reid et al., 1988), the intestinal tract of humans (Drago et al., 1997) and pigs (Kmet et al., 1997), and between related lactobacilli from the crops of chickens (Clemans et al., 1999). Forestier et al., 2001 showed that the addition of the human probiotic Lactobacillus casei subsp. rhamnosus before, during or after the incubation with three human pathogens to an intestinal cell line was shown to reduce the adhesion success of the pathogens. It has been hypothesised that the presence of the probiont may restrict the access of pathogens to tissue receptors by steric hindrance (Forestier et al., 2001) or by blocking the receptor with specific adhesin analogues (Tuomola et al., 1998). Kos et al., 2003 established coaggregation between L. acidophilus M92 and two other potential probiotic strains, particularly Ent. faecium L3, which could increase their colonization potential if they were to be used in mixed culture as probiotics. Vine et al. 2004b suggest that one potential explanation for this increase is that the probiotic bacteria may have modified the mucus structure creating more or better attachment sites for the pathogens.
2.2.6 Biofilm formation

Biofilms are architecturally complex communities of microorganisms in which the cells are held together by an extracellular matrix, typically containing exopolysaccharides (EPS), proteins and even nucleic acids (Costerton et al., 1995). Colonization, followed by biofilm formation, starts by the adhesion of a single cell or cell aggregates at the surface. Microbial attachment and biofilm formation are influenced by number of factors including biological factors hydrodynamic features and environmental conditions (Elhariry, 2008). Bacterial biofilm develop on many abiotic surfaces (plastic, glass, metal and minerals) and biotic surfaces (plants, animals and humans) in three developmental steps (Lembke et al., 2006).

The first step includes adhesion of planktonic bacteria to surfaces. This initial attachment is either mediated by electrostatic contacts or relies on interaction of bacterial surface structures (proteins and carbohydrates) with inert or protein-carbohydrate-coated surfaces. The second step is the proliferation of the primary colonizers and the maturation of the biofilm. During this step, either bacteria multiply without releasing progeny cells, or primary colonizers recruit and coaggregate planktonic members of the same species or other species. At the same time, most biofilm bacteria produce extracellular polymeric substances, which stabilize the biofilm architecture.

In a final third step, previously sessile members of the mature biofilm detach and act as primary colonizers at different sites (Lembke et al., 2006). For residents of a biofilm, the communal lifestyle offers considerable advantages over the planktonic mode of growth. For example, bacteria living as a biofilm are significantly more tolerant to antibiotics and biocides and they enjoy shelter from environmental stresses, including attack by the host immune response. Furthermore, the close proximity of cells facilitates
horizontal gene transfer and sharing of metabolic by-products within the biofilm community (de Kievit, 2009).

Biofilms of *Bacillus* sp. are important contaminants in many food industry settings and are recognized as a serious problem. Members of the *Roseobacter* are the first to attach to surfaces in coastal environment and several members of the clade have been suggested as probiotic bacteria (Bruhn *et al*., 2005). Spores are highly resilient dormant cell types that are to withstand extremes of temperature, radiation and chemical assault. If spores were produced with a biofilm matrix on food and contact surfaces those spores may have greater resistance to environmental stresses including sanitizers.

2.3 Materials and Methods

2.3.1 Microorganisms used in the study

Selected five sporeformers from different natural sources as per chapter I was used for this study. Organisms under study includes MBTU PBBM1, CI-2, CD-4, S-7, and CD-1. Our preliminary studies revealed that these five sporeformers possess basic probiotic characters such as acid, bile tolerance, resistance in artificial gastric and intestinal fluid and selective antagonism to enteric pathogens such as *Salmonella* *typhi*, *Salmonella* *paratyphi* A and *Vibrio cholerae*.

