Chapter II
Evaluation of \textit{in vitro} and \textit{in vivo} Probiotic characteristics of \textit{Enterococcus faecium} MBTU-P1F1

2.1. Introduction

The mucosal surfaces of the gastrointestinal tract (GIT) are particularly susceptible to adherence and colonization of food borne pathogens. Pathogen infections are initiated by adhesion and further involved in invasion through multiple mechanisms that require filamentous structures, cell surface proteins, site-specific ligands or biofilms (Xu \textit{et al.}, 2009). Because adhesion is necessary for the initiation of host–pathogen interactions, preventing pathogenic bacterial adhesion to the epithelial cells of GIT is an effective strategy for reducing the risk of food borne illness. Probiotics can beneficially affect the host by improving its intestinal microbial balance (Fuller, 1989). The major functions of probiotics include production of antimicrobials, stimulation of mucosal immunity, modulation of intestinal microflora and competitive exclusion of pathogens (Ketley, 1997; Ng \textit{et al.}, 2009). Therefore, the use of probiotic strains is realistically considered as an alternative for improving intestinal microbial balance because of their non pathogenic, safe and health beneficial properties (Fuller, 1989). However, to exert the beneficial effects for human health, the number of probiotics that reach the GIT and their viability is highly important.

The definition for probiotics proposed by Fuller, Salminen and FAO/WHO emphasises probiotic viability as a reasonable measure of probiotic activity. But there are situations such as improved digestion of lactose, immune system modulation activities and anti-hypertensive effects where probiotic activity has been linked to non-viable cells, cell components,
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Enzyme activities, or fermentation products (Sanders and Huis in’t Veld, 1999). Before reaching the intestinal environment (the place where they need to colonize and exert their action), probiotic strains must stand several technological (strain viability and maintenance of desirable characteristics during product manufacture and storage) and biological (low pH in the stomach, bile salts) barriers. It has been suggested that probiotic strains can be utilized and can only be utilized if they fulfil the following criteria: (Knorr, 1998; Vinderola et al., 2008; Harzallah and Belhadj, 2013).

General /safety aspects - origin, safety, acid and bile resistance etc

Technological aspects - Production and processing characteristics

Functional aspects - intrinsic microbiological properties (adhesion to mucosal surface and health effects)

Desirable physiological criteria - Immunomodulation, antagonistic activity towards gastrointestinal pathogens, cholesterol metabolism, lactose metabolism.

Hence in the present chapter focus has been given in evaluating the technological, functional and safety aspects of the potential probiotic strain Enterococcus faecium MBTU-P1F1.

Among technological criteria, strain viability and maintenance of desirable characteristics during product manufacture and storage are necessary for probiotic strains.

Viability and activity of bacteria are important considerations, because the bacteria must survive in the food during shelf life and during transit through the stomach and intestine. It is important that viability and
stability of the desirable characteristics of the strain must be maintained during commercial production and in the final product.

The protective role of probiotic bacteria against gastrointestinal pathogens and the underlying mechanisms has received special attention recently. Probiotic bacteria can form a natural barrier against pathogen and provide significant human health protection against infection. The clinical applications of probiotics have been related to the management of gastrointestinal infections caused by pathogenic microorganisms. The rapid emergence of antibiotic resistance in pathogenic strains and the adverse consequences of antibiotic treatment on the protective microflora have led to the development of alternative therapies based on bacterial replacement. Competition for adhesion sites and nutritional sources is regarded as a possible mechanism of antagonistic action. Several studies have suggested that adhesive probiotic bacteria could prevent the attachment of pathogens and stimulate their removal from the infected intestinal tract (Lee et al., 2000). Lee and Salminen (1995) reported that successful probiotic bacteria should be able to survive gastric conditions and colonize the intestine by adhering to the intestinal epithelium (Fernandez et al., 2003). And therefore adhesion to intestinal epithelium is an important functional criterion for the selection of probiotic strains. Adhesion is a complex multistep process in which both non-specific mechanisms and specific ligand receptor play a role. Bacterial traits such as structural properties of the cell surface and autoaggregation ability are related to adhesiveness of bacterial strains.

Physicochemical interactions such as cell surface hydrophobicity, autoaggregation and coaggregation can mediate bacterial adhesion. The cell surface property and cell binding properties such as aggregation ability and coaggregation with pathogens are essential functional criteria in identifying
potentially adherent bacteria with properties suitable for commercial application.

Autoaggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cells, and coaggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms (Reid et al., 1998; Del Re et al., 2000). Autoaggregation has been correlated with adhesion, which is known to be a prerequisite for colonization and infection of the gastrointestinal tract by many pathogens. The coaggregation properties of probiotic strains with pathogens as well as their ability to displace pathogens are of importance for therapeutic manipulation of the aberrant intestinal microbiota (Collado et al., 2008). Consequently the ability to aggregate and coaggregate is desirable properties for probiotics in health promoting foods.

Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces. The composition, structure and forces of interactions related to bacterial adhesion have been investigated. The adhesive probiotic bacteria could prevent the attachment of pathogens and stimulate their removal from the infected intestinal tract (Lee et al., 2000). Successful probiotic bacteria should be able to survive gastric conditions and colonize the intestinal epithelium (Lee and Salminen, 1995). Adherence to intestinal mucosa is the first step in gut colonization (Khalil et al., 2007). Gut colonization prolongs the time the microorganism can influence the gastrointestinal immune system and microbiota of the host and hence is important for a beneficial health effect (Forestier et al., 2001). There is good evidence that probiotic strains can inhibit the growth and adhesion of a range of enteropathogens and such studies are useful in characterizing probiotic organisms. Characteristics such as adhesion to cells, production of
bacteriocins, acids and hydrogen peroxide and the ability to inhibit adhesion of pathogens are important to confer probiotic effects (Reid et al., 2003). The physicochemical properties and adhesion ability of probiotic strains can be evaluated using the hydrophobicity, autoaggregation, coaggregation and adhesion assay.

In vitro tests are useful to gain knowledge of strains and the mechanism of probiotic effect. Appropriate target specific in vitro tests that correlate with in vivo results are recommended. Suitable animal models exist to provide substantiation of in vitro effects and determination of probiotic mechanism. Balb/c mice are among the most widely used inbred strains in animal experimentation. They serve as a mammalian animal model and share 99% of their genes with humans. Balb/c mice meet determined taxonomic equivalency to humans so as to react to diseases or its treatment in a way that resembles human physiology as needed.

Bacterial biofilms are complex, mono or poly microbial communities adhering to biotic or abiotic surfaces. This adaptation has been implicated as a survival strategy. The cells embedded in an extracellular matrix where they interact with each other and the environment. This ecosystem provides a safe home for the members of the community. The biofilm formation has been documented as survival strategy of pathogens. Production of biofilms is associated with virulence in most of the potential pathogens. Enterococci in biofilms are more highly resistant to antibiotics than planktonically growing enterococci and hence the potential impact of biofilm formation could be significant. Enterococcus faecalis produces biofilm more often than Enterococcus faecium and biofilm formation could be an important factor in the pathogenesis of enterococcal infection (Muhamed and Huang, 2007).
Examination of biofilm formation is part of the safety assessment of enterococcal strains.

Any inability to compete for attachment sites on the mucus of the gut wall suggests that these bacteria may not multiply sufficiently fast to compensate for being flushed from the mucus during gut evacuation. A probiotic may colonize the intestinal tract and prevent the proliferation of pathogenic or opportunistic bacteria if certain of its growth characteristics are superior to that of the pathogen. Therefore, screening of candidate probiotics preferably requires that growth characteristics be also considered with the other selective criteria such as antagonism, production of beneficial compounds and attachment.

Although Enterococci are widely distributed in the environment principally inhabiting the human and animal gastrointestinal tract, some strains are potential human pathogens. Human infections caused by Enterococci outside the healthcare setting are very uncommon and consist of endocarditis, urinary tract infections, or pelvic infections resulting from contamination by the fecal microbiota (Murray, 2000). Enterococci have been used as probiotics because of their possible health promoting capacities. While the probiotic benefits of some strains are well established, the emergence and the increased association of Enterococci with human disease and multiple antibiotic resistances have raised concern regarding their use as probiotics (Foulquie et al., 2006). The use of Enterococci as probiotics remains a controversial issue. Some Enterococci carry potential virulence factors and pathogenic traits which are strain specific. Safety assessment of Enterococcal strains based on the absence of any possible pathogenic properties is therefore essential. Among the microbial additives currently used in animal nutrition, nearly one third contain strains of Enterococcus
faecium (EFSA Journal, 2012). 10 preparations with 9 different strains of Enterococcus faecium are authorized as additives in feeding stuffs in the European Union (European Commision, 2004). In the view of the FEEDAP Panel (European Panel on Additives and Products or Substances used in Animal Feed) any strain of Enterococcus faecium need to be assessed for the susceptibility to Ampicillin and the presence of the virulence markers such as resistance to Vancomycin, the surface protein esp involved in biofilm formation, and the hyl-like gene for glycosyl hydrolase which facilitate intestinal colonisation leading to lethality in murine peritonitis model.

2.2 Review of Literature

Probiotics can benefit the human host by acting as a first line of defence against disease causing pathogens by improving the intestinal flora and there is a necessity that probiotic strains be viable and metabolically active within the gastrointestinal tract as well as during commercial manufacture (Kailaspathy and Chin, 2000). Following section provides a brief review of the evaluation of the in vitro and in vivo probiotic properties of a suitable probiotic strain as well as its safety assessment for use in functional foods.

2.2.1 Viability in milk based media

Kurmann and Rasic, (1991) suggested that apart from the probiotic activity, a LAB selected for dairy products should be able to ferment milk relatively quickly either on their own or in combination with other strains. It should multiply to achieve a large number of cells in fermented product and should have high tolerance to lactic acid to remain viable during storage of the product. This can set the stage to promote a selective advantage to suppress the growth of pathogenic and spoilage microorganisms (Knorr,
1998). When probiotic bacteria are to be added as adjunct cultures to fermented dairy products it must be taken into consideration that living bacteria will interact with their environment and evaluation of technological traits such as growth and survival in milk based media and during product manufacture are important considerations when bacterial strains are to be used in food applications (Charteris et al., 1998; Vinderola et al., 2008). The determination of haemolytic activity is considered a safety aspect for the selection of probiotic strains (FAO/WHO, 2002).

