Chapter 1
Isolation, screening and selection of potential Lactic Acid Bacteria strain from infant feces

1.1 Introduction

Bacteria have a reputation for causing diseases and there is a tendency to regard all microorganisms as harmful; to equate bacteria with germs. The number of non-pathogenic species far exceeds the number of pathogenic species and many of the non-pathogens are in fact useful, even essential for the continued existence of life on earth. One example of a beneficial group of microorganisms is those which inhabit the gastrointestinal tract of animals.

There is, in the gut, a very complex population of microorganism which exists in a symbiotic relationship between the host, where the bacteria get food and a suitable environment for growth and the host animal acquires protection against some forms of disease. Microflora of the gastrointestinal tract plays a crucial role in the anatomical, physiological and immunological development of the host. It stimulates the immune system to respond rapidly to infection with pathogens and through bacterial antagonism it inhibits the colonization of the gut by harmful or pathogenic bacteria. Microflora of the gastrointestinal tract consists of species belonging to the families Bacteroides, Fusobacterium, Butyribrio, Clostridium, Bifidobacterium, Eubacterium and Lactobacillus. Enterococcus and Escherichia coli constitute less than 1% of all intestinal microorganisms. A dominant flora represents 90% of the population essentially composed of Bifidobacteria and Lactobacillus (Cebra, 1999). Within hours after birth, bacteria ingested during the birthing process rapidly colonize the gastrointestinal tract and become responsible for priming
the gastrointestinal immune system. It is these beneficial organisms that have attracted attention as possible probiotics. The most commonly used organisms in probiotic preparations are the Lactic Acid Bacteria (*Lactobacilli, Streptococci* and *Bifidobacteria*). These are found in large numbers in the gut of healthy animals and do not appear to affect them adversely. They are in the words of the America FDA, Generally Regarded as Safe (GRAS) (Fuller, 1989).

![Distribution of beneficial bacteria in the human gastrointestinal tract](Image)

*Fig. 1.1* Distribution of beneficial bacteria in the human gastrointestinal tract
Probiotics are defined as live microorganisms that, when ingested, produce some therapeutic or preventive health benefit, an important benefit being their capacity to prevent or curtail infectious diseases. By definition, they also must be of human origin and have some demonstrable health promoting benefits proven by clinical studies. A lot of research had been carried out on probiotics and it is increasing steadily since then, but much of it is in Europe, Asia and of recent in South Africa. Presently, probiotics are available in a variety of food products. The most frequently used bacteria in these products belong to Lactic Acid Bacteria (LAB) and *Bifidobacterium*. The success of probiotics in recent times, fuelled by failure of pharmaceutical agents, consumer demands for natural products and scientific validity of the concept, has led to the entrance of products onto the market that are close to meeting the standards set by FAO/WHO (Anukam and Reid, 2007).

**Evolution of the concept of probiotics**

Since ancient times, probiotic foods such as fermented products and cultured milk have been around. But it has been only of late that there has been a great demand for probiotics with the markets flooding with probiotic supplements and foods. History provides us a few clues on how different cultures promoted their intestinal health before modern times. The first records of ingestion of live bacteria by humans are over 2000 years ago. The role of fermented milk in human diet was known even in Vedic times. Years ago, people used fermented foods like yoghurt and sauerkraut - as support for intestinal and overall health.

- **During Roman times**, people ate sauerkraut because of its taste and benefits to their overall health.
In ancient Indian society, it became commonplace (and still is) to enjoy a before-dinner yoghurt drink called a lassi. These Indian traditions were based on the principle of using sour milk as a probiotic delivery system to the body.

Bulgarians are known both for their health which has been attributed to the high consumption of fermented milk and kefir.

In Asian cultures, pickled fermentations of cabbage, turnips, eggplant, cucumbers, onions, squash and carrots still exist today.

People of the Ukraine consume probiotic from foods like sauerkraut, raw yogurt, and buttermilk.

The interesting thing was most of these dietary habits were born from tradition, and no one really knew or understood why they were so healthy to the digestive system. Well, things changed in the early 1900s, when Russian scientist Elie Metchnikoff (Nobel Prize, 1908) proposed that the Balkan population enjoyed excellent health due to consumption of large quantities of fermented milks containing beneficial bacteria.

The word probiotic was used initially as an anonym of the word antibiotic. The origin of the first use can be traced back to 1953 when Kollath used it to describe the restoration of the health of malnourished patients by different organic and inorganic supplements. In 1965 Lilly and Stillwell defined probiotics as microorganisms promoting the growth of other microorganisms. The word probiotics was used by Parker in 1974 for fecal suspensions from healthy adult animals. These preparations were used in those periods for supplementing the diet of animals in the same species with deficient flora and thereby to improve their resistance. Parker defined probiotics as: ‘Organisms and substances which contribute to intestinal
microbial balance’ (Anukam and Reid, 2007; Oyetayo and Oyetayo, 2005). In 1989, Fuller modified the definition as: ‘A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’. This revised definition stresses the importance of live cells as an essential component of the probiotic preparation (Fuller, 1989).

Table 1.1 List of probiotic products available in market

<table>
<thead>
<tr>
<th>Probiotic bacteria</th>
<th>Product</th>
<th>Manufacturer</th>
<th>Health benefit</th>
</tr>
</thead>
</table>
| *Bifidobacterium longum*  
*Lactobacillus gasseri*  
*Bifidobacterium bifidum* | Flora  
Mend  
Prime  
Probiotic | Thorne Research | Supports digestive and immune health, Enhances balance of good bacteria in health |
| *Lactobacillus casei Shirota* | Yakult | Yakult | Maintenance of gut flora, immunomodulation, improvement of bowel habits and constipation |
| *Bacillus subtilis and Enterococcus faecium* | Medilac | Hanmi Pharmaceutical Co., Ltd | Maintenance of gut flora, Improves digestive health |
| *Escherichia coli 1917* | Mutafior | Ardeypharm | Immune stimulation |
| *Lactobacillus rhamnosus,*  
*Bifidobacterium bifidum,Bifidobacterium longum* | Probiotic immune | Zenith Nutrition | Immune stimulation, enhances beneficial gut flora |
| *Lactobacillus casei F19* | Cultura | Arlafood | Improves digestive health, Immune stimulation, Reduces weight gain, reduces antibiotic associated diarrhea |
The various beneficial and therapeutic applications of probiotics can be listed as -

**Beneficial effects:**
- Maintenance of normal intestinal microflora
- Enhancement of immune system
- Reduction of lactose intolerance
- Reduction of serum cholesterol levels
- Anticarcinogenic activity

**Therapeutic applications:**
- Prevention of urogenital infection
- Alleviation of constipation
- Protection against traveller's diarrhoea
- Prevention of infantile diarrhoea
- Reduction of antibiotic-induced diarrhoea
- Prevention of hypertension
- Protection against colon/bladder cancer
- Prevention of osteoporosis
Considering the impressive list of potential health-promoting benefits, there continues to be considerable interest in the use of probiotics as biotherapeutic agents. Due to heightened awareness given among consumers that probiotic-containing foods are generally perceived as “safe” and “natural,” the global market for dairy-based products marketed for the prophylaxis or alleviation of gastrointestinal disorders has increased.
Recent scientific investigation has supported role for probiotics as a part of healthy diet for humans and animals and may be an avenue to provide a safe, cost effective barrier against microbial infection (Parvez et al., 2006). There are moves all over the world to isolate and characterize novel strains of LAB with probiotic activities with the focus that they might be incorporated into health promoting functional foods. Ongoing basic research will continue to identify and characterize existing strains of probiotics, identifying strain-specific outcomes, determine optimal doses needed for certain results and assess their stability through processing and digestion. With the recognition and endorsement by the United Nations and World Health Organization, the call that “Efforts should be made to make probiotic products more widely available, especially for relief work and population at high risk of morbidity and mortality” is to be made practical.