2.3.1.1 Growth conditions

Vegetative cells of isolates were cultured in nutrient broth for 12 hrs while their spores were cultured in Difco sporulation medium for 48 hrs. The enteric pathogens were separately cultured in nutrient broth for 6hrs. All cultures were incubated at 37°C.
2.3.2 Adhesion Property

2.3.2.1 Mucosal adhesion assay

2.3.2.1.1 Treatment of bacteria prior to adhesion

Spores were prepared in Difco sporulation medium and they were harvested by centrifugation at 1500g for 15min at 4°C. Vegetative cells of isolates were propagated in nutrient broth overnight at 37°C. Vegetative forms were harvested by centrifugation at 10,000 g, for 10 min at 4°C. Cell pellets were separately washed twice with phosphate buffer saline. The optical density of the bacterial suspensions at 600 nm was adjusted with PBS to 0.5 ± 0.02 giving a viable count that varied between $10^6$ and $10^8$ CFU/ml.

2.3.2.1.2 Preparation of intestinal mucous (Ouwhand et al., 1999)

Human intestinal mucous was obtained from the faeces of healthy new-borns 15 - 36 months of age. Faecal samples were suspended in ice-cold PBS containing 0.5 g/l NaN3 to prevent bacterial growth. The suspension was shaken for 1 h at 4°C and centrifuged for 30 min at 4°C at 15,000 g. From the clear supernatant, mucous was precipitated twice with ice-cold ethanol (final concentration of 60%), dissolved in ultra pure water, and then resuspended in HH buffer (HEPES HANKS buffer, pH 7.4) as 10 mg/ml.

2.3.2.1.3 In vitro adhesion assay (Vesterlund et al., 2005)

The crystal violet method was used to determine adhesion ability of spores and vegetative cells of the test isolates. Mucous stock suspension was prepared by dissolving 10 mg/ml in HH buffer. Micro titer polystyrene plate wells were coated with 150µl of mucous stock suspension. The test cultures (spores and vegetative forms of the test isolates) were added at a volume of 100µl to respective wells. The greater volume of the mucous compared to the volume of the added bacteria was used to avoid the contact of the stain with
the polystyrene. Spores and vegetative cells of test isolates were allowed to adhere at 37°C for 1h, and the non adherent bacteria were removed by washing the wells three times with 250µl of PBS. The adherent bacteria were fixed at 60°C for 20 min and stained with crystal violet (100µl/ well, 0.1 % solution) for 45 min. Wells were subsequently washed five times with PBS to remove excess stain. The stain bound to the bacteria was released by adding 100 µl of citrate buffer (20 mmol-1; pH4.3). After 45min incubation at room temperature, the absorbance values at 640 nm were determined using the microtiter plate reader. Stained mucous without bacteria was considered as negative control. Results were expressed by subtracting the absorbance value of this negative control from absorbance value recorded for all samples according to Vesterlund et al. (2005). Each determination was done in triplicate.

2.3.3 Cell surface properties

2.3.3.1 Hydrophobicity (Jayesh et al., 2011)

Hydrophobicity test of spore formers and enteric pathogens were performed by using xylene - a nonpolar solvent. Spores and vegetative cells of test isolates from respective culture medium (see section 2.3.1.1) were centrifuged at 3000 g for 15 min at 4°C. Pellets were resuspended in deionized water and optical density was adjusted to 0.5 at 610 nm. 1 ml of xylene was added to 3ml of bacterial suspension and incubated for 15m’ at room temperature. The mixture was then thoroughly mixed in a vortex for 2 minutes. After the two layers had separated completely on standing for about 20 minutes at room temperature, OD of the lower aqueous phase was analyzed with a spectrophotometer at 610nm and compared with initial OD at 610 nm.
Percentage of hydrophobicity was calculated as:

\[
\frac{\text{OD}_i - \text{OD}_e}{\text{OD}_i} \times 100
\]

Where OD\textsubscript{i} is the initial OD of bacterial suspension and OD\textsubscript{e} is the reading of the aqueous phase after microbial adhesion to xylene. Each determination was done in triplicate.