### 2.2.2 Cell surface property- hydrophobicity

Hydrophobicity is the long range noncovalent interaction defined as the attraction among apolar or slightly polar cells or other molecules immersed in an aqueous solution (Rajesh and Vandana, 2009). It was reported that the proteins, glycoproteins, teichoic of lipoteichoic acids on the cell surface of bacteria play important role in the autoaggregation and hydrophobicity of the strain. The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue (Kiely and Olson, 2000). There was considerable difference in the hydrophobicity of 19 *Lactobacillus* strains as reported by Schillinger et al., 2005). Pelletier et al. (1997) reported relatively higher hydrophilic cell surface nature of 8 strains of *Lactobacillus casei*. High cell surface hydrophobicity may favour colonisation of mucosal surface and play a role in adhesion of bacteria to epithelial cells and extracellular matrix of the host tissue. Cell surface hydrophobicity methods measure bacterial adhesion to a hydrophobic substratum, such as to hydrocarbons (xylene, chloroform or ethyl acetate) in Microbial Adhesion to Hydrocarbon (MATH), or to sepharose beads with covalently bound hydrophobic moieties in Hydrophobic interaction Chromatography (HIC). According to Del Re et al. (2000) hydrophobicity
results obtained by one method need not be compared with those obtained by another method since these methods measures the bacterial adhesion to hydrophobic substrates and do not measure the intrinsic microbial cell surface hydrophobicity. Hydrophobic cell surface is demonstrated by high adherence to xylene and is related to the presence of proteinaceous material at the cell surface whereas hydrophilic surfaces are associated with the presence of polysaccharides (Collado et al., 2008). Vlkova et al. (2008) demonstrated no significant correlation between hydrophobicity and aggregation properties in Bifidobacteria strains whereas Collado et al., 2007 revealed positive correlation between aggregation ability and hydrophobicity in Bifidobacterial strains. Bacterial hydrophobicity/hydrophilicity are related to certain proteins associated with cell walls and cell membranes and are major physico-chemical property of their surface and is associated with adhesion to host tissue and to inert surfaces (Mamo, 1989). 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. LAB commonly expresses cell surface hydrophobicity as measured by the salt aggregation test, contact angle and adhesion to xylene (Wadström et al., 1987; Strus et al., 2001).

Ouwehand et al. (1999) observed no correlation between cell surface hydrophobicity and the ability to adhere to intestinal mucus. Furthermore, all tested strains had relatively 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).

2.2.3 Cell Binding Properties-Microbial aggregation

Microbial aggregation is a widespread phenomenon in both natural and engineered aquatic systems. In these systems, microorganisms cluster together, forming fairly stable, contiguous, multi-cellular associations. Microbial aggregation is interpreted as a result of interactions of naturally produced extra-cellular polymeric substances (EPSs) and microbial cells; EPSs, which are excreted by microbial cells, play a key role in adsorbing to and bridging between cell surfaces. Microbial autoaggregation and coaggregation, leading to biofilm or floc formation, widely occur in natural systems (Feichen, 2007). Bacterial autoaggregation refers to bacteria forming aggregates with genetically identical cells (Grady et al., 1999) and bacterial coaggregation refers to bacteria forming aggregate with genetically distinct cells. 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).

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). In order to manifest beneficial effects, probiotic bacteria need to achieve an adequate mass through aggregation, consequently, the ability of probiotics to aggregate is a desirable property.

Coaggregation property helps to determine the interbacterial adhesion and Reid *et al.* (1998) suggested that inhibitor producing LAB, which coaggregate with pathogens, may constitute an important host defence mechanism against infection in urogenital tract. Similar protective mechanism could operate in the gastrointestinal tract as suggested by Spencer and Chesson, (1994). Organisms with the ability to coaggregate with other bacteria such as pathogens have an advantage over non-coaggregating organisms which are more easily removed from the intestinal environment (Collado *et al.*, 2008). The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains used as probiotics. 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).

2.2.4 **Adhesion to intestinal mucosa**

Bacterial adhesion is important in various areas of environmental, medical and industrial research. It is crucial in establishing many natural
microbial communities (Mamo, 1989). Among the criteria for selecting probiotic microorganisms with beneficial effects for the host, the ability to adhere to host tissues is important (Ouwhend et al., 1999). Adhesion to intestinal mucosa is considered a prerequisite for successful colonisation and is important for immune modulation by the probiotics. 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 (Wadstrom et al., 1987; Schillinger et al., 2005). For effective mechanism of probiotic action, the probiotic bacteria should be properly adhered to intestinal mucosa. 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, 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 whilst in a diseased gut it may reduce the possibility of pathogen translocation when the host’s defence mechanisms are impaired (Apostolou, 2001).

Ability to adhere to intestinal mucosa and colonization potential in the gastrointestinal tract are common criteria for classifying a viable bacterial strain as a probiotic (Kailaspathy and Chin, 2000).

It has been proposed by Fuller (1989) that probiotics could maintain the healthy intestinal microbiota through competitive exclusion and
antagonistic action against pathogenic bacteria in the animal intestine (Fernandez et al., 2003). Adhesion ability of probiotics to intestinal mucus is an important prerequisite for colonization of the host intestinal tract (Ouwehand et al., 1999). Knowledge about the adhesive properties of the probiotic organisms can give information about its possibility to colonize and modulate the immune system.

Zanoni et al., 2008 reported that the ability of probiotic bacteria to reach survive and persist in the environment in which it is intended to act have to be assessed to ensure optimal functionality and expression of health promoting physiological functions. In vitro tests still remain valuable and can provide scientific insight into characteristics of probiotic organisms as recommended by FAO/WHO. According to Schillinger et al. (2005), resistance to gastric acid and physiological concentrations of bile and adherence to intestinal epithelial cells are required characteristics for effective probiotic microorganisms. Surface hydrophobicity and aggregation properties may be relevant for adhesion purposes and colonization (Collado et al., 2008; Xu et al., 2009).

Bacterial adhesion is complex process. Adhesion to the mucosa is, however, influenced by the flow rate, competition for nutrients, adhesion sites and specific physicochemical properties of the probiotic (Wadstrom 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; Vida 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).

### 2.2.5 Biofilm formation

Biofilm is a population of cells attached irreversibly on various biotic and abiotic surfaces, and encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids (Costerton, 1995). Biofilm formation is a complex developmental process involving attachment and immobilization on a surface, cell-to-cell interaction, microcolony formation, formation of a confluent biofilm, and development of a three dimensional biofilm structure (O’Toole et al., 2000). Bacteria in a biofilm behave differently from their free floating (planktonic) counterparts. According to the National Institutes of Health, biofilms are medically important, accounting for over 80% of microbial infections in the body. A mature biofilm can tolerate antibiotics at concentrations of 10–1000 times more than are required to kill planktonic bacteria. Bacteria in biofilms are resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts. Bacteria in biofilms colonize a wide variety of medical devices, such as catheters, artificial cardiac pacemakers, prosthetic heart valves and orthopaedic appliances, and are associated with several human diseases, such as native valve endocarditis, burn wound infections, chronic otitis media with effusion and cystic fibrosis (Costerton et al., 1999). Enterococci in biofilms are more highly resistant to antibiotics than planktonically growing Enterococci, thus the potential impact of biofilm formation could be significant.
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).

2.2.6 Growth Profile

It is assumed that a probiotic will be most competitive \textit{in vivo} if it has a short lag phase and a fast growth rate. Fast growth and ability to attach to the intestinal tract can be considered together with the probiotic’s ability to produce substances antagonistic to pathogens as a mode of competition for probiotic strain. A bacterium with a short lag period and short doubling time has a better chance of out competing other bacteria based on their growth characteristics. It has been suggested by Vine \textit{et al.} (2004) that lag period and doubling time are the most appropriate criteria for the comparison of growth between probiotic strains and pathogens.

2.2.7 \textit{In vivo} probiotic characterization

According to the Guidelines for the evaluation of probiotics for food use by FAO/WHO, the functional characterization of probiotic strains includes \textit{in vitro} and animal studies. A serious consideration related to the use of probiotics is whether they are opportunistic pathogens, especially in immune compromised hosts. Probiotic bacteria are likely to be safe for immune competent and immune deficient host but they should be tested for safety in suitable animal model (Wagner \textit{et al.}, 1997a). According to guidelines by WHO, there is a need for refinement of \textit{in vitro} and \textit{in vivo} tests to better predict the ability of probiotic microorganisms to function in humans. Animal models exist to provide substantiation of \textit{in vitro} effects and determination of probiotic mechanism.
2.2.8 Safety assessment for the use of *Enterococcus faecium* as probiotic

The genus *Enterococcus* is the most controversial among LAB. *Enterococcus faecium* is a species of bacteria that has been characterized as part of the normal gastrointestinal microbial flora in animals and humans. Although it is a naturally occurring bacterium that grows in human and animal intestinal contents some strains are potential human pathogens. Despite the concerns for *Enterococcus faecium* as an opportunistic pathogen, it has long been used as human or animal probiotic (Franz *et al.*, 1999). Carefully selected and researched strains of a *Enterococcus faecium* are well-documented as safe and effective probiotics.

2.2.8.1 Opsonophagocytic assay

Opsonophagocytic killing is considered to be an important test to assess the safety of Enterococcal strains (Hufnagel *et al.*, 2003).

Opsonophagocytic killing is used as an *in vitro* test to detect a protective immune response against bacterial pathogens (Pier *et al.*, 1994), as all the antibodies that provide antibacterial immunity are opsonic. *Enterococcus faecalis* and *Enterococcus faecium* can posses acapsular polysaccharide that is the target for opsonic antibodies (Huebner *et al.*, 1999).

2.2.8.2 Ampicillin Resistance

Isolation of *Enterococcus faecium* from healthcare-associated infected sites has increased markedly over the past 15-20 years and coincident with this increase, it was recognised that hospital-associated isolates of *Enterococcus faecium* were more frequently resistant to Ampicillin and Piperacillin than those found in the community setting.
Currently, about 70% of Enterococcus faecium isolates in US hospitals are Vancomycin resistant while 90% are Ampicillin resistant.