Lactic Acid Bacteria (LAB) constitutes an integral part of the healthy gastrointestinal microecology and influence host metabolism (Gibson and Fuller, 2000). LAB is among the most numerous of the commensal microflora inhabiting the human large intestine and plays an important role in the maintenance of gut homeostasis and is beneficial to health. The isolation of Bifidobacterium species has assumed considerable importance, recently as a consequence of interest in the potential health-promoting properties of this genus with large industrial and medical importance (Hadaji et al., 2005). Lactobacillus and Bifidobacterium are the most common probiotic bacteria used as food adjuvants.

These bacteria, usually found in decomposing plants and lactic products, produce lactic acid as the major metabolic end-product of carbohydrate fermentation. This aspect partially enables LAB to outcompete other bacteria in a natural fermentation, as they can withstand the increased
acidity from organic acid production (e.g., lactic acid). This property has linked LAB with food fermentations, as acidification inhibits the growth of spoilage agents. Proteinaceous bacteriocins are produced by several LAB strains and provide an additional hurdle for spoilage and pathogenic microorganisms. Furthermore, lactic acid and other metabolic products contribute to the organoleptic and textural profile of a food item. The industrial importance of the LAB is further evinced by their generally recognized as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces.

Some of the beneficial effects of Lactic Acid Bacteria consumption include: (i) improving intestinal tract health; (ii) enhancing the immune system, synthesizing and enhancing the bioavailability of nutrients; (iii) reducing symptoms of lactose intolerance and (iv) and decreasing the prevalence of allergy in susceptible individuals. Strains isolated from the human intestinal tract are generally recommended suitable for probiotic use in human because some health-promoting benefits may be species specific, and microorganisms may perform optimally in the species from which they were isolated (Vinderola et al., 2008; Stanton et al., 2003)

Probiotic cultures of foreign origin are being used in currently available food products. As there are inherent differences in gut microflora of the Indian population as compared to the western population, the effects of such cultures on the Indian population may not be same as that on the western population. The isolation and characterization of new strains is still desirable, in developing countries mainly, for the formulation of probiotic foods since there is still a restricted access to probiotic strains to small dairy plants (Vinderola et al., 2008).
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Many *in vitro* tests are performed when screening for potential probiotic strains. The first step in the selection of a probiotic LAB strain is determination of its taxonomic classification, which may give an indication of the origin, habitat and physiology of the strain. The ability to survive in the conditions prevailing in the gastrointestinal tract, anti haemolytic property and antibacterial property are widely accepted screening tests for selection of probiotic strains. All these characteristics have important consequences on the selection of the novel strains (Morelli, 2007).

Mucous epithelial surfaces of GIT where the host is confronted with a range of different microorganisms from the outside environment are suitable places to start often infection with pathogens. These surfaces are not unprotected and different mechanisms are involved in the permanent and effective surveillance. Mucous surfaces are protected by many defense mechanisms that ensure a permanent and effective protection. They include the production of secretory IgA, the production of mucous, cytoprotective peptides, defensins etc. Indigenous microflora markedly affects the structure of the host mucous, its function and the development of the whole immune system. The protective microflora creates the barrier effect against problematic pathogens and produces regulatory factors such as short chain fatty acids, protein exotoxins, lytic agents and bacteriocins. This effect also includes the competition for receptors and metabolic substrates (Herich and Levkut, 2002). Bacterial colonization of the intestine undergoes changes depending on age and other factors such as environment, stress, diet and medicine. Reinforcement of the gut microecology can greatly improve health status of the host as well as protection against gastrointestinal pathogens. This can be achieved through probiotic supplementation. Probiotic bacteria stand on guard to protect us from pathogenic bacterial, viral and yeast
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invaders. Probiotic bacteria can be used as alternative to antibiotics. *Lactobacilli, Bifidobacteria* and *Streptococci* have been extensively used as prophylactic agents for traveller’s diarrhoea caused by enterotoxigenic *E. coli* and are used as therapeutic agents against diarrheal diseases caused by Rotavirus and *Clostridium difficile* (George and Cummings, 1999; Cunningham-Rundles *et al.*, 2000). The strongest evidence of the beneficial effect of probiotics has been established with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB-12 for prevention and *Lactobacillus reuteri* SD 2222 for treatment of acute diarrhoea mainly caused by Rotaviruses in children (Reid *et al.*, 2003). Bacteriocins from Lactic Acid Bacteria have attracted special interests from the aspect of their potential use as safe and natural food preservatives (biopreservatives) and antimicrobials (Deegan *et al.*, 2006; Zendo *et al.*, 2008). The important bacteriocins such as nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins and plantaricins characterized from LAB have acquired a status as potential antimicrobial agents because of their potential as food preservatives and antagonistic effect against important pathogens (Aly *et al.*, 2006). The lantibiotic nisin which is produced by *Lactococcus lactis* spp. is the most thoroughly studied bacteriocin and is applied as an additive in food worldwide (Delves Broughton *et al.*, 1996).

Enteric fever is a major public health problem in India, accounting for more than 300,000 cases per year, *Salmonella typhi* being the most common etiologic agent, but *Salmonella paratyphi* A, the other causative agent, causes more asymptomatic infections than *S. typhi* (Richens, 1996). Multidrug resistance in *S. typhi*, especially to quinolones has also been reported and therefore continued surveillance and monitoring of antimicrobial sensitivity of these strains are needed (Kumar *et al.*, 1997;
Saxena and Sen, 1966). These two pathogens are also significant as major contaminant in food industry.