2.3.3.2 Auto aggregation (Del Re et al., 2000)

Autoaggregation assay was performed with spore formers and enteric pathogens. For the assay, spores and vegetative cells of bacteria were grown according to the growth conditions as described in section 2.3.1.1. The vegetative cells were harvested by centrifugation at 5000 g for 15 min, at 4°C and spores were harvested by centrifugation at 1500 g for 15 min, at 4°C. The cells were washed twice and re-suspended in phosphate buffered saline (PBS) to give viable counts of approximately $10^8$ CFU/ml. Four ml of the cell suspension was mixed for 10 s in a sterile tube to determine autoaggregation during 5h of incubation, at room temperature. The upper suspension was used in each hour by transferring 0.1 ml to another 3.9 ml of phosphate buffer solution, and the optical density at 600 nm was measured. Tests were carried out in triplicate and the results were averaged.

The autoaggregation percentage was calculated by the formula:

\[
1 - \frac{A_t}{A_0} \times 100
\]

where $A_t$ represents the absorbance at time t = 1, 2, 3, 4 or 5, and $A_0$ the absorbance at t = 0. Aggregation abilities of microorganisms were screened by visual observation.
2.3.3.3 Coaggregation (Del Re et al., 2000)

Coaggregation assays were done with selected sporeformers and enteric pathogens. Coaggregation of vegetative forms and spores of five selected isolates were separately tested with all the three indicator pathogens. Equal volumes (2 ml) of each test isolate and each indicator pathogen were separately taken and transferred to a sterile test tube. Suspensions were mixed together in pairs by vortexing for 15 s. Control tubes contained 4 ml of each bacterial suspension on its own. The absorbance at 600 nm of the suspensions was measured after mixing and after 5 h of incubation at room temperature. Absorbance of samples was taken in the same way as in the autoaggregation assay. Tests were carried out in triplicate and the results were averaged.

The percentage of coaggregation was calculated using the equation of Handley et al. (1987) as

\[
\text{Coaggregation (\%)} = \frac{(A_x + A_y)^2 - A(x + y)}{A_x + A_y} \times 100
\]

where x and y represent each of the different isolates in the control tubes, and (x + y) the mixture of isolates tested for coaggregation.

2.3.3.4 Quantification of biofilm formation by bacterial strains on polystyrene (Rode et al., 2007)

Microtitre-plate method for determining bacterial adhesion to plastic surface was applied in the present study. Briefly, the wells of sterile 96-well polystyrene microtitre-plates were filled with 230µl of nutrient broth. 20µl of overnight cultures of the selected isolates and the three indicator strains in nutrient media were added into respective wells. The negative control wells contained nutrient broth only. The plates were incubated aerobically for 24 h
at 37°C. The content of the microtitre-plate was poured off and the wells was washed three times with 300µl of phosphate buffer. The remaining attached bacteria were fixed with 250µl of methanol per well. After 15 min, microtitre-plate were emptied, air dried and stained with 250µl/well of 1% crystal violet for 5min. The excess of stain was rinsed off by placing the microtitre-plates under running tap water. After the microtitre-plate were air dried, the dye bound to the adherent cells was extracted with 250µl of 33% (v/v) glacial acetic acid per well. The absorbance of each well was measured at 570 nm using a microtiter ELISA reader. Based on the absorbance ($A_{570}$) produced by the bacterial films, strains were classified into four categories (Christensen et al., 1985; Stepanovic et al., 2000): $A = A_c =$ no biofilm producer (0); $A_c < A = (2A_c) =$ weak biofilm producer (+); $(2A_c) < A = (4A_c) =$ moderate biofilm producer (++); $(4A_c) < A =$ strong biofilm producer (+++), where the cutoff absorbance $A_c$ was the mean absorbance of the negative control. All tests were carried out in triplicate and the results were averaged