It is now recognised that Enterococcus faecium consists of two distinct subpopulations, or clades, that may have diverged many hundreds of thousands of years ago. These clades have been differentiated by Multi-Locus Sequence Typing (MLST), by sequence comparisons of individual shared core genes, by the presence of insertion sequence IS16, and other acquired elements, and in their resistance to Ampicillin. One subpopulation termed clade (B) consists predominantly of isolates from the faeces of healthy individuals, and is characterised by susceptibility to Ampicillin. The other subpopulation (clade (A)) contains most of the Ampicillin resistant clinical isolates (EFSA Journal, 2012).

Resistance to Ampicillin of hospital associated isolates (often with MICs >128mg/L confers cross resistance to Piperacillin and very high level resistance to Cephalosporins. This β-lactum resistance, together with resistance, to Vancomycin, provides a selective advantage to a resistant organism in the hospital environment, where Vancomycin, Cephalosporins and Piperacillin are commonly used (Murray, 2000).

2.2.8.3 Penicillin Binding Protein (PBP) and Ampicillin resistance

Cell wall synthesis enzymes referred to as penicillin binding proteins (PBPs) shared by the two clades of Enterococcus faecium exists in two allelic forms, pbp5-S and pbp5-R. Among sequenced isolates, most Enterococcus faecium isolates from human infections (which belong to the hospital-associated clade (A) have the pbp5-R form of this gene, while pbp5-S characterises isolates of the community-associated clade (B). Enterococcus faecium strains with an MIC of Ampicillin of > 4 mg/L had the pbp5-R
sequence while the *Enterococcus faecium* strains with an MIC of < 4 had the *pbp5*-S sequence; those *Enterococcus faecium* with an Ampicillin MIC = 4 had either the *pbp5*-S or the *pbp5*-R sequence. Thus, an MIC ≤ 2 mg/L appears to reliably exclude the clade that contains most isolates from human infection. Thus, an MIC ≤ 2 mg/L appears to reliably exclude the clade that contains most isolates from human infection (clade (A)) and excludes strains that might have a selective advantage in the GI tract if an individual was given Ampicillin, Amoxicillin or similar antibiotics (Rice *et al*., 2004).

2.2.8.4 Virulence factors and markers associated with hospital strains

The following virulence factors and markers are now considered the most relevant for the assessment of safety:

**IS16 (hospital associated strain marker)**

IS elements are the simplest transposable elements encoding only the enzyme(s) necessary for their own transposition. Enterococci harbour numerous mobile genetic elements and IS16 can be found e.g. as flanking the transposon Tn1547, which confers resistance to vancomycin in *Enterococcus faecalis*. IS16 is a specific marker for hospital-associated subpopulations of *E. faecium* (clade (A)). Vancomycin Resistant Enterococci (VRE) are highly resistant to all standard anti-enterococcal drugs and constitute a serious risk group (Foulquie, 2006).

**Esp (pathogenicity island (PAI) marker)**

Esp is a large (approximately 200 kDa) surface protein of *Enterococcus faecium* that is covalently linked to the cell wall. The *esp* gene is part of a large pathogenicity island which also carries genes for its mobilisation. The *esp* gene has an important role in biofilm formation of *Enterococcus faecium* (Heikens *et al*., 2007) and has been experimentally
proven to contribute to endocarditis (Heikens et al., 2011) and urinary tract infections in animal models. The esp gene is common among Ampicillin and Vancomycin resistant Enterococcus faecium isolates (Rice et al., 2003). Esp was first described in a clinical Enterococcus faecalis MMh594 isolate and studies on the distribution of this surface protein revealed a significant enrichment in infection derived Enterococcus faecalis Shankar et al. (1999). The incidence of Esp in food isolates differs for Enterococcus faecium and Enterococcus faecalis. The incidence of esp gene in Enterococcus faecalis is higher but is hardly found in Enterococcus faecium (Foulquie et al., 2006). Muhamed and Huang (2007) reported that esp gene does not appear to be necessary nor sufficient for the production of biofilm in Enterococcus faecalis and Enterococcus faecium.

**hyl-like gene**

HylEfm was initially described as a hyaluronidase but recently annotated as a putative glycosyl hydrolase. Glycosyl hydrolases facilitate intestinal colonisation in many bacterial organisms. These hyl plasmids have been shown to increase colonisation of mice GI tracts and to increase lethality in a murine peritonitis model and, thus, might contribute to the success of at least some members of the hospital-associated clade. Strains from the community-associated clade almost never have very large plasmids containing a hyl-like gene; while hospital-associated strains often (~ 30% in one study) harbour this gene (Rice et al., 2003).

The purpose of safety assessment is to exclude Enterococcus faecium strains belonging to the hospital-associated clade from the use in animal nutrition because of the hazard they present to a vulnerable subpopulation of consumers.
Prior to the safety assessment, the strain must be identified as *Enterococcus faecium* using appropriate molecular methods. Then the MIC for Ampicillin should be determined:

If the MIC $> 2$ mg/l, the strain is considered unsafe and should not be used as a feed additive. If the MIC $\leq 2$ mg/l, the absence of the genetic elements IS16, *hylEfm*, and *esp* should be established. If none of the three genetic elements are detected, then the strain is considered safe for use as a feed additive.

With the background of the literature review the objectives of the chapter II include:

Examining the desirable probiotic capabilities (*in vitro* and *in vivo*) of the *Enterococcus faecium* MBTU-P1F1 isolated and characterized in chapter 1 such as

- Cell surface and adhesion abilities in comparison with the enteric fever pathogens, *Salmonella typhi* and *Salmonella paratyphi A*.
- Colonization of the strain in the gastrointestinal tract (GIT) of Balb/c.
- The effect of colonization on the health status and as measured by body weight and histopathological examination.
- Comparison of the growth pattern of the test strain with that of the enteric fever pathogens and susceptibility to antibiotics.
- Safety assessment of the strain (at phenotypic level) for animal and human use.
2.3. Materials and Methods

2.3.1 Microorganisms used in the study

A: Test strain: The potential probiotic strain *Enterococcus faecium* MBTU-P1F1, isolated from infant feces in chapter 1

B: The enteric fever pathogens, *Salmonella typhi* (MTCC 734), *Salmonella paratyphi* A (MTCC 735) obtained from MTCC Chandigarh.

2.3.2 Examination of desirable probiotic characters (*in vitro*) of the LAB isolate *Enterococcus faecium* MBTU-P1F1

2.3.2.1 Evaluation of viability and shelf life of the LAB

2.3.2.1.1 Growth in skim milk: (Vinderola et al., 2008)

Overnight culture of *Enterococcus faecium* MBTU-P1F1 was centrifuged at 10,000 rpm for 10 min at 4°C and pellets washed twice with buffered phosphate saline (PBS) at 7.4 pH. Cells were resuspended in skim milk (10 g%). 1 ml from the cell suspension was inoculated to 10 ml sterile skim milk, followed by incubation (37°C aerobiosis). Coagulation of milk, changes in milk pH and colony count were recorded at 12, 24 and 48 h of incubation.

2.3.2.1.2 Viability in milk acidified with lactic acid

Cells of overnight culture of the test strain was harvested by centrifugation (10000 rpm for 10 min at 4°C), washed and resuspended in PBS. The 1 ml cell suspension was transferred to 10 ml skim milk previously acidified with lactic acid to pH 4 and 5. Skim milk without lactic acid addition was used as control. Cultures were stored at 5°C. Colony counts on MRS agar were performed on days 0 and 30 (Vinderola et al., 2008).
2.3.2.2. Cell Surface properties

2.3.2.2.1 Measurement of hydrophobicity of Enterococcus faecium MBTU-P1F1

The test was determined according to the method of Perez et al. (1998) with modifications. The test was performed by using hydrocarbons, xylene (a nonpolar solvent) and chloroform (an acidic solvent). Overnight culture of the test strain Enterococcus faecium MBTU-P1F1 was harvested by centrifugation at 12,000 x g for 5 min, washed twice in 50 mM K_2HPO_4 (pH 6.5) buffer and finally resuspended in the same buffer. The cell suspension was adjusted to an absorbance of value of approximately 1.0 at 560 nm with the buffer and 3 ml of the bacterial suspensions were put in contact with 0.6 ml of n-hexadecane for 15 min at room temperature and vortexed for 120 s. The two phases were allowed to separate for 20 min at 37°C. The aqueous phase was carefully removed and the absorbance at 560 nm was measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%). H% was calculated with the formula H%=[(A_0-A)/A_0] x 100, where A_0 and A are the absorbance before and after extraction with the hydrocarbons. The experiment was done in triplicate.

2.3.2.2.2 Autoaggregation assay of Enterococcus faecium MBTU-P1F1 and the enteric fever pathogens

Autoaggregation of the test strain Enterococcus faecium MBTU-P1F1 and the pathogens, Salmonella typhi and Salmonella paratyphi A were performed as per Del Re et al. (2000) with modifications. Overnight culture of the test strain in MRS and the pathogens in nutrient broth were harvested by centrifugation at 5,000 g for 15 min. Cells were washed twice and resuspended in 50mm K_2HPO_4 buffer at pH 6.4. The optical Density of each of the organism was adjusted at 600 nm as 0.5. 4 ml of cell suspension of
each organism was mixed by vortexing for 10 s and autoaggregation was determined during 5 h of incubation at room temperature (RT). 0.1 ml of the upper suspension was transferred at different time interval to another tube with 3.9 ml of the buffer and absorbance measured at 600 nm. The test was done in triplicate and the results were averaged. The autoaggregation percentage was calculated by the formula: 1- (A_t/A_0) X 100, where A_t represents the absorbance at time t = 1, 2, 3, 4 or 5, and A_0 the absorbance at t = 0. The experiment was done in triplicate.

2.3.2.2.3. Coaggregation assay: (Del Re et al., 2000)

Here cell suspension of the isolate and the enteric fever pathogens were prepared as in 2.3.2.2.2. Equal volumes of the test isolate and each of the pathogens were mixed together in pairs by vortexing for 15 s. Control tubes were set up at the same time, containing 4 ml of each bacterial suspension on its own. The absorbance (A) was measured at 600 nm after mixing and after 5 h of incubation at room temperature. Absorbance was determined for the mixture and for the bacterial suspensions alone. Results are expressed as the percentage reduction after 5 h in the absorbance of a mixed suspension compared with the individual suspension. Each determination was done in triplicate.