The words ‘probiotics’ and ‘antibiotics are Greek, meaning- ‘for life’ and ‘against life’. Antibiotics suppress and kill both the harmful bacteria and beneficial bacteria. A single course of antibiotics is capable of wiping out beneficial strains of bacteria for more than six months. On the other hand, probiotics are for life which helps to preserve the intestinal bacterial microflora and boost natural immunity. The use of antibiotics should be regulated and should be under strict supervision. Probiotics are capable of boosting our body’s immunity against infection, generating antimicrobial substances that affect the metabolic system in a positive way by increasing the production of vitamins, improving digestion and lactose absorption. Probiotics are promising alternative among individuals who have adverse reactions to antibiotics because they help to resist the gastrointestinal aggression brought on by antibiotics (Mangiante et al., 1999; Saavedra, 1999). Charteris et al. (1998) have reported that antibiotic/probiotic combinations for conditions such as diarrhoea, female urinary and genital tract infection as well as infective endocarditis is being advocated by researchers. We cannot completely avoid the use of antibiotics, but is best to avoid them for simple infections and try alternative therapies. Fermented milk products which are good sources of probiotic are recommended for children and adults to reinforce their microflora and thereby resist infections. Multiple antibiotic resistances are a continual threat in the battle against once-treatable infections. Prevention of infections before they occur is clearly the better alternative. Certain probiotics may be a safe, cost-effective approach that adds a barrier against or resistance to microbial infection. A high percentage of aquatic products, dairy products as well as poultry and
meat produced in many countries bear antibiotic residue. As the world community continues to demand for animal food products with no antibiotics whenever possible, probiotics are find an increasingly greater role in future.

Factors such as the increasing levels of multidrug resistance among pathogenic organisms, the increasing demands of consumers for natural substitutes for drugs and the emergence of scientific and clinical evidence showing the efficacy and effectiveness of some probiotic strains are now leading physicians to examine probiotics and other alternatives to pharmaceutical remedies (Reid et al., 2003). Over the world, the research of novel probiotic strains is important in order to satisfy the increasing request of the market and to obtain new functional products. These new functional products must contain probiotic cultures more active and with better probiotic characteristics comparing to those already present on the market.

1.2 Review of Literature

1.2.1 Origin and Historical development of Probiotics

Probiotics also known as ‘friendly bacteria’ in lay terms, are live, non-pathogenic microorganisms that benefit the consumer’s digestive system by restoring the naturally existing gastrointestinal microflora, preventing the colonization of the intestine by pathogens, and consequently, improving the immune system. Although there is reference to sour milk or fermented cultures as far back as in the Bible, Elie Metchnikoff is regarded as the grand father of modern probiotics. He made a land mark observation that the regular consumption of LAB in fermented dairy products, such as yoghurt, was associated with enhanced health and longevity in Bulgarian peasant population (Fuller, 1989; Burns and Rowland, 2000). Later he was convinced that yoghurt contained the organisms necessary to protect the
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intestine from the damaging effects of other harmful bacteria. Bulgarian physician Stamen Grigorov demonstrated how healthy bacteria in yoghurt helped digestion and improved the immune system and discovered the ‘Bulgarian bacillus’ mentioned by Metchnikoff (Anukam and Reid, 2007). In his book “The Prolongation of Life” published in 1907, Metchnikoff suggested that the dependence of the intestinal microbes on the foods makes it possible to adopt a measure to modify the flora in our bodies and to replace the harmful microbes by useful microbes (Metchnikoff, 1907). At the time of Metchnikoff, Henry Tissier, a French paediatrician, observed that children with diarrhoea had in their stools a low number of bacteria characterized by a peculiar Y shaped morphology. These Bifidobacteria were, abundant in healthy children (Anukam and Reid, 2007). The term probiotics was first introduced into the scientific literature in 1965 by Lilly and Stillwell to represent ‘substances secreted by one organism which stimulate the growth of another. In 1974, Parker defined probiotics as: ‘Organisms and substances which contribute to intestinal microbial balance’. He described it as animal feed supplements which had a beneficial effect in the host animal by affecting its gut flora (Fuller, 1989; Oyetayo and Oyetayo, 2005). Fuller modified the definition in 1989 as live microbial supplements which beneficially affects the host animal by improving its microbial balance. This revised definition stresses the importance of live cells as an essential component of the probiotic preparation (Fuller, 1989). Probiotics have more recently been defined as ‘microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being of the host’ (Salminen et al., 1998). The first clinical trials on the beneficial effect of probiotic were performed in the 1930’s on the effect of probiotics on constipation (Koop-Hoolihan, 2001). The most recent definition for
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probiotics was given by Schrezenmier and De Vrese, (2001) who defined probiotics as viable microbial food supplements which beneficially influence the health of the host. The health promoting effect of probiotic agents has been clearly pointed out in this definition.

1.2.2 Composition of probiotic preparations

The most extensively studied and widely used probiotics are the Lactic Acid Bacteria, particularly the *Lactobacillus* and *Bifidobacterium* spp. These are found in large numbers in the gut of healthy animals and are in the words of the America FDA, Generally Regarded As Safe (GRAS) (Parvez et al., 2006). Fuller listed the following organisms as species used in probiotics preparation: *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Enteroccocus feacium*, *Enterococcus feacalis*, *Bifidobacterium species*. With the exception of *Lactobacillus Bulgaricus* and *S. thermophilus*, all the other organisms are intestinal strains (Fuller, 1989; Oyetayo and Oyetayo, 2005). Organisms other than LAB which are currently being used in probiotic preparations include *Bacillus* sp. *Saccharomyces cerevisiae* and *Saccharomyces boulardii* and *Aspergillus oryzae* (Parvez et al., 2006). Probiotic preparations may be made up of a single strain or may contain any number up to eight strains. They may either be included in pelleted feed or granules, capsules, paste or spray depending on the condition to be treated (Fuller, 1989).

1.2.3 Modes of action of probiotics

The beneficial effects of probiotics may be mediated by direct antagonistic effect against specific groups of organisms resulting in a decrease in numbers or by an effect on their metabolism or by stimulation of immunity (Oyetayo and Oyetayo, 2005). Probiotic agents exert a beneficial effect through a wide array of actions. These include resistance to colonization, production of
antimicrobial substances, inhibition of pathogen adhesion, degradation of toxins, stimulation of local and peripheral immunity, stimulation of brush border enzyme activity, stimulation of secretory Ig A, and prevention of microbial translocation. Pathogens are unlikely to develop resistance against probiotic agents due to these varied modes of actions (Wadher et al., 2010). Mechanisms of action of probiotics that have been suggested by Lichtenstein, (2003) include receptor competition, effects on mucin secretion and/or immunomodulation of gut associated lymphoid tissue, increased immunosuppressive and decreased proinflammatory mediators. Most probiotics have the capability to produce substances which have direct antimicrobial action. The antibacterial compounds synthesized and secreted by probiotics include organic acids (acetic acid, lactic acid, butyric acid), antimicrobial peptides, mucins, bacteriocins, fatty acids etc. (DeVuyst and Vandamme, 1994; Kailasapathy and Chin, 2000). Probiotics reduce gut pH by stimulating the Lactic acid producing microflora (Langhendries et al., 1995). The metabolic end products of their growth such as lactic acid and acetic acid can lower the pH of the intestinal contents and create conditions less favourable for harmful bacteria (Parvez et al., 2006). They compete for binding and receptor sites that pathogens occupy (Fujiwara, 1997; Kailasapathy and Chin, 2000) and also for available nutrients and other growth factors (Rolfe, 2000). It has been proposed that the mechanism of probiotic therapy is through promotion of the non immunologic and immunologic gut defence barrier function (Isolauri et al., 2001). Probiotic flora exerts their beneficial effect through release of various enzymes which can enhance the bioavailability of nutrients (Parvez et al., 2006).
Table 1.2 Therapeutic applications of probiotics and the proposed mechanism of action