2.3.3.5 Selection of superior adhesive isolate and its confirmation for Biofilm production

Most adhesive isolate was selected and biofilm production was confirmed by Scanning Electron Microscopy (SEM) studies as described by Lembke et al., 2006. 1 ml of overnight culture of MBTU PBBM1 was inoculated into nutrient broth containing sterile glass pieces of 0.5 mm$^3$ dimension and incubated for 48h. After incubation the glass pieces were fixed for 1 hr in a solution containing 2.5% gluteraldehyde and were washed in 0.1 M sodium acetate buffer (pH 7.3). Samples were dehydrated through a graded series of ethanol, dried, coated with platinum and examined using JEOL 6390 (Japan).
2.4 Results

2.4.1 Adhesion Assay

Adhesion to intestinal mucosa is a prerequisite for probiotic colonization in gastrointestinal tract. The five selected sporeformers and the three indicator pathogens were tested for their ability to adhere on intestinal mucous. Spores and vegetative cells of the isolates showed different adhesion rate and spores were found to be more adherent to intestinal mucous than vegetative cells. Among the selected isolates, spores of MBTU PBBM1 (OD$_{640} = 0.23 \pm 0.05$) was found to adhere significantly better than the three enteric pathogens. Among enteric pathogens Vibrio cholera (OD$_{640} = 0.19 \pm 0.02$) showed a better adhesion ability to intestinal mucous than other two indicator pathogens. (See figure 2.1 & 2.2).
2.4.2 Cell Surface Properties

2.4.2.1 Hydrophobicity

Hydrophobic nature of sporeformers and enteric pathogens were measured on the basis of their affinity towards xylene. Spores of all isolates were hydrophobic than their vegetative cells. From the results it is evident that the vegetative cells are non hydrophobic in nature except the isolate S-7. Among the five sporeformers, spores of MBTU PBBM1 showed a higher hydrophobicity of 58.4 ± 2.3%. Among enteric pathogens Vibrio Cholera registered a high percentage of hydrophobicity 52.2 ± 1.4% than other indicator pathogens. The hydrophobicity of spores formers and enteric pathogens are shown in figure 2.3.
2.4.2.2 Autoaggregation

On the basis of sedimentation characteristics, autoaggregation capability was tested on the five selected sporeformers and the three indicator enteric pathogens Salmonella typhi, Salmonella paratyphi A and, Vibrio cholera. Vegetative cells of all five isolates were found to be more autoaggregating than that of its spores (See figure 2.4, 2.5). Spores of all isolates showed a moderate autoaggregation. Among the isolates MBTU PBBM1 showed highest autoaggregation both in its vegetative (38±2.4%) and spore (23.4±3.2%) form. Among the enteric pathogens Vibrio cholera showed a higher autoaggregation of 26±1.6%. Refer figure 2.6.
Figure 2.4: Autoaggregation of spores

Figure 2.5: Autoaggregation of vegetative cells
2.4.2.3 Coaggregation

The coaggregations of the five selected isolates were tested with three enteric pathogens *Salmonella typhi*, *Salmonella paratyphi A* and *Vibrio cholera*. Results are expressed in Table 2.1, as the percentage reduction after 5 hrs in the absorbance of a mixed suspension compared with the individual suspension. Coaggregation was shown by the vegetative cells of isolates towards enteric pathogens. Among the vegetative cells, MBTU PBBM1 and CI-2 were found to be coaggregating with all the three enteric pathogens. Other isolates showed selective coaggregation with enteric pathogens. Spores of all the five isolates were found to be non coaggregating with enteric pathogens (Results not tabulated).
Table 2.1: Coggregation of vegetative cells of Sporeformers

<table>
<thead>
<tr>
<th>Name of Organisms</th>
<th>Percentage of Co aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salmonella typhi</td>
</tr>
<tr>
<td>BM-3</td>
<td>6.4±0.15</td>
</tr>
<tr>
<td>CD-4</td>
<td>0</td>
</tr>
<tr>
<td>CI-7</td>
<td>0</td>
</tr>
<tr>
<td>S7</td>
<td>0</td>
</tr>
<tr>
<td>CI-2</td>
<td>2.67±0.64</td>
</tr>
</tbody>
</table>

2.4.2.4 Quantification of biofilm formation

At the optimum growth temperature 37°C, MBTU PBBM1 had the maximum ability to form biofilm, than CI-2 and S-7 which displayed moderate biofilm productions. Among the selected isolates CI-7 and CD-4 showed the weakest ability to form biofilm. Among the enteric pathogen V. cholerae was the highest biofilm producer, while Salmonella typhi and Salmonella paratyphi A showed moderate biofilm productions at their optimum growth temperature 37°C. See table: 2.2.