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

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

Where A_x and A_y represent the absorbance of the test strain and each of the different pathogens in the control tubes, and A(x + y) that of the mixed bacterial suspensions (Test strain and pathogen) for coaggregation.
2.3.2.2.4 In vitro adhesion assay

2.3.2.2.4.1 Treatment of bacteria prior to adhesion

*Enterococcus faecium* MBTU-P1F1 was propagated in MRS broth and the pathogens *Salmonella typhi* and *Salmonella paratyphi A* in nutrient broth, overnight at 37°C. Cells of each of the bacterial strains were harvested by centrifugation (10,000 g, for 10 min) at 4°C, and washed twice with PBS and optical density 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.

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

Human intestinal mucous was obtained from feces of healthy newborn 15 - 36 months of age. Fecal samples were suspended in ice-cold PBS containing 0.5 g/l NaN3 to prevent bacterial growth. The suspension were shaken for 1 h at 4°C and centrifuged for 30 min at 4°C at 15,000 g. From the clear supernatant, the 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.

2.3.2.2.4.3 Adhesion assay (Vesterlund et al., 2005)

The crystal violet method was used to determine adhesion ability of test isolates. Mucous stock suspension was prepared by dissolving 10 mg/ml in HH buffer. The test cultures were added as a volume of 100 µl into microtitre polystyrene plate wells previously coated with 150 µl of human intestinal mucous. 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. Bacteria were adhered 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; pH 4.3). After 45 min incubation at room temperature, the absorbance values at 640 nm were determined using microtiter plate reader. Stained mucus 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.

### 2.3.2.2.5 Biofilm Formation

#### 2.3.2.2.5.1 Quantification of biofilm formation by *Enterococcus faecium* MBTU-P1F1 on polystyrene

Microtitre plate method for determining bacterial adhesion to plastic surface was applied in the present study according to Rode et al. (2007). 230 µl of MRS medium was added to three wells in a 96-well polystyrene microtitre - plate. 20 µl cultures of the test strain *Enterococcus faecium* added into each well separately. The negative control wells comprised of 250 µl MRS broth only. The plates were incubated aerobically for 24 h at 37°C temperature. The content of the microtitre-plate was poured off and the wells were washed three times with 300 µ1 of phosphate buffer. The remaining attached bacteria were fixed with 250 µl of methanol per well. After 15 min, the wells were emptied, air dried and stained with 250 µl/well of 1% crystal violet for 5min. The excess of stain was rinsed off by placing the microtitre-plate under running tap water. After the wells were air dried, the dye bound to the adherent cells was extracted with 250 µl of 33% (v/v) glacial acetic acid per well. The absorbance of each well was measured at 570 nm using a UV-Vis spectrophotometer. Based on the absorbance ($A_{570}$) produced by the
bacterial films, strains were classified into four categories: $A = A_c = \text{no biofilm producer (0); } A_c < A = (2A_c) = \text{weak biofilm producer (+); } (2A_c) < A = (4A_c) = \text{moderate biofilm producer (++); } (4A_c) < A = \text{strong biofilm producer (+++), where the cut off absorbance } A_c \text{ was the mean absorbance of the negative control. All tests were carried out in triplicate and the results were averaged.}$

### 2.3.2.2.5.2 Scanning Electron Microscopy analysis of *Enterococcus faecium* MBTU-P1F1

Scanning Electron Microscopy (SEM) of the LAB isolate was performed as described by Lembke *et al.* (2006). 1 ml of overnight culture of the LAB isolate was inoculated into MRS broth containing sterile glass pieces of 0.5 mm$^3$ dimension and incubated for 48 h. After incubation the glass pieces were fixed for 1 h 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 SEM analysis was performed using JEOL 6390 (Japan).

### 2.3.2.3 Susceptibility to antibiotics

#### 2.3.2.3.1 Study of resistance of *Enterococcus faecium* MBTU-P1F1 to commonly prescribed antibiotics by Kirby Bauer disc diffusion method

Antibiotic resistance of the test strain was determined by agar diffusion technique in Muller Hinton Agar with the following antibiotic discs: Ampicillin 10mcg, Chloramphenicol 30mcg, Gentamycin 10mcg, Tetracycline 30mcg, Ciprofloxacin 30mcg, and Erythromycin 10 mcg.
2.3.2.3.2 Minimum inhibitory concentration of Ampicillin

The minimum inhibitory concentration (MIC) is the least amount of antimicrobial that will inhibit visible growth of an organism after overnight incubation.

2.3.2.3.2.1 Inoculum

Overnight culture of the test organism was standardized using 0.5 McFarland so as to get a concentration of $10^5$ colony forming unit/ml.

2.3.2.3.2.2 Stock solution of Ampicillin

A stock concentration of 200 µg/ml was prepared in sterile distilled water.

2.3.2.3.2.3 Determination of MIC of Ampicillin towards Enterococcus faecium MBTU-P1F1

From the stock solution, dilutions were made to get a concentration ranging from 0.2 g/l to 4 g/l.

The tubes containing 10 ml of the antibiotic solution were inoculated with 100 µl of the test organisms. Tubes were incubated overnight at 37°C. After incubation tubes were observed for the visible growth and optical density was measured at 560 nm.

2.3.3 Growth curve analysis of Enterococcus faecium MBTU-P1F1 in comparison with enteric fever pathogens

Overnight cultures of the test strain and the pathogens were prepared. 10 ml of Enterococcus faecium MBTU-P1F1 was inoculated into a conical flask containing 90 ml of MRS broth. Similarly 10 ml from the cultures of Salmonella typhi and Salmonella paratyphi A were also transferred to 90 ml of nutrient broth in separate flasks. The flasks were incubated at 37°C. Aliquots were taken every hr for 24 h and the turbidity was measured
spectrophometrically at 630 nm. Growth curve was made for the test strain and the pathogens from the readings. The growth rate and doubling time of the test strain and the pathogens were calculated from the graph.

### 2.3.4 Examination of Plasmid profile in Enterococcus faecium MBTU-P1F1

Small-scale preparation of plasmid DNA was performed using rapid alkaline lysis procedure of Maniatis et al. (1982).

Small-scale preparation of plasmid DNA was performed using rapid alkaline lysis procedure.

1) A single bacterial colony was inoculated into 2 ml of MRS medium and the cultures were incubated overnight with vigorous shaking.

2) 1.5 ml of the culture was poured into a micro centrifuge tube and centrifuged at 12,000 rpm for 30 S at 4°C.

3) The supernatant was removed leaving the bacterial pellet as dry as possible.

4) The bacterial pellet was resuspended in 100 µl of ice cold solution I (see Appendix A) by vigorous vortexing.

5) 200 µl of freshly prepared solution II (see Appendix A) was added. The tube was closed tightly and the contents were mixed by inverting the tube rapidly five times. The tube was stored on ice.

6) 150 µl of ice-cold solution III (see Appendix A) was added. The tube was closed and vortexed gently in an inverted position for 10 sec to disperse solution III through the viscous bacterial lysate. The tube was stored on ice for 3-5 min.
7) Centrifugation was done at 12,000 rpm for 5 min at 4°C in a microfuge. The supernatant was transferred to a fresh tube.

8) The DNA was precipitated with 2 volumes of ethanol at room temperature and mixed by vortexing. The mixture was allowed to stand for 2 min at room temperature.

9) The samples were centrifuged at 12,000 rpm for 5 min at 4°C in a microfuge.

10) The supernatant was removed by gentle aspiration. The tubes were kept in an inverted position on a paper towel to allow all of the fluid to drain away.

11) The DNA pellet was rinsed with 1 ml of 70% ethanol at 4°C. The supernatant was removed as described in step 10 and the pellet was dried in air for 10 min.

12) DNA was redissolved in 50 µl of TE (pH 8.0), vortexed briefly, and stored at -20°C. The isolated DNA was run on an agarose gel (0.8% w/v) prepared using TBE buffer. The electrophoresis was carried out at 75 V for 1 h. Lambda DNA / Hind III Double Digest was used as the marker.

2.3.5. Examination of in vivo probiotic characteristics of Enterococcus faecium MBTU-P1F1 isolated from infant feces

2.3.5.1 Animal model

Male Balb/c mice (8 week old, (20±0.8)g were used in the study. Balb/c mice were obtained from small animal breeding station under Kerala Agricultural University, Department of Veterinary and Animal Sciences University, Thrissur and the animals were kept in animal house of School of Biosciences, Mahatma Gandhi University. Animals were housed in
polypropylene cages and were given standard sterile dry pellet (Sai Feeds, Bangalore, India) and sterile drinking water ad libitum. The animals were maintained at a controlled condition of temperature of 26-28°C with a 12 h light: 12 h dark cycle. Bedding in cages was changed every day. Care and use of animals under study were followed according to the institutional guidelines of Mahatma Gandhi University.

2.3.5.2 Preparation of inoculum for in vivo study

Enterococcus faecium MBTU-P1F1 was cultured in MRS for 48 h at 37°C in the presence of 5% CO₂. The culture was centrifuged at 1500 g for 10 min at 4°C and washed with PBS thrice and re suspended in sterile PBS. For assessment of the number of viable bacteria, suitable dilutions of the bacterial suspensions were inoculated onto MRS agar plates and CFU were counted after 24 h at 37°C. Suitable dilution of the culture supernatant containing 10⁸ viable cells per ml was chosen and turbidity was measured spectrophotometrically at 560 nm.

2.3.5.3 Experimental design for evaluation of in vivo probiotic characteristics

Mice were assigned into 2 groups, comprising of one experimental group and one control group. Each group consisted of six mice. The two groups were caged individually animals in each group were given autoclaved food and water. Beddings of the cages and water bottles were cleaned and changed each day.

2.3.5.4 Examination of the normal flora of the animals.

Freshly voided fecal samples from the experimental group and control group were collected. Fecal samples were homogenised in sterile saline and serially diluted. 50μl aliquot from each sample was plated onto to
a simple media (nutrient agar), Differential media (Mac Conkey agar) and selective media (MRS agar). Colonies with distinct morphology were subjected for Gram reaction.

2.3.5.5 Oral treatment

The experimental group was given daily oral administration of 1ml of the bacterial suspension in sterile PBS ($10^8$ viable cells) by orogastric gavage with a ball tip needle. The control group received 0.1ml of PBS alone. The treatment was given consecutively for 20 days.