<table>
<thead>
<tr>
<th>Beneficial Effects</th>
<th>Mode of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aid in lactose digestion</td>
<td>Bacterial lactase acts on lactose in small intestine</td>
</tr>
<tr>
<td>Resistance to enteric pathogens</td>
<td>Adjuvant effect increasing antibody production Systemic immune effect Colonization Resistance Alteration of intestinal condition to be less favourable for pathogenicity (pH, short chain fatty acids, bacteriocins, H₂O₂)</td>
</tr>
<tr>
<td>Anti colon cancer effect</td>
<td>Anti mutagenic activity Alteration of pro cancerous enzymatic activity of colonic microbes Stimulation of immune function Influence of secondary bile salt concentration</td>
</tr>
<tr>
<td>Small bowel bacterial overgrowth</td>
<td><em>Lactobacillus</em> sp. influence the activity of over growth flora, decreasing metabolic production, Antibacterial characteristics</td>
</tr>
<tr>
<td>Immune system modulation</td>
<td>Strengthening of nonspecific and antigen specific defense against infection and tumours, Adjuvant effect in antigen specific immune response, Regulating / influencing TH1/TH2 cells and production of cytokines</td>
</tr>
<tr>
<td>Prevention of Allergic reaction</td>
<td>Prevention of antigen translocation into blood stream</td>
</tr>
<tr>
<td>Blood lipid and heart diseases</td>
<td>Assimilation of cholesterol by bacterial cell, Alteration of activity of bile salt hydrolase enzyme, Anti oxidative effect</td>
</tr>
<tr>
<td>Antihypertensive effect</td>
<td>Bacterial peptidase action on milk protein results in antihypertensive tripeptides, cell wall components act as ACE inhibitors</td>
</tr>
<tr>
<td>Urogenital infection</td>
<td>Adhesion to urinary and vaginal tract cells, competitive exclusion, Inhibitor production hydrogenperoxide and biosurfactants</td>
</tr>
<tr>
<td>Infection caused by <em>Helicobacter pylori</em></td>
<td>Competitive colonization</td>
</tr>
<tr>
<td>Hepatic encephalopathy</td>
<td>Competitive exclusion, inhibition of urease producing gut flora</td>
</tr>
<tr>
<td>Gastrointestinal Disorder (Irritable Bowel Syndrome, Constipation)</td>
<td>Modulation of the Microflora</td>
</tr>
<tr>
<td>Nutrient synthesis and bioavailability</td>
<td>Release of various enzymes and vitamins into the intestinal lumen</td>
</tr>
</tbody>
</table>
1.2.4 Probiotics in animal and aquatic use

Probiotics are well known and routinely used additives in the main livestock species. They claim to improve gut health by stabilizing gut flora being their effect reflected in a better overall health status, welfare and performance of the animals.

The use of probiotics for poultry is mainly aimed to prevent and combat digestive disorders. This includes competitive exclusion of potentially pathogenic bacteria (Salmonella, Escherichia coli and Clostridium perfringens), antimicrobial secretions (bacteriocins) as well as the stimulation of an immune response that contributes to the maintenance or reinstallation of intestinal health. Increase in the natural antibody production in the serum and gut in poultry for some antigens was observed by Haghighi et al. (2006) by administering a probiotic mixture consisting of Lactobacillus acidophilus, Bifidobacterium bifidum and Streptococcus faecalis. Probiotics may induce changes in the gastrointestinal tract in terms of histological structure and regulation of mucus secretion. Dietary inclusion of a microbial feed additive (Lactobacillus salivarius and Lactobacillus reuteri) slightly increased the growth performance and improved intestinal nutrient absorption with an associated improvement of intestinal architecture (Awad et al., 2009).

In bovines the use of probiotics has the main purpose of preventing and combating digestive disorders (especially diarrhea in livestock during lactation), to influence the ruminal metabolism of nutrients and to stimulate activities to ruminal microorganisms which helps to maintain health and improve productive performance (Corcionivoschi et al., 2010).

Ruminal acidosis cause discomfort, anorexia, reduced digestibility and milk production in cows. Probiotics can act as bioregulators and prevent
reduction of ruminal pH by increasing the use of lactic acid by some ruminal bacteria (Nocek et al., 2003). Supplementation of Streptococcus faecium or of a mixture of Streptococcus faecium, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum and Lactobacillus plantarum in the diets of lambs, infected with E.coli, led to a reduction of pathogenic strains with an improvement in growth performance (Lema et al., 2001). Anu and Keerthi, (2012) have isolated and characterized Bacillus subtilis from the natural flora of cow’s milk for its probiotic capabilities and possible application for animal use.

In aquaculture, probiotics are administered by feed and/or as a water additive. The supplementation of probiotics through feed is a better method to ensuring the efficiency of the probiotic bacteria in the gastrointestinal tract of fish. However, their use in fish feed production is still scarce.

The application of probiotics in aquaculture practices has already gained momentum, and nowadays, numerous microorganisms, both from indigenous and exogenous sources are used as probiotics. The commonly used probiotics in fish culture practices belong to Saccharomyces, Clostridium, Bacillus, Enterococcus, Lactobacillus, Shewanella, Leuconostoc, Lactococcus, Carnobacterium, Aeromonas and several other genera (EPA Position papers). Aparna and Keerthi, (2012) characterized the antimicrobial principal of four species of potential probiotic strains isolated from the major flora of the ornamental fish Poecilia reticulata.

Widespread interest in probiotic bacteria is the results of their medicinal properties reported both for human and animal subjects. Probiotics have been employed in the feeding of farm animals such as pigs, poultry,
ruminants as well as fish for a long time and it is expected that they would replace antibiotics or supplement their use (Goderska and Czarnecki, 2007).

1.2.5 Lactic Acid Bacteria (LAB)

Lactic Acid Bacteria consist of a number of bacterial genera within the phylum Firmicutes. The genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are recognized as LAB (Holzapfel, 1997; Shea Beasly, Ph.D. Thesis, 2004). Lactic acid producing Gram positive bacteria but belonging to the phylum Actinobacteria are genera such as *Propionibacterium* and *Bifidobacterium* (Gibson and Fuller, 2000; Holzapfel et al., 2001).

The Lactic Acid Bacteria (LAB) comprise a clade of Gram-positive, low-GC, acid-tolerant, generally non-sporulating, non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics. The Lactic Acid Bacteria can only obtain ATP by fermentation, with carbon being the main source of sugars. Two main hexose fermentation pathways are used to classify LAB genera (Khalid, 2011).