Table 2.2: Ability of the isolated bacterial strains and indicator strains to form biofilm on polystyrene surface

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biofilm production at 37°C incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBTU PBBM1</td>
<td>+++</td>
</tr>
<tr>
<td>CI-2</td>
<td>++</td>
</tr>
<tr>
<td>CI-7</td>
<td>+</td>
</tr>
<tr>
<td>S7</td>
<td>++</td>
</tr>
<tr>
<td>CD-4</td>
<td>+</td>
</tr>
<tr>
<td>V. cholerae</td>
<td>+++</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>++</td>
</tr>
<tr>
<td>Salmonella paratyphi A</td>
<td>++</td>
</tr>
</tbody>
</table>
2.4.2.5 Selection of superior adhesive isolate and its confirmation for biofilm production

Based on adhesion and cell surface properties, isolate MBTU PBBM1 was selected as superior one, among the five tested isolates, than enteric pathogens. Scanning electron microscopic studies confirms the capability of MBTUPBBM1 for biofilm production. Scanning electron micrographs revealed biofilm formations on the surface of the glass pieces. See Figure 2.7.

Figure 2.7: Scanning electron micrograph of Biofilm produced by MBTU PBBM1

2.5 Discussion

Enteric pathogens are of great importance because they cause infections both in man and animals. Comparative studies on the adhesion and cell surface properties of sporeformers and enteric pathogens are scarce in the field of probiotic research. Adhesion and cell surface properties such as hydrophobicity, autoaggregation together with coaggregation properties with potential pathogens can be used for preliminary selection of probiotic
bacteria. Hydrophobicity, autoaggregation and mucin adhesion are important attributes which help in the attachment to various substrata that explain the probiotic nature of the microorganism (Anil et al., 2010). In this study five selected sporeforming isolates, which possessed basic probiotic qualities were selected and examined for their adhesion ability and cell surface properties against enteric pathogens. Otera et al. 2004 found that through adhesion ability and colonization on tissues, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage on cell receptors. Spore and vegetative phases of isolates possess a different rate of adhesion potentials, which indicates that cell surface properties were involved in adhesion process. Compared to spores, vegetative cell phase of selected isolates remains to be less adhesive on intestinal mucous. Increased adhesion of the spore phase of the isolates, on intestinal mucosa than their corresponding vegetative cells may be due to their hydrophobic nature. This result agrees with the study of Borja et al., 2009 who found that spores adhered better than the corresponding vegetative cells on mucin. The bacterial adhesion to hydrocarbons has been extensively used for measuring cell surface hydrophobicity in lactic acid bacteria (Kos et al., 2003; Vinderola et al., 2004) and Bifidobacteria (Perez et al., 1998; Collado et al., 2008). Adherence of organisms to xylene, a non polar solvent, demonstrates hydrophobic nature of the isolates. Increased hydrophobic nature of spores than their corresponding vegetative cells may be due to presence of hydrophobic proteins present in spore coat. Doyle et al 2000 found that agents which disrupt protein structure modified the hydrophobicity of spores, suggesting that the spore coat also has hydrophobic sites. Results from the present study on hydrophobicity and adhesion, revealed a positive correlation between hydrophobicity and adhesion capability of selected five isolates. This result