2.3.5.6 Persistence Studies.

Freshly voided fecal pellets were collected from mice in each group individually at 3, 5, 7, 15 and 20 days during the oral administration of probiotic bacteria. 1gm of feces was weighed and homogenised and serially diluted in sterile saline water. 50 µl aliquots from each sample was placed on MRS agar. Plates were incubated at 37°C for 48 h with 5% CO$_2$ and the colonies were examined. The number of viable bacteria was recorded as colony forming units (CFU) per gram of fecal pellet. 10 days after the treatment, fecal pellets were collected and examined as above.

2.3.5.7. Confirmation of gastrointestinal tract colonization by probiotic strain (Wagner et al., 1997a)

After 20 days of probiotic treatment, the animals in the experimental group were sacrificed. Colonization of the gastrointestinal tract of Balb/c mice was assayed by counting colonies of viable bacteria (CFU) recovered from the contents of the stomach, small intestine and colon of mice that has been sacrificed. The contents were washed out of the organs with sterile water and serially diluted in normal saline and 100 µl aliquots were inoculated onto MRS agar.
2.3.5.8 Measurements of general health, growth, assessment of pathogenicity and translocation of the test strain to other organs

The weight of each of the animals in the experimental and control group were measured on a Sartorius balance before the commencement of the treatment and after the completion of treatment. Feed and water intake, behavioural changes, treatment related illness or death (diarrhoea or related diseases), unhealthy symptoms and difference in hair lustre were monitored throughout the experimental period. Also the gross pathology and weight of the internal organs such as liver, spleen and kidney were measured and compared with the control animals after they have been sacrificed. Tissue of liver, kidney and spleen were homogenised in PBS, serially diluted, and were inoculated on MRS medium. The plates were incubated for 48 h in CO\textsubscript{2} incubator in the presence of 5% CO\textsubscript{2}.

2.3.5.9 Histopathological evaluations (Wagner et al., 1997a)

Colonic sections were fixed in 10% formaldehyde in pH 7.4 phosphate buffered saline. The fixed tissues were dissected and embedded in paraffin. Five micrometer sections were placed onto slides and stained with hematoxylin and eosin. The tissue sections were evaluated in a phase contrast microscope (Q capture Pro\textsuperscript{TM} Olympus BX 43) by a pathologist for evidence of infection, inflammation, accumulation of leukocytes, epithelial erosion, and mucosal thickness. Photomicrographs were produced with a Nikon automatic camera attached imaging software.

2.3.6 Phenotypic assessment of safety of the strain Enterococcus faecium MBTU-P1F1 isolated in the study for animal and human use

Safety assessment was based on the following tests

1. Biofilm formation (2.3.2.2.5) associated with Esp (pathogenicity island (PAI) marker)
2. Plasmid profile

3. Susceptibility to Vancomycin associated with IS16 (hospital associated strain marker)

4. Susceptibility to Ampicillin (2.3.2.3.) and Minimum inhibitory concentration of Ampicillin (2.3.2.3.2)

5. Effect of colonization of the strain on the health status of animal model (2.3.5.6, 2.3.5.8 and 2.3.5.9) associated with hyl-like gene

2.4 Results

Results from chapter I revealed that the potential probiotic in the present study had appreciable growth at pH 2, 3 and 4 as well as in the presence of 0.3, 0.5 and 1% bile salts. These characteristics together with its viability in simulated gastric juice at pH 2 and 3 determine the transit tolerance capacity of the selected strain *Enterococcus faecium* MBTU-P1F1. Further results proving the diverse probiotic capabilities of the test strain are as follows.

2.4.1 Evaluation of viability and shelf life of *Enterococcus faecium* MBTU-P1F1 in skim milk

Although, the most important characteristics of probiotic bacteria are their beneficial effects on host health, evaluation of technological traits such as growth and survival in milk-based media and during product manufacture and shelf life are important considerations for selection of strains for food applications. The ability of the test strain to coagulate skim milk and to change the pH of the milk was examined for 48 h in aerobically. The results are summarized in Table 2.1. We did not find much decrease in the colony count of the strain when grown in skim milk for 48 h. The pH of the milk was 5, 4 and 3.5 when checked at 12, 24 and 48 h respectively. Similarly
when the isolate was maintained in acidified milk only very low decrease in cell counts was observed pH 4 and 5 for 30 days as indicated in Fig. 2.1. These results indicate resistance of the isolate to lactic acid as well as its viability during shelf life.

Table 2.1 Viability of *Enterococcus faecium* MBTU-P1F1 and pH change in skim milk

<table>
<thead>
<tr>
<th>Time in h</th>
<th>pH</th>
<th>CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>5</td>
<td>28±8x10^5</td>
</tr>
<tr>
<td>24</td>
<td>4</td>
<td>13±2x10^5</td>
</tr>
<tr>
<td>48</td>
<td>3.5</td>
<td>11±2x10^5</td>
</tr>
</tbody>
</table>

**Fig. 2.1** Viability of *Enterococcus faecium* MBTU-P1F1 in skim milk acidified to pH 4 and 5 with lactic acid. Viability is expressed as the log value of the CFU/ml of the test strain. Bars represent mean ± standard deviation of triplicates.
2.4.2 Cell surface properties

Results on the study of the cell surface properties of Enterococcus faecium MBTU-P1F1 are summarized in Table 2.2, Fig. 2.2, Fig. 2.3 and Table 2.3. As indicated in Table 2.2, Enterococcus faecium MBTU-P1F1 showed 0% adherence to xylene while 26% adherence to chloroform. Examination of autoaggregation ability of the test strain and the pathogens Salmonella typhi and Salmonella paratyphi A revealed that our test strain is 98% autoaggregating (Fig. 2.2) and the property is more than that of the pathogens. More over Enterococcus faecium MBTU-P1F1 coaggregates Salmonella typhi 6% and Salmonella paratyphi A 9% (Table 2.3). It is seen from the results of in vitro adhesion assay presented in Fig. 2.3 that the test strain is more adherent to intestinal mucous than the pathogens Salmonella typhi and Salmonella paratyphi A (Fig. 2.3). The quantitative analysis to examine the production of biofilm revealed that the test strain Enterococcus faecium MBTU-P1F1 does not produce biofilm (result not tabulated). Again we did not observe production of biofilm by the strain in Scanning Electron Microscopy, further confirming the result Fig. 2.4.

Table 2.2 Hydrophobicity assay of Enterococcus faecium MBTU-P1F1, tested using the solvents xylene and chloroform expressed as percentage of adherence to the solvents.

<table>
<thead>
<tr>
<th>% of Hydrophobicity</th>
<th>Xylene</th>
<th>Chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>26 ± 1</td>
</tr>
</tbody>
</table>

Table 2.3 Coaggregation ability of Enterococcus faecium MBTU-P1F1 with the enteric fever pathogens

<table>
<thead>
<tr>
<th>Coaggregation (%)</th>
<th>Enterococcus faecium MBTU-P1F1 + S. typhi</th>
<th>Enterococcus faecium MBTU-P1F1 + S. Paratyphi A</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 ± 1.01</td>
<td></td>
<td>9 ± 1.01</td>
</tr>
</tbody>
</table>
**Fig: 2.2** Autoaggregation of *Enterococcus faecium* MBTU-P1F1 and the pathogens expressed as % of aggregation of cells after 5h.

**Fig. 2.3** Adhesion of *Enterococcus faecium* MBTU-P1F1 and the pathogens to intestinal mucous. Bars represent mean ± standard deviation of three determinations.
2.4.3 Susceptibility to Antibiotics

The antibiogram of Enterococcus faecium MBTU-P1F1 is given in the Table 2.4. The test strain is sensitive to Ciprofloxacin, Chloramphenicol, Carbenicillin, Tetracycline and Vancomycin whereas it is resistant to all other drugs except for Ampicillin. Susceptibility of Enterococcus sp to Ampicillin is of considerable significance especially when they are to be characterized as probiotic for animal or human use. Here we found that our probiotic strain shows moderate susceptibility towards Ampicillin and hence determination of minimum inhibitory concentration of Ampicillin became essential. The result of MIC summarized in Table 2.5 reveals that the growth of Enterococcus faecium MBTU-P1F1 has been completely inhibited by all the different concentrations of Ampicillin starting from 0.4g/l to 4 g/l.
Table 2.4  Study of susceptibility to antibiotics of *Enterococcus faecium* MBTU-P1F1 by Kirby Bauer Method. (R: resistant, S: sensitive, MS: intermediate)

<table>
<thead>
<tr>
<th>Antibiotics and disc potency</th>
<th>Zone size in mm</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (10mcg)</td>
<td>13</td>
<td>MS</td>
</tr>
<tr>
<td>Amikacin (30mcg)</td>
<td>8</td>
<td>R</td>
</tr>
<tr>
<td>Ciprofloxacin (30mcg)</td>
<td>19</td>
<td>S</td>
</tr>
<tr>
<td>Chloramphenicol (30mcg)</td>
<td>18</td>
<td>S</td>
</tr>
<tr>
<td>Erythromycin (10mcg)</td>
<td>8</td>
<td>R</td>
</tr>
<tr>
<td>Gentamycin (10mcg)</td>
<td>8</td>
<td>R</td>
</tr>
<tr>
<td>Carbenicillin (100mcg)</td>
<td>7</td>
<td>R</td>
</tr>
<tr>
<td>Methicillin (5mcg)</td>
<td>7</td>
<td>R</td>
</tr>
<tr>
<td>Kanamycin (30mcg)</td>
<td>9</td>
<td>R</td>
</tr>
<tr>
<td>Tetracyclin (10mcg)</td>
<td>19</td>
<td>S</td>
</tr>
<tr>
<td>Streptomycin (25mcg)</td>
<td>9</td>
<td>R</td>
</tr>
<tr>
<td>Vancomycin (30mcg)</td>
<td>18</td>
<td>S</td>
</tr>
</tbody>
</table>

Table 2.5  Evaluation of Minimum Inhibitory Concentration of Ampicillin towards *Enterococcus faecium* MBTU-P1F1

<table>
<thead>
<tr>
<th>Concentration of Ampicillin in g/l</th>
<th>Turbidity (OD at 600nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>0.008</td>
</tr>
<tr>
<td>0.8</td>
<td>0.001</td>
</tr>
<tr>
<td>1.2</td>
<td>0.012</td>
</tr>
<tr>
<td>1.6</td>
<td>-0.004</td>
</tr>
<tr>
<td>2</td>
<td>-0.006</td>
</tr>
<tr>
<td>2.4</td>
<td>-0.006</td>
</tr>
<tr>
<td>2.8</td>
<td>-0.016</td>
</tr>
<tr>
<td>3.2</td>
<td>-0.030</td>
</tr>
<tr>
<td>3.6</td>
<td>-0.028</td>
</tr>
<tr>
<td>4</td>
<td>-0.024</td>
</tr>
</tbody>
</table>
2.4.4 Growth curve analysis

The growth curve pattern of *Enterococcus faecium* MBTU-P1F1 was determined in MRS medium for 24 h. This was compared with the growth pattern of the pathogens *Salmonella typhi* and *Salmonella paratyphi* in nutrient broth for 24 h. and the result is summarized in Fig. 2.5. Even though the lag phase of *Enterococcus faecium* MBTU-P1F1 is found to be slightly longer than the pathogens, it is evident from the graph that the growth rate of the test strain is much higher than the pathogens. The doubling time of *Enterococcus faecium* MBTU-P1F1 can be determined (Time taken for the OD value to double) from the graph as 2.5 h where as that of the pathogens can be seen as 5 h. More over it can be studied from the graph that the test strain has much higher biomass than either of the pathogens.