Under conditions of excess glucose and limited oxygen, homolactic LAB catabolize one mole of glucose in the Embden-Meyerhof-Parnas pathway to yield lactic acid. Representative homolactic LAB genera include *Lactococcus, Enterococcus, Streptococcus, Pediococcus*, and group I Lactobacilli (Todar’s Online Textbook of Bacteriology). Heterofermentative LAB use the pentose phosphate pathway, alternatively referred to as the pentose phosphoketolase pathway to yield lactic acid and ethanol. Obligate heterofermentative LAB include *Leuconostoc, Oenococcus, Weissella* and group III Lactobacilli.
Since they do not use oxygen in their energy production, Lactic Acid Bacteria happily grow under anaerobic conditions, but they can also grow in oxygen's presence. They are protected from oxygen byproducts (e.g. H$_2$O$_2$) because they have peroxidases. These organisms are aerotolerant anaerobes (Michaela et al., 2009). Laboratory media used for LAB typically include a carbohydrate source, as most species are incapable of respiration. LAB is catalase negative. Because of the low energy yields, Lactic Acid Bacteria often grow more slowly than microbes capable of respiration, and produce smaller colonies of 2-3 mm. The Lactic Acid Bacteria have limited biosynthetic ability, requiring preformed amino acids, B vitamins, purines, pyrimidines and typically a sugar as carbon and energy source. A rich medium is usually employed when cultivating lactics. These multiple requirements restrict their habitats to areas where the required compounds are abundant (animals, plants and other multicellular organisms) (Microbiology laboratories).

The classification of Lactic Acid Bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentration and acid or alkali tolerance. Lactic Acid Bacteria can grow at temperatures from 5-45°C and not surprisingly are tolerant to acidic conditions, with most strains able to grow at pH 4.4 (Khalid, 2011).

*Enterococcus* is gram-positive cocci that form pairs or chains. They are distributed widely in the environment, particularly in feces of vertebrates. Enterococci are tolerant of a wide range of environmental conditions: extreme temperature (10-45°C), pH (4.5-10.0) and high sodium chloride
concentrations. Strains can grow in the presence of 6.5% NaCl and in the presence of 40% bile (Franz et al., 2003). *Enterococcus faecium* is one of a large number of Lactic Acid Bacteria that are used extensively in functional foods for humans, cheese products and as a food supplement for animals. A major advantage of *Enterococcus faecium* is that it can survive the passage through the stomach without loss of vitality.

Bifidobacteria are normal inhabitants of the human and animal colon. Breast fed newborns are colonized with Bifidobacteria within days after birth. Bifidobacteria are Gram positive anaerobes which are non-motile, non-sporeforming and catalase negative. They have various shapes, such as short, curved rods, club shaped rods and bifurcated Y-shaped bifid rods and hence the name Bifidobacteria. Bifidobacteria were first isolated from the feces of breast-fed infants. They are saccharolytic organisms that produce acetic acid and lactic acid without generation of CO₂. Many species of Bifidobacteria have been isolated and those which are used as probiotics include *Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium animalis, Bifidobacterium thermophilum, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis* and *Bifidobacterium lactis* (Bergey’s manual of systemic bacteriology, Vol.2).

### 1.2.6 Sources of Lactic Acid Bacteria

The most common organisms of choice as probiotics are LAB and *Bifidobacterium*. Isolation and study of new strains from the intestinal flora may lead to the development of novel probiotic products. Lactic Acid Bacteria (LAB) and Bifidobacteria dominate the microbiota of full term neonates especially when breast fed with a health promoting effect on the child (Arici *et al.*, 2004) where human milk is source of Lactic Acid Bacteria
for the infant gut. Strains of human origin are most suitable for human use because some health promoting benefits may be species specific, and microorganisms may perform optimally in the species from which they were isolated (Vinderola et al., 2008). LAB were first isolated from milk. They can be found in fermented products as meat, milk products, vegetables and bakery products. According to Holzapfel et al. (2001) they have been detected in soil, water, manure and sewage. LAB exists in human and in animal (Schrezenmer and De Vrese, 2001). They are part of the microbiota on mucous membranes such as the intestine, mouth, and skin, urinary and genital organs of both humans and animals beneficially influencing the ecosystems. Honey bees possess an abundant, diverse and ancient LAB microbiota in their honey crop with beneficial effects for bee health, defending them against microbial threats. The indigenous bacterial flora in the honey stomach is dominated by Lactobacillus and Bifidobacterium phylotypes. The LAB flora in the honey stomach includes Lactobacillus kunkeei, Bifidobacterium asteroids and Bifidobacterium coryneforme. LAB found in the GIT of Honey bee species Apis cerena indica, adult workers, collected from Apiculture farm in Athirampuzha, Kottayam, possess all essential probiotic qualities (Raghavan et al., 2013).

LAB has gained much interest in various applications in food and fermentation industries, in pharmaceuticals, probiotics and as biocontrol agents. Due to their antioxidant and antimicrobial properties LAB finds application in food biopreservation (Harzallah and Belhadj, 2013). In the food Industry, LAB is widely used as starters to achieve favorable changes in texture, aroma, flavor and acidity (Leroy and De Vyust, 2004).
1.2.7 Isolation and Screening for Lactic Acid Bacteria

Since they do not use oxygen in their energy production, lactic acid bacteria happily grow under anaerobic conditions, but they can also grow in oxygen's presence. These organisms are aerotolerant anaerobes. Laboratory media used for LAB typically include a carbohydrate source, as most species are incapable of respiration. LAB is catalase negative. Because of the low energy yields, Lactic Acid Bacteria often grow more slowly and produce smaller colonies of 2-3 mm. The Lactic Acid Bacteria have limited biosynthetic ability, requiring preformed amino acids, B vitamins, purines, pyrimidines and typically a sugar as carbon and energy source. A rich medium such as de Man Rogosa Sharpe (MRS) and Trypticase Phytone Yeast (TPY for Bifidobacterium) are usually employed when cultivating lactics. They grow optimally between 5 and 45°C. Screening for LAB is based on Gram’s reaction, catalase and oxidase tests as well as spore staining (Khalil et al., 2007; Hadaji et al., 2005).

1.2.8 Antibiotics vs Probiotics

Antibiotics have been around for years, and are beneficial for us in many ways. Even though correct usage of antibiotics reduces complications and mortality rates due to infections, over use and abuse of antibiotics has an undesirable effects on our bodies. Antibiotic-associated disruption of gastrointestinal flora tract can lead to diarrhea, dehydration and mineral-salt imbalance, particularly among small children. Triple-antibiotic therapy is used to aggressively eradicate Helicobacter pylori and using probiotics between antibiotic courses repopulates the digestive tract with friendly bacteria and effectively inhibiting colonization of H. pylori (Kabir et al., 1997).
In 1994, the World Health Organisation deemed probiotics to be the next most important immune defences system when commonly prescribed antibiotics are rendered useless by antibiotic resistance (Levy, 2000). The use of probiotic resistance is termed as a microbial interference therapy (Parvez et al., 2006). With increasing understanding that beneficial microbes are required for health, probiotics may become a common therapeutic tool used by health care practitioners in the not-too-distant future (Wadher et al., 2010).