was in support to the studies of Wadstrom et al., 1987. He found a correlation between adhesion ability and hydrophobicity, in some *Lactobacilli*. Spores of MBTU PBBM1 showed an increased adhesion capability and hydrophobicity than all the three enteric pathogens revealing the probiotic character of the strain to be used as probiotic. Among enteric pathogens *Vibrio cholera* registered a higher hydrophobicity than that of the other two enteric fever pathogens. Aggregation studies suggest that, both vegetative and spore forms of all selected five isolates are capable for autoaggregation. Autoaggregation nature of isolates were found to be more in its vegetative cell phase than its spores. All the five selected isolates showed a different rate of autoaggregation and the isolate MBTU PBBM1 possessed a better autoaggregation capability than other four isolates. Among enteric pathogens *Vibrio cholera* registered a higher autoaggregation than other two enteric pathogens. A relationship between autoaggregation and adhesion ability has been reported for some *Bifidobacterial* species (Perez et al., 1998; Del Re et al., 2000). Collado et al. (2007) showed that autoaggregation of *lactic acid bacteria* (LAB) correlates with their adhesion ability. Present study does not support a positive correlation between aggregation properties and adhesion potentials. To quantify interbacterial adherence, a coaggregation assay was developed, which established coaggregation between selected isolates and enteric pathogens (*Salmonella typhi*, *Salmonella paratyphi* A and *Vibrio cholera*). Coaggregation between native bacteria and pathogens has been considered as a way to exclude pathogenic bacteria from their hosts (Spencer and Chesson, 1994). It has been suggested that inhibitor producing LAB, which coaggregate with pathogens, may constitute an important host defence mechanism against infection in the urogenital tract (Reid et al., 1988) and in gastrointestinal
tract (Spencer and Chesson, 1994). Coaggregation with potential gut pathogens could therefore contribute to the probiotic properties ascribed to LAB. Furthermore, many authors have reported that the coaggregation abilities of *Lactobacillus* species might enable it to form a barrier that prevents colonization by pathogenic bacteria. (Boris *et al*., 1997; Schauder and Bassler, 2001). Coaggregation ability of probiotic strain helps in excluding the pathogens before to the proper adhesion. Vegetative cells of selected isolates showed selective coaggregation with enteric pathogens. Non coaggregative pattern of spores of isolates clearly refers that cell wall structure, composition and physicochemical properties of bacteria play a key role in aggregation traits. Present results indicated that cell surface properties such as hydrophobicity and aggregation properties of the isolates change according to their spore and vegetative phase. This may be due to the morphological peculiarities of vegetative cell wall. Among the isolates MBTU PBBM1 possessed a better coaggregation with the enteric pathogens *Salmonella typhi*, *Salmonella paratyphi* A and *Vibrio cholera*. Adhesion and cell surface properties of *Vibrio cholera* were found to be more than that of enteric fever pathogens *Salmonella typhi*, *Salmonella paratyphi* A. Biofilm production helps in making a dynamic equilibrium of the isolate in their niche. In nature, the bulk of bacterial biomass is believed to exist as an adherent community of cells called a biofilm (de Kievit, 2009). Bacterial biofilms are microbial depositions on surfaces in aqueous environments. Quantitative analysis of the biofilm production showed that, among five selected isolates (at identical conditions), MBTU PBBM1 had the highest ability to form biofilm. Among enteric pathogens *Vibrio cholera* registered a higher biofilm production than other two enteric pathogens. Biofilm formation capability was confirmed for the most adhesive probiotic isolate
with good cell surface properties. So biofilm production of MBTU PBBM1 was studied by SEM analysis. Biofilm production capability enhances adhesion and colonization traits of probiotic isolate. Chapter 2 summarizes that, among the five selected sporeformers, the isolate MBTU PBBM1 possesses superior adhesion and cell surface properties than enteric pathogens. This revealed the ability of MBTU PBBM1 for gastrointestinal adhesion and competitive exclusion of enteric pathogens. From the *in vitro* studies (Chapter 1 and Chapter 2) it was concluded that the isolate MBTU PBBM1 has basic probiotic properties which are essential for gastrointestinal tract adhesion and colonization. Hence the further studies were aimed at the identification and characterization of this potential candidate bacterium.