![Growth Curve](image)

**Fig. 2.5** Comparison of the growth curve pattern of *Enterococcus faecium* MBTU-P1F1 and the pathogens *Salmonella typhi* and *Salmonella paratyphi*. A
2.4.5 Plasmid profile

Study revealed the presence of singe plasmid in the test strain Fig. 2.6.

![Plasmid profile of Enterococcus faecium MBTU-P1F1](image)

Fig. 2.6  Plasmid DNA profile of *Enterococcus faecium* MBTU-P1F1

2.4.6 *In vivo* probiotic characteristics

2.4.6.1 Examination of the normal gut flora of the animals before and after the oral administration of *Enterococcus faecium* MBTU-P1F1

Colonies with distinct morphology were observed on all MacConkey agar and nutrient agar but not on MRS medium. Both Gram positive and Gram negative isolates were observed on nutrient agar where as only Gram negative isolates were found on MacConkey agar. Interestingly when the fecal flora was examined after 20 days of oral probiotic administration. Growth of Gram positive isolates having the same colony morphology and microscopic appearance as the test strain on MRS agar and almost limited no of colonies on the other plates were observed.
2.4.6.2 Colonization of gastrointestinal tract (Balb/c mice) by \textit{Enterococcus faecium} MBTU-P1F1

Colonies of the LAB strain started appearing in the fecal samples of the treated mice after 7 days of oral administration of the test strain. Colonization became stable after 14 days of treatment (Fig. 2.7). The ability of \textit{Enterococcus faecium} MBTU-P1F1 to colonize the gastrointestinal epithelial wall of mice model was confirmed when appreciable no of colony forming units of the test strain were obtained in the MRS medium from the intestinal contents after the animals have been sacrificed.

![GIT Colonization](image)

Fig. 2.7 Gastro intestinal tract colonization of \textit{Enterococcus faecium} MBTU-P1F1 in Balb/c mice. Log value of the colony forming units of the LAB isolate recovered from stool samples of mice during the oral dosage period is represented.

2.4.6.3 Safety of viable oral administration of \textit{Enterococcus faecium} MBTU-P1F1 on the health status of Balb/c mice and translocation to other organs

All the mice treated with the test strain remained healthy throughout the duration of the study. There were no signs of hair ruffling, weight loss, diarrhoea, rectal prolapse or loss appetite. No adverse clinical signs were
observed in any of the mice during the period of experimentation. No diarrhea or other treatment related sickness or no death was recorded. General health appearance did not differ significantly between mice fed with probiotics strain and control. Mice in both the groups exhibited almost similar growth and body weight (Fig. 2.8). Also no change was observed in the general behaviour or food and water intake by the animals. We did not observe difference in the gross pathology of the internal organs between the test animals and the control ones. No significant difference was observed in the gross pathology and weight of the vital organs (Kidney, Spleen, Liver) indicating absence of hepatomegaly or splenomegaly (Fig. 2.9). Moreover we did not observe the growth of the test strain when the tissues of liver spleen and kidney were cultured. The result indicates that the test strain has not translocated to other organs from the gastrointestinal tract.

![Figure 2.8](image)

**Fig. 2.8** Body weight of test mice during the three weeks of oral administration of *Enterococcus faecium* MBTU-P1F1 in comparison with the control group.
Fig. 2.9  Effect of oral administration *Enterococcus faecium* MBTU-P1F1 as probiotic on the weight of the vital organs of test mice when compared to control mice.

### 2.4.6.4 Histopathological evaluation

Fig. 2.10a, 10b, 10c and 10d represent the photomicrographs of the intestinal section of the test group mice and the control group mice. Examination of tissue sections revealed the following observations: Increased colonization by Gram positive bacteria was observed in the treatment group with no signs of infection, inflammation, epithelial erosion, or abscesses. Thickness of the mucosal layer was unaffected indicating absence of infection after oral administration of the potential probiotic strain *Enterococcus faecium* MBTU-P1F1 for 20 days. Also increased neutrophil infiltration was observed in the treatment group than the control mice.
Fig. 2.10a & 10b Histopathological evaluation of the intestine of the test group mice which received 10^8 viable cells of *Enterococcus faecium* MBTU-P1F1 as probiotic
(A) Enhanced bacterial colonization of intestinal mucosa
(B) No sign of epithelial erosion, inflammation or mucosal thickening.

Fig. 2.10c & 10d Histopathological evaluation of the intestine of the control group mice

2.4.7 Phenotypic Assessment of safety of the strain *Enterococcus faecium* MBTU-P1F1 for animal and human use

Safety assessment of the test strain was evaluated based on the following observations.

The test strain *Enterococcus faecium* MBTU-P1F1 was not found to produce biofilm as given in the results (2.4.2). Production of biofilm can form a virulence determinant of *Enterococcus* strains and in some cases be associated with *esp* gene which is part of a large pathogenicity island in
Enterococcus sp. From the disc diffusion assay to detect the antibiotic susceptibility of the strain, it was found that the strain is sensitive to Vancomycin. Sensitivity to Vancomycin is an important criterion to be studied while considering Enterococcus faecium as probiotic. Further the strain was found to be moderately sensitive to Ampicillin and hence the Minimum Inhibitory concentration (MIC) of the drug was evaluated. The result clearly indicated that Enterococcus faecium MBTU-P1F1 is completely inhibited by Ampicillin at a concentration as low as 0.4g/l, which is much lower than 2g/l. According to the recommendation by the European Food Safety Authority any strain of Enterococcus faecium with MIC of Ampicillin $\geq 2g/l$ is unsafe for use as probiotic. Finally no adverse effects were found in the test mice after 20 days of oral treatment with Enterococcus faecium MBTU-P1F1 strain characterized in the study.

2.5 Discussion

Lactic Acid Bacteria are among the most numerous of the commensal microflora inhabiting the human large intestine and are generally thought to play an important role in the maintenance of gut homeostasis, immune status and are beneficial to health. For the characterization of a novel probiotic strain for application in fermented or dairy or pharmaceutical product, or in functional foods, it must be taken into consideration that living bacteria will interact with their environment. Hence LAB strains need to be screened for their capacity to tolerate the conditions of the upper gastrointestinal tract, they should have technological traits important during product manufacture, should have desirable cell surface properties required for adhesion to gastrointestinal tract and to prevent or reduce adhesion by pathogens, and most importantly antibacterial property as well as health promoting attributes. Evaluation of the
above mentioned properties are desirable while selecting a suitable probiotic strain on account of the fact that these characters are strain specific. The chapter II has examined most of the essential and desirable probiotic features of the test strain *Enterococcus faecium* MBTU-P1F1 isolated from infant feces including the safety assessment at the phenotypic level.

Viability and activity of the bacteria are important considerations for probiotic strains as the bacteria must survive in the food during product manufacture, shelf life and resist the barriers during transit through the GIT. It is essential that products sold with any health claims meet the criterion of a minimum $10^6$ CFU /ml probiotic bacteria at the expiry date, because the minimum therapeutic dose per day is suggested to be $10^8 – 10^9$ cells (Kurmann and Rasic, 1991). It has been reported by Varnam and Sutherland, (1994) that strains of Bifidobacteria used in commercial products neither survive gastric transit nor product activity during storing. Kailaspathy and Chin, (2000) considered it misleading to describe probiotic yoghurt as having health promoting properties unless minimum level of viable cells is present at the expiry date. Our candidate strain *Enterococcus faecium* MBTU-P1F1 showed appreciable viability when grown in skim milk for 48 h at 37ºC. Moreover we observed only a very low decrease in the cell counts of the LAB when stored in skim milk acidified with lactic acid at pH 4 and 5 for 30 days of at 4ºC. The result indicates the ability of the strain to survive in milk based media. This property becomes important as most of the functional foods incorporating probiotic bacteria are prepared in dairy products. The result highlights the resistance of the isolate to lactic acid which contributes to its viability during shelf life.