1.2.9 Enteric fever and significance in India

Typhoid fever is a systemic infection caused by *Salmonella enterica* serotype *Salmonella typhi*. The disease remains an important public health problem in developing countries. In 2000, it was estimated that over 2.16 million episodes of typhoid occurred worldwide, resulting in 216000 deaths and that more than 90% of this morbidity and mortality occurred in Asia. Typhoid fever is an important cause of morbidity and mortality in many developing countries. Enteric fever is endemic in all parts of India and still constitutes significant health hazard. The resistance of *Salmonella enterica* subspecies *Enterica serovar Typhi* (*S. typhi*) to Chloramphenicol was first reported in India from Kerala, where a substantial outbreak took place in 1972 (Kumar et al., 2008). Since then multidrug-resistant strains of *S. typhi* have escalated into a worldwide problem. The steadily increasing multidrug resistance in *S. typhi* strains is a cause of grave concern in India, where such strains are endemic in many parts. There are reports of increasing isolation rates of *S. paratyphi* A from India as well as Asia with the recent escalation of drug resistance in *S. paratyphi* (Kothari et al., 2008).
1.2.10 Selection of probiotic strains

The definition for probiotics proposed by Fuller, Salminen and FAO/WHO emphasizes probiotic viability as a reasonable measure of probiotic activity (Anukam and Reid, 2007). 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, enzyme activities or fermentation products (Vinderola and Reinheimer, 2003). It is important that probiotic bacteria should survive, proliferate and colonize in the site where it is presumed to be functioning. It is possible that only host specific microbial strains are able to compete with the indigenous microflora and to colonize the niches. The probiotic strain must be tolerated by the immune system and not stimulate the formation of antibodies against the probiotic strain. So the host must be immunotolerant to the probiotic. Probiotic strain can act as an adjuvant and stimulate the immune system (Harzallah and Belhadj, 2013). New probiotic bacteria should be selected based on their functional properties which include tolerance to gastrointestinal environment (presence of low pH, proteolytic enzymes and bile salts) lack of pathogenicity, adhesion to intestinal epithelium, immunity stimulation, antagonistic action towards pathogens, antitumoral activity (Vlkova et al., 2008). Also determination of haemolytic activity is considered a safety aspect for the selection of probiotic strains (FAO/WHO, 2002).

The following selection criteria are to be considered for a Lactic Acid Bacteria to be used as probiotic:
• The ability to exert beneficial effect on the host;

• To withstand into a food stuff at a high cell counts and remain viable throughout the shelf life of the product;

• To withstand transit through the GI tract;

• Adhere to the intestinal epithelial cell lining and colonize the lumen of the tract;

• Be capable of surviving and metabolizing in the gut;

• Produce antimicrobial substances towards pathogens;

• Should stabilize the intestinal microflora and be associated with health benefits;

• Probiotics must have a good shelf life in food or preparations, containing a large number of viable cells at the time of consumption, and

• Be non-pathogenic and nontoxic in their preparation;

• Be isolated from the same species as its intended host;

• Have good sensory properties;

• Have antibiotic resistance pattern.

1.2.11. Characteristics of probiotics

Bacteria which constitute part of probiotic products must be capable of settling the gastrointestinal tract of the host. Holzapfel et al., 1997 suggests it as reasonable to carry out initial selection of probiotic strains based on their resistance to the unfavorable physiological factors in the gastrointestinal tract. According to the guide lines by FAO/WHO for the
evaluation of probiotics in food, the most widely used *in vitro* tests are resistance to gastric acidity and bile salts as based on survival and growth studies (Pinto *et al.*, 2006, Goderska and Czarnecki, 2007). Successful colonization in the intestinal tract with a probiotic is usually transient and many probiotics fail to colonize as the gastrointestinal tract has many defenses that inhibit colonization. These include gastric acid, duodenal bile, mucin, and the gut immune system. Successful probiotics are resisting these insults and transiently take up residence in the gut (Vanderhoof and Young, 2002). According to Maruo *et al.* (2006), ability to survive through the gastrointestinal tract human origin is required for bacterial strains to be effective probiotic microorganisms.

### 1.2.11.1 Acid tolerance

Charteris *et al.* (1998) reported that about 2.5 l of gastric juice at a pH of approximately 2.0 is secreted each day in the stomach. This can cause destruction of most microorganisms ingested. Resistance to human gastric transit is an important selection for probiotic microorganisms (Charteris *et al.*, 1998). Goderska and Czarnecki, (2007) have emphasized that in the variable pH environment that exist in the gastrointestinal tract, differences between strains in the bacteria survivability is more apparent. Prasad *et al.* (1998) were able to select only a few strains with satisfactory acid resistance out of 200 strains of Lactobacilli and Bifidobacteria.

### 1.2.11.2 Bile tolerance

The relevant physiological concentrations of human bile range from 0.3% to 0.5% (Dunne *et al.*, 1999). It is generally considered necessary to evaluate the ability of potentially probiotic bacteria to resist the effects of bile acids (Collins *et al.*, 1998; Dunne *et al.*, 1999). There have been reports
that Lactobacilli and Bifidobacteria exhibit strain variation in their tolerance to bile salts (Vinderola and Reinheimer, 2003).

1.2.11.3 Antibacterial activity

The ability of Lactic Acid Bacteria to inhibit the growth of various Gram positive and Gram negative bacteria is well known. The inhibitory property could be attributed to various mechanisms. The protective effects of probiotics against intestinal infections have been shown in animal models. Mechanism of action includes the production of acids, hydrogen peroxide or antimicrobial substances, competition for nutrients or adhesion receptors, antitoxin actions, and stimulation of the immune system (Marteau et al., 2001). 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). Probiotic supplementation is thought to provide a significant microbial stimulus for the immature immune system thereby conferring protection against atopic diseases. Antagonistic properties could be very useful in probiotic products. In 1994, the World Health Organisation deemed probiotics to be the next most important immune defences system when commonly prescribed antibiotics are rendered useless by antibiotic resistance (Kailaspathy and Chin, 2000; Levy, 2000). The use of probiotic resistance is termed as a microbial interference therapy (Parvez et al., 2006). There are many scientific proofs to enable probiotics to be used as treatments for gastrointestinal infections. The use of *Saccharomyces boulardii* and *Enterococcus faecium* SF 68 has shown to prevent or shorten the duration of antibiotic associated diarrhoea. The use of *Saccharomyces boulardii* prevents recurrence of *Clostridium difficile* associated diarrhoea (Marteau et al., 2001).
1.2.12 Identification of Lactic Acid Bacteria

The first step in the selection of a probiotic LAB strain is the determination of its taxonomic classification, which may give an indication of the origin, habitat and physiology of the strain (Morelli, 2007).

According to the guidelines of FAO and WHO for the evaluation of probiotics in food, the first consideration is to identify and characterize the organism to the genus and species level with internationally accepted methods, such as sequencing of DNA encoding 16s rRNA (Wang et al., 2002) The second consideration for particular strains that are being targeted for probiotic use is to have clear and consistent strain designation. This will allow physicians and consumers to track publications associated with that strain has probiotic effects (Reid et al., 2003).

The classification of Lactic Acid Bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures and configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance. Even for some of the newly described genera of Lactic Acid Bacteria, additional characteristics such as fatty acid composition and motility are used as the basis of classification (Khalid, 2011). The basis for the classification of Lactic Acid Bacteria in different genera has essentially remained unchanged since the work of Orla-Jensen. An important characteristic used in the differentiation of the Lactic Acid Bacteria genera is the mode of glucose fermentation under standard conditions, i.e., nonlimiting concentrations of glucose and growth factors (amino acids, vitamins and nucleic acid precursors) and limited oxygen availability. Under these conditions, Lactic Acid Bacteria can be divided into two groups: homofermentative, which
convert glucose almost quantitatively to lactic acid, and heterofermentative, which ferment glucose to lactic acid, ethanol/acetic acid, and CO$_2$. Growth at certain temperatures is mainly used to distinguish between some of the cocci. Enterococci grow at both 10°C and 45°C, Lactococci and Vagococci at 10°C, but not at 45°C. Streptococci do not grow at 10°C, while growth at 45°C is dependent on the species. Salt tolerance (6.5% NaCl) may also be used to distinguish among Enterococci, Lactococci/Vagococci, and Streptococci, although variable reactions can be found among Streptococci. Enterococci are characterized by growth at both high and low pH.

1.2.13 Molecular characterization of probiotic strain

*Enterococcus faecium* is one of the most phenotypically heterogenous of all the Enterococcal species (Teixeria *et al.*, 1995). Species identification and antimicrobial susceptibility profiles are necessary for recognizing colonization patterns and analysis of epidemiologic data. Various techniques have been used for the species identification of *Enterococcus faecium*. According to Cheng *et al.*, 1997 species identification of *Enterococcus faecium* by the highly conserved sequence EM1A and EM1B (Rec G gene that codes for ATP dependent DNA helicase) has been proved highly specific.

1.2.14 Genus *Enterococcus* and application in food and health

*Enterococcus* is a genus of Lactic Acid Bacteria of the phylum Firmicutes. Enterococci are Gram-positive cocci that often occur in pairs (Diplococci) or short chains, two species are common commensal organisms in the intestines of humans: *Enterococcus. faecalis* (90-95%) and *Enterococcus faecium* (5-10%). Enterococci are facultative anaerobic organisms, *i.e.*, they are capable of cellular respiration in both oxygen-rich
and oxygen-poor environments and grow optimally at 35°C. Though they are not capable of forming spores, but are tolerant to a wide range of environmental conditions. The genus can grow at temperatures ranging from 10 to 45°C, at pH 9.6, in the presence of 6.5% sodium chloride and they survive heating at 60°C for 30 min. Also most Enterococci can hydrolyse aesculin in the presence of 40% bile salts (Foulque et al., 2006). Enterococci typically exhibit gamma-hemolysis on sheep's blood agar.

Moreover, although Enterococcus faecalis, Enterococcus faecium, and Enterococcus durans are frequently isolated from human faeces, they are much less prevalent in livestock, such as pigs, cattle, and sheep (Franz et al., 1999). Rare clusters of infections occur with other species, including Enterococcus casseliflavus, Enterococcus gallinarum, and Enterococcus raffinosus. Enterococci are not only associated with warm blooded animals, but they also occur in soil, surface waters and on plants, vegetables, and insects. The contribution of Enterococci to the organoleptic properties of fermented food products and their ability to produce bacteriocins (enterocins) are important characteristics for their application in food technology. In recent years, the reports about Enterococci used as starter cultures or co-cultures (adjuncts) have increased considerably. The major part of this kind of research has focused on the applicability of Enterococcus strains, which harbor interesting biochemical and technological properties. A well-studied Enterococcus strain used as probiotic is Enterococcus faecium SF 68, which is produced in Switzerland (Cylactin\LBC SF68 ME10, F. Hoffmann-La Roche Ltd., Basel, Switzerland). It has been proposed to be clinically effective in the prevention of antibiotic-associated diarrhea and in the treatment of diarrhea in children (Foulque et al., 2006). Enterococcus faecium SF 68 has been tested in adults with acute diarrhea in two hospitals in Belgium (Buydens and
Debeuckelaere, 1996). Although this type of diarrhea was self-limited (>95% recovered by 6 days), the use of the Enterococci shortened the duration of diarrhea by 1-3 days. Enterococcus faecium SF 68 has also been studied as a feed probiotic. Enterococcus faecium SF 68 used in a dry dog food significantly enhanced cell-mediated and humoral immune functions in dogs (Benyacoub et al., 2003). In Denmark, a fermented milk product that contains Enterococcus faecium SF 68 (GAIO; MD Foods, Aarhus, Denmark) has been sold for several years because of its hypocholesterolemic effect on individuals (Agerbaek et al., 1995).

The objectives of Chapter I include-

Isolation of bacteria from the normal gut flora of human infant.

Screening for LAB

Selection of a suitable LAB isolate based on preliminary probiotic characteristics.

Biochemical and molecular identification of selected strain.

1.3 Materials and Methods

1.3.1 Bacterial strains

Escherichia coli (MTCC 901), Klebsiella sp (3384), Salmonella typhi (MTCC 734), Salmonella paratyphi A (MTCC 734), Vibrio cholerae (MTCC 3906) All the strains were obtained from (Microbial Type Culture Centre) MTCC Chandigarh

1.3.2 Source of samples: Fresh fecal samples from human infants who were new born, breast fed and delivered by vaginal route.

1.3.3 Collection of samples: Freshly voided fecal samples from 25 newborn infants were collected in tubes containing pre-reduced saline (0.9%
NaCl) with cysteine-HCl (as followed by Hadaji et al., 2005). Sterile test tubes and sterile swabs (Hi media) were provided to the hospital. Freshly voided stool samples were transferred to the tubes using the swabs by hospital personnel (staff nurse in pediatric wing). The samples were immediately transported to the laboratory at 4°C and assayed within 1 h.

1.3.4 Isolation of bacterial strains (Lactic Acid Bacteria/Bifidobacterium) and culture conditions

The sample was homogenized in the saline (0.9% NaCl), and the suspension was inoculated onto Trypticase Phytone Yeast (TPY) solid media. All plates were incubated at 37°C for 48 h in CO₂ incubator (Galaxy Biotech) supplied with 5% CO₂ and sufficient moisture. Well isolated colonies were selected on the basis of colony morphology and purified by streaking on TPY (Appendix-A) solid medium Khalil et al. (2007).

1.3.5 Screening of the isolates

Colonies were examined for Gram’s reaction, motility by hanging drop preparation, spore formation, catalase production and oxidase production tests. They were also checked for their ability to coagulate skimmed milk.

1.3.5.1 Gram’s staining

The purified selected isolates were Gram stained by routine protocols.

1.3.5.2 Spore staining

Smears of the isolates were prepared and heat fixed and spore staining was performed using malachite green and saffranin by routine protocol.

1.3.5.3 Catalase Test

Catalase test was conducted by routine protocol.
1.3.5.4 Oxidase test

Oxidase test was conducted by routine protocol.

1.3.5.5 Motility test

Hanging drop preparation for motility was conducted by routine protocol.