Examination of the hydrophobicity / hydrophilicity of cell surface give information on the structural properties responsible for aggregation
and adhesion. Wadstrom et al. (1987); Perez et al. (1998); and Del Re et al. (2000) reported that autoaggregation and adhesion of bacteria to different surfaces may be affected by physicochemical characteristics of the cell surface such as hydrophobicity. *Enterococcus faecium* MBTU-P1F1 showed 0% adherence to the apolar solvent xylene indicating the absence of proteinaceous material at the cell surface. The adherence of our test strain to the acidic solvent, chloroform reveals the hydrophilic cell surface property associated with the presence of polysaccharides. Kos et al. (2003) has reported that *Enterococcus faecium* L3 showed more hydrophilic cell surface properties and are strong electron donors. Our results are in agreement with the observations made by Savage, (1992) and Ouwehand et al. (1999) who found no correlation between cell surface hydrophobicity and the ability to adhere to intestinal mucus. However Wadstrom et al. (1987) observed that *Lactobacillus* strains, isolated from porcine small intestine, showing high surface hydrophobicity and adhered in high numbers to porcine enterocytes. Aggregation substance (Agg) is a pheromone inducible surface protein that may contribute to the pathogenesis of enterococcal infection. Munday et al. (2000) reported that Agg increases hydrophobicity of the enterococcal surface and prevent or delay fusion with lysosomal vesicles and is a virulence determinant in Enterococci. 0% hydrophobicity of our test strain indicates the absence of such a virulence factor proving the nonpathogenic trait of the strain. More over presence of polysaccharide (as revealed by adherence to chloroform) on the cell surface can contribute to susceptibility of the strain to opsonophagocytic killing. The property is regarded as a method to assess the safety of *Enterococcus* sp (Hufnagel et al., 2003).
Bacterial aggregation between microorganisms of the same strain (autoaggregation) or between genetically different strains (coaggregation) is of considerable importance in several ecological niches, especially in the human gut, where probiotics are to be active. In order to manifest beneficial effects, probiotic bacteria need to achieve an adequate mass through aggregation, and hence the ability of probiotics to aggregate is a desirable property (Collado et al., 2008). In the present study we have detected the 98% autoaggregation by the test strain, 71% by *Salmonella typhi* and 82% by *Salmonella paratyphi* A. Even though the pathogens also showed appreciable autoaggregation, the property was much higher for the test strain with 98% autoaggregation. Autoaggregation of probiotic strains appear to be necessary for adhesion to intestinal epithelial cells.

Investigations by several workers have related aggregation ability to cell adherence properties (Kos et al., 2003). 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) also showed that autoaggregation of LAB correlates with their adhesion ability. Our study too support a positive correlation between autoaggregation ability and adhesion potentials when we put the results of autoaggregation (Fig. 2.2) and *in vitro* adhesion assay (Fig. 2.3) together.

Coaggregation assay was performed to measure the interbacterial adherence between the test strain and the enteric fever pathogens. Coaggregation ability of probiotic strain helps in excluding the pathogens prior to the proper adhesion. The mechanism of coaggregation involves interaction between proteinaceous and polysaccharides molecules on the cell surfaces of the aggregating bacterial cells. Our test strain *Enterococcus faecium* MBTU-P1F1 coaggregated *Salmonella typhi* 6% and *Salmonella*
paratyphi 9%. Anu and Keerthi, (2012) reported that the potential probiotic Bacillus subtilis MBTU PBBM1 isolated from cow’s milk can coaggregate enteric pathogens, Salmonella typhi, Salmonella paratyphi A and Vibrio cholera. Reid et al. (1998) suggested that LAB which coaggregate with pathogens and are capable of producing antimicrobial compounds form an important host defence mechanism against infection in the urogenital tract. And Spencer and Chesson, (1994) reported that a similar mechanism could operate in the gastrointestinal tract. Similarly the present study found the inhibitory action of the Lactic Acid Bacteria Enterococcus faecium MBTU-P1F1 against the pathogens Salmonella typhi and Salmonella paratyphi A (Chapter 1). Our findings in the present chapter indicate capability of the strain to coaggregate with the pathogens. Hence coaggregation of the pathogens together with production of antimicrobial compounds enhances the ability of the test strain Enterococcus faecium MBTU-P1F1 to exclude pathogenic bacteria from their hosts. Collado et al. (2008) reported that coaggregation property are related to autoaggregation abilities. 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). Organisms with the ability to coaggregate with other bacteria especially pathogens may have an advantage over non coaggregating organisms, which are more easily removed from the intestinal environment. Coaggregation with gut pathogens could therefore contribute to the probiotic properties ascribed to the test strain.

Del Re et al. (2000) reported that adhesion is a multistep process in which both non-specific mechanisms and a specific ligand receptor play a role. Bacterial adhesion is initially based on non specific physical
interactions between two surfaces, which then enable specific interactions between adhesions and complementary receptors (Kos et al., 2003, Perez et al., 1998). According to Del re et al. (2000), Kos et al. (2003) adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract, preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem.

The ability to adhere to epithelial cells and mucosal surfaces has been suggested as an important property of probiotic bacterial strains and according to Ouwehand et al. (1999) it is an important property for colonisation by preventing wash-out in the small intestine where flow rates are relatively high. Lin et al. (2006) reported that adhesiveness to intestine influences the time of bacteria retention in intestine and the functional activity of bacteria. Adherence of bacteria to intestinal epithelium is known to be a prerequisite for colonization and infection of the gastrointestinal tract by many pathogens Collado et al., 2008). In the present study we performed an in vitro assay using intestinal mucous of human origin to examine the adhesion ability of the test strain and the enteric fever pathogens. It was observed that the test strain Enterococcus faecium MBTU-P1F1 has the potential to adhere to human intestinal mucous the source of the sample (infant feces) supports this finding. Also the test strain has more ability to adhere to mucosal surface than the pathogens providing it with the competitive advantage in preventing colonisation and establishment of infection by intestinal pathogens. Khalil et al. (2007) observed strong adhesion to intestinal mucous for the strains Enterococcus faecium (P166) and Enterococcus durans (P174). Adhesion of probiotic bacteria to the intestinal mucosa has been shown to enhance their antagonistic activity
against pathogens (Coconnier et al., 1993) and is an important prerequisite for colonization the gastrointestinal tract and prevents immediate elimination by peristalsis. Adhesion to intestinal mucosa has also been suggested to enhance the ability to stimulate the immune system (Ouwehand et al., 1999). A direct correlation between the adhesive ability of probiotic strains and the serum antibody titres have been observed in some studies Ouwehand et al. (1999) argued adhesion as a potential virulence factor of the of highly adhesive strains, also that once microorganisms have adhered to the intestinal mucus layer, they will only be removed if that part of the mucus layer is sloughed off into the lumen. We have also performed in vivo studies to substantiate the result obtained in the in vitro assay.

Results from the quantitative analysis followed by SEM analysis indicated the absence of biofilm formation by our isolate. Biofilm formation constitutes an essential mechanism for colonization by pathogenic microorganisms. Enterococci in biofilms are highly resistant to antibiotics and biofilms form an important factor in the pathogenesis of enterococcal infection (Mohamed and Huang, 2007). The result of biofilm formation in our study can be explained by the less pathogenic trait of our isolate which in turn could be attributed to its human origin. Other investigators have reported similar results and suggest that Enterococcus faecalis (95%) isolates produce a biofilm more often than Enterococcus faecium (29%) (Di Rosa et al., 2006). There are many factors affecting biofilm formation among different bacteria such as growth media, presence of glucose, osmolarity, temperatures, carbondioxide etc. There are many reports on the role of esp gene (large pathogenicity island in Enterococcus sp) in biofilm formation by Enterococci. It has been proposed a strong association between the presence of an esp gene and greater levels of biofilm formation in Enterococcus
faecalis (Mohamed et al., 2004). According to Foulquie et al. (2006) *esp* gene is hardly found in the food isolates of *Enterococcus faecium*. However there are also reports of biofilm production in *Enterococcus faecium* contributing to endocarditis (Heikens et al., 2011).

One of the important features of probiotic strains is their resistance to antibiotics, especially when they are to be used after antibiotic therapy. Nedelcheva et al. (2010) that LAB which are involved in probiotics and probiotic foods must be able to survive in the conditions of gastrointestinal tract and be resistant to antibiotics. A key requirement for probiotic strains is that they should not carry transmissible antibiotic resistance genes (Zhou et al., 2005) and high sensitivity differences of LAB strains have been reported (Delgado et al., 2007) in recent investigations. In the present study we tested the susceptibility of the test strain towards 12 antibiotics. It was found that the test strain was resistant to Erythromycin, Gentamicin, kanamycin, Streptomycin, Amikacin and also to the β lactam drug, Methicillin. But the test strain was moderately sensitive to Ampicillin and was sensitive to Tetracyclin, Chloraphenicol, Ciprofloxacin and most importantly towards the glycopeptides, Vancomycin. Ampicillin, Gentamicin and Vancomycin are the most clinically relevant antibiotics to cure infections with multiple antibiotic resistant strains. Sensitivity to Vancomycin and extensive use of Vancomycin has steadily raised the percentage of invasive nosocomial Enterococci displaying high level Vancomycin resistance (Endtz et al., 1999). Vancomycin resistant Enterococci are resistant to all standard anti-enterococcal drugs are considered as a serious risk group. Vancomycin resistance is associated with the genetic element IS16, a virulence marker associated with hospital strains.
According to the guidance on the assessment of *Enterococcus faecium* put forward by the European Food Safety Authority (EFSA), absence of the genetic element IS16 should be established before it can be used as probiotic. Since the test strain was found to be moderately sensitive to Ampicillin, we estimated the Minimum Inhibitory Concentration (MIC) of Ampicillin required to inhibit the growth of the test strain. Interestingly we found complete inhibition of the test strain at a concentration of Ampicillin as low as 0.4g/ml. Again as per the guidance on the assessment of *Enterococcus faecium* put forward by the European Food Safety Authority (EFSA), determination of MIC of Ampicillin is considered crucial in assessing the safety of *Enterococcus faecium* strains. Strains with Ampicillin MICs < than 4g/l have the *pbp5* – S form of the gene encoding Penicillin binding protein which is characteristic of community associated non pathogenic *Enterococcus faecium* isolates. The result clearly proves the non pathogenic nature of our test strain.

Our findings clearly reveal that the test strain had lesser doubling time and greater growth rate than the pathogens, *Salmonella typhi* and *Salmonella paratyphi* A. Also it can be learned that the test strain has a longer stationary phase extending more than 24 h. In addition the growth pattern gives the information that the biomass of the strain is much higher than the pathogens. 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. By attaching to the intestinal mucosa, probiotics can extend their time within the gut thereby influencing the gastrointestinal microflora of their host. It has been reported by Monaghan Gagliardi and
Streicher, (1999) that candidate probiotic bacteria may only produce antimicrobial metabolites during the stationary growth phase (Vine et al., 2004). The lesser doubling time and the greater growth rate and biomass will definitely be an advantage for our candidate probiotic strain to establish itself in the gastrointestinal tract of the host and to out compete the enteric fever pathogens Salmonella typhi and Salmonella paratyphi A during the onset of an infection. Similar study was conducted by Aparna and Keerthi, (2012) where they compared the growth curve pattern of potential probiotic strains from majorflora of Poecelia reticulata with fish pathogens. Moreover the longer stationary phase can be related to production various metabolites, most of which can add to the antimicrobial property of the probiotic candidate. The higher biomass will also facilitate enhanced production of antimicrobial metabolites.

Taken together the results of adhesion ability, growth pattern and the antibacterial activity against the enteric fever pathogens, it can be concluded that the test strain Enterococcus faecium MBTU-P1F1 when used as a probiotic can prevent the pathogenic invasion by these pathogens.

Presence of a single plasmid was detected in the test strain, Enterococcus faecium MBTU-P1F1. Since the test strain was found sensitive to Vancomycin and Ampicillin and since we did not find any adverse health reaction in the animal model, further evaluation of the plasmid was not proceeded in the study.

As per the guidelines of WHO, in vivo studies are mandatory for substantiation of in vitro results. Male Balb/c mice were chosen as the animal model for the in vivo studies. They serve as a mammalian animal model and meet determined taxonomic equivalency to humans so as to react to diseases
or its treatment in a way that resembles human physiology as needed. The dosage for oral treatment was selected as $10^8$ viable cells per ml of normal saline for each mouse per day. Probiotic concentrations ranging between $10^8$ and $10^9$ CFU/mouse/day are sufficient to efficiently colonize the intestinal mucosa of rodent. This dosage is biologically relevant since it is based on a daily intake of about 3,600 billion bacteria for an adult human weighing 70 kg (Bassaganya-Reira et al., 2012). The experiment consisted of two groups—group 1 (Test mice) and group 2 (Control mice) with six mice each.

The animals in the test group were given 1ml (containing $10^8$ viable cells) of test strain, *Enterococcus faecium* MBTU-P1F1 in saline, orally for 20 days. The animals in the control group received 1ml of normal saline. Ability of the test strain to colonize the gastrointestinal tract of Balb/c mice was monitored by periodic examination of quantitative cultures of serially diluted feces collected from the animals during and after oral treatment of the test strain. Colonies of the test strain started appearing on MRS plates from the 7th day of treatment and the number of colonies characteristic of the test strain increased with the no of days of treatment proving colonization of the gastrointestinal tract by the test strain. Also we noticed a decrease in the number of Gram negative isolates in the Nutrient agar plate and in the MacConkey agar plates.

Satisfactory number of colony forming units of the test strain was recovered from the fecal pellet, when examined 10 days after the completion of the oral treatment with the test strain. The gastrointestinal tract colonization of the test strain was further confirmed by culturing the contents of the intestine after the animals have been sacrificed. Colonization of gastrointestinal tract by oral administration of probiotic bacteria has been shown in many studies by Wagner et al. (1997a and b). 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. The beneficial immunological effects can be best achieved if the microorganisms colonise the intestinal surface mucus layer. This makes them affect the intestinal immune system, displace enteric pathogens, provide antioxidants and antimutagens and possibly other effects by cell signalling (Ljungh and Wadstrom, 2005). It was studied in the *in vitro* adhesion assay study that the test strain possessed adhesion ability to intestinal mucous and the result has been proved in the animal model.

The animals belonging to the test group as well as the control group remained healthy throughout the experiment period. We did not observe any difference with regard to the general behaviour, food and water intake or general health appearance in the test animals. There was no weight loss, diarrhoea or any adverse clinical signs. Also no difference in the gross pathology of the internal organs was observed between the test animal and control ones with no signs of hepatomegaly or splenomegaly. All these findings support the safety of viable administration of the test strain *Enterococcus faecium* MBTU-P1F1 as a probiotic. Assessment of pathogenicity is one of the important components of probiotic safety studies, the indicators for which include splenomegaly and hepatomegaly (Zhou *et al.*, 2000; Zhu *et al.*, 2009).

Histopathological evaluation of the gastrointestinal tract revealed increased colonization of the intestinal mucous layer by Gram positive bacteria than the control ones. Colonization of the gastrointestinal tract of the animal model, Balb/c mice by the test strain did not create any signs of inflammation, abscesses, epithelial erosion or mucosal thickening. All these
observations give information on the safety of test strain for use as a probiotic.

Enterococci are an essential part of the endogenous gut microbiota of humans and animals, where they are believed to play a key role in the balance of the microbiota and thereby showing great potential as probiotics (Izquierdo et al., 2008). Enterococci are also promising for the biopreservation of food, especially by means of bacteriocin production. Despite the concerns for Enterococci as opportunistic pathogens, they have long been used as human and animal probiotics (Franz et al., 2007). According to Murray, (2000) human infections caused by Enterococci outside the healthcare setting are very uncommon. Human isolates of Enterococcus faecium have been recognised as belonging to two clade, the hospital associated clade and the community acquired associated clade. According to the Guidance on the safety assessment of Enterococcus faecium put forward by the European Food Safety and Authority, strains of Enterococcus faecium belonging to the hospital associated clade must be excluded from the use in animal nutrition. As per the guidance, a strain of Enterococcus faecium can be used only if–

a) the MIC of Ampicillin is $\leq 2$ mg/ml

b) none of the following genetic elements should be detected.

- IS16 (Hospital associated strain marker)
- hylefm (Plasmid encoded gene for glycosyl hydrolase)
- esp IS16 (Pathogenicity island marker).

Safety of the test strain, Enterococcus faecium MBTU-P1F1 was evaluated based on the following observations.
The test strain was found to be moderately sensitive to Ampicillin in the disc diffusion assay. Therefore the Minimum Inhibitory Concentration (MIC) of the drug was evaluated. The result clearly indicated that *Enterococcus faecium* MBTU-P1F1 was completely inhibited by Ampicillin at a concentration as low as 0.4 g/l, which is much lower than 2 g/l.

The test strain *Enterococcus faecium* MBTU-P1F1 was not found to produce biofilm. Production of biofilm can form a virulence determinant of *Enterococcus* strains and in some cases be associated with *esp* gene which is part of a large pathogenicity island in *Enterococcus* sp. According to Heikens *et al.*, 2007 the *esp* gene has an role in biofilm formation of *Enterococcus faecium*. We did not observe the formation biofilm in the quantitative analysis or in the SEM analysis of the test strain. The result of biofilm formation can be related to the absence of the *esp* gene which in turn can indicate the safety of the strain for use as probiotic.

From the disc diffusion assay to detect the antibiotic susceptibility of the strain to commonly prescribed antibitics, it was found that the strain was sensitive to Vancomycin. Sentitivity to Vancomycin is an important criterion to be studied while considering *Enterococcus faecium* as probiotic. Vancomycin resistant Enterococci are resistant to all standard anti-enterococcal drugs and form a serious risk group (Endtz *et al.*, 1999; Foulquie *et al.*, 2006). IS16 is the genetic element which flanks the transposon Tn1547. This transposon confers resistance to Vancomycin in *Enterococcus faecalis* and is a marker for hospital associated subpopulations of *Enterococcus faecium*. Sensitivity to Vancomycin observed for the test strain can be associated with the absence of this genetic element.
Finally no adverse effects were found in the test mice during or after oral treatment with the test strain characterized in the study. The effect of the test strain on the health status of the test mice can be associated with the absence of hyl-like gene coded in very large plasmids. The hyl genes have shown to increase lethality in murine peritonitis model. However we did not observe any sign of infection or morbidity in the test group animals treated with Enterococcus faecium MBTU-P1F1 and according to Rice et al., (2003), this gene is never present in community associated clade. The hyl$_{efm}$ gene is predominantly seen in Vancomycin and Ampicillin resistant strains which come under hospital associated clade. Vancomycin sensitivity and the MIC of Ampicillin has proved that the strain belong to the community associated clade. Safety assessment in the in vivo studies would further rule out the possible presence of the virulence marker hyl$_{efm}$.

Taken together, the phenotypic assessment based on the results of MIC for Ampicillin, biofilm formation, susceptibility to Vancomycin and finally the in vivo studies favour the safety of the strain Enterococcus faecium MBTU-P1F1 for animal and human use.

Conclusion

After having selected and identified a potential candidate strain with preliminary and essential probiotic capabilities (chapter1), in vitro studies were carried out to determine the desirable properties required during product manufacture and the ability to adhere to the intestinal mucosa. The cell surface property of the strain, its ability to autoaggregate and to coaggregate the enteric fever pathogens were determined. These properties are of considerable importance in ecologically niches such as human gut, where probiotics are to be active (Collado et al., 2008). Our studies revealed
the hydrophilic cell surface property of the research strain. It showed higher autoaggregation than the enteric fever pathogens, *Salmonella typhi* and *Salmonella paratyphi* A and coaggregates them at room temperature in 50 mM K$_2$HPO$_4$ buffer. It was found to be more adherent to intestinal mucosal cells than the pathogens. The chapter further reveals the capability of the strain in colonizing gastrointestinal mucosa of Balb/c mice and thereby the influence on the health status of the animal model. Here Balb/c mice were given oral administration of the probiotic candidate. The ability of the strain in achieving colonization in the gastrointestinal mucosa, effect on the gross pathology, infection, inflammation in the mucosal epithelium, translocation to other organs are described. The observations were compared with that of an untreated control group. The ability of *Enterococcus faecium* MBTU-P1F1 to colonize the gastrointestinal epithelial wall of mice model was confirmed when appreciable no of colony forming units were obtained in selective media from the intestinal contents, after the experimental animals have been sacrificed. The result was further supported by histological evaluation of the intestinal wall. Enhanced colonization of the gastrointestinal mucosa with no signs of infection or inflammation was observed. The survivability and colonization in the digestive tract are considered critical factors to ensure optimal functionality and expression of the beneficial health effects by probiotics. The chapter also presents the growth curve pattern of the isolate in comparison with the enteric fever pathogens to project the advantage of the strain with its short doubling time and fast growth rate in outcompeting the pathogens. The Final part of the chapter II explains the safety assessment of the strain *Enterococcus faecium* MBTU-P1F1 isolated from infant feces for use in animal and human nutrition. No mortality or lethality was observed in balb/c mice after
colonization of the gastro intestinal tract with the strain. A critical or important criterion to examine while using *Enterococcus faecium* as probiotic for human or animal use is the absence of resistance to the antibiotics Vancomycin and Ampicillin. Our research strain is sensitive to Vancomycin, and the MIC of Ampicillin was found to be less than 2mg/l. Also *Enterococcus faecium* MBTU-P1F1 do not form biofilm in *in vitro* tests. These assessments have revealed the absence of potential virulent factors associated with the strain and hence can be considered safe for use.