1.3.5.6 Milk coagulation

Overnight cultures of each of the isolate was centrifuged and pellets were washed twice with buffered phosphate saline (PBS). Cells were resuspended in skim milk (10 g %) and 1ml was inoculated to 10 ml of sterile skim milk, followed by incubation for 72 h at 37°C.

The isolates which were positive for Gram reaction, negative for catalase and oxidase tests and coagulated milk were subjected to further studies.

1.3.6 Evaluation of probiotic characteristics of the Lactic Acid Bacteria isolates

1.3.6.1 Acid tolerance test: (Khalil et al., 2007 with modifications).

Overnight cultures of the test isolates were inoculated (1% v/v) in MRS (Man Rogosa Sharpe) broth previously adjusted to pH values 2, 3 and 4 with IN HCl or NaOH. The cultures were incubated aerobically at 37°C for 6 h. Culture turbidity was monitored at 650 nm after, 6 h of incubation. The control comprised of MRS broth adjusted to pH 6.5. The experiment was conducted in triplicate. Further, the viability was checked by plating the organisms on solid agar plate after 24 h. Results are expressed as the mean and standard deviation of three determinations.
1.3.6.2 Bile tolerance test: (Walker and Gilliland, (1993) with modification)

Overnight cultures of the LAB isolates (2% v/v) were inoculated in MRS broth containing 0.3%, 0.5% and 0.1% bile salt and incubated aerobically at 37°C for 6 h. The pH of control and test cultures were adjusted to 6.5 with 1N HCl or NaOH. The control comprised of MRS broth without bile. Absorbance was measured at 560 nm. The experiment was conducted in triplicate. Results are expressed as the mean and standard deviation of three determinations.

1.3.6.3 Resistance to artificial gastric fluid (Fernandez et al., 2003)

Artificial gastric fluid was prepared with 125 mmol/l NaCl; 17 mmol/l KCl; 45 mmol/l NaHCO₃ and 3 g/l pepsin. The final pH was adjusted with HCl to pH 2 and 3 and with NaOH to pH 7. Cultures of LAB isolates incubated at 37°C for 24 h were centrifuged at 10,000 rpm for 10 min at 4°C. Cell pellet thus obtained was washed in sterile saline and 1ml of the cell suspension in saline was added to 10 ml of artificial gastric fluid. Aliquots were taken at 180 min and growth was measured at 560 nm. The cells were also tested for viability after 24 h by streaking on solid media.

The experiment was conducted in triplicate. Results are expressed as the mean and standard deviation of three determinations.

1.3.7. Stock culture maintenance

Stock culture of the selected LAB isolates were maintained in MRS broth containing 30% glycerol and stored at -20°C.
1.3.8  Anti haemolytic property of the selected Lactic Acid Bacteria isolates

The selected LAB isolates were streaked on blood agar plates (MRS medium with 5% fresh blood) and incubated for 24 h at 30°C. Strains that produced green hued zones around the colonies (α-haemolysis) or did not produce any effect on the blood plates γ-haemolysis were classified as non haemolytic. Strains displacing blood lysis zones around the colonies were considered β haemolytic. The experiment was conducted in triplicate.

1.3.9.  Study of Antibacterial activity of the selected LAB isolates

1.3.9.1 Agar diffusion assay

Antibacterial activity of the selected Lactic Acid Bacteria isolates towards known pathogens like *Salmonella typhi*, *Salmonella paratyphi A*, *Vibrio cholera* and strains of pathogens such as *Escherichia coli* and *Klebsiella pneumoniae* (all strains obtained from MTCC) was studied by agar diffusion test (Herreros *et al*., 2005). Medium used for the diffusion tests were Muller Hinton medium. The pathogens were inoculated to nutrient broth and incubated aerobically for 4 -5 h and the turbidity was matched with 0.5 McFarland tube. Each of the selected LABs was grown in MRS broth for 24h. Antibacterial activity was assayed in the extracellular culture filtrate obtained by centrifugation (4°C) at 10000 rpm for 10 min. The pH of the culture supernatant of the isolates was checked and the antagonistic action of the supernatant was tested by the above method in the following ways: a) using the supernatant with its original pH, b) using the supernatant after adjusting the pH to 4, 5 and 6.5.
1.3.9.2 Growth of the enteric fever pathogens at low pH.

Overnight cultures of the pathogens *Salmonella typhi* and *Salmonella paratyphi A* were inoculated into nutrient broth adjusted to pH 4 and 5. The tubes were incubated overnight at 37°C.

1.3.9.3 Mixed culture method to test the inhibition of the enteric fever pathogens

The selected LAB strain and the pathogens (*Salmonella typhi* and *Salmonella Paratyphi A*) were incubated separately in MRS and nutrient broth respectively. Aliquots of LAB culture was mixed with equal volumes of each of the pathogen culture and incubation was resumed. Samples from the mixed cultures were plated after 24 h on MRS media and MacConkey agar. The plates were incubated at 37°C overnight and observed for growth of the organisms in respective selective media.

1.3.10 Identification of the selected Lactic Acid Bacteria isolate

1.3.10.1 Fructose-6 - phosphate phosphoketolase (F6PPK) Test (Scardovi, 1986)

The most direct and reliable characteristics assigning an organism to the genus *Bifidobacterium* is that based on the demonstration of F6PPK in cellular extracts. Genus *Bifidobacterium* can be distinguished from other bacterial groups by the metabolic pathway, the bifidus shunt, whose key enzyme is Fructose – 6 - phosphate phosphoketolase (F6PPK). The demonstration of the F6PPK activity serves as a taxonomic tool in the identification of the genus. The experiment was conducted with *E. coli* as the negative control. The following reagents were used for the test: 1) 0.05 M phosphate buffer (pH 6.5) containing cysteine HCl (500mg/l), 2) A solution containing NaF, (6 mg/ml) and K or Na iodoacetate, (10mg/ml), 3) Hydroxylamine HCl (13.9 g/100ml of water) freshly neutralized with 2N
NaOH to pH 6.5, 4) Trichloroacetic acid (TCA), 15% (w/v) in water, 5) 4 M HCl, 6) FeCl$_3$ 6H$_2$O 5% w/v in 0.1 M HCl, 7) Fructose 6- phosphate (Na salt 70% purity), 80 mg/ml in water. 10 ml of overnight culture of the selected LAB isolate in MRS and E.coli in nutrient broth were centrifuged at 4°C (10,000 rpm). The harvested cells from each sample was washed twice with buffer 1 and resuspended in 1 ml of the same buffer. The cells were disrupted by sonication in the cold and 0.25 ml of each of reagents 2 and 7 were added to the cells extract. After 30 min incubation at 37°C, the reaction was stopped with 1.5 ml of reagent 3. After 10 min at room temperature 1ml of each of the reagents 4 and 5 were added. The mixture was left at room temperature before the final addition of 1 ml of the colour-developing reagent 6. Immediate formation of reddish violet colour was taken as positive for *Bifidobacterium* genus.

1.3.10.2 **Physiological characterization of the Lactic Acid Bacteria isolate**

The selected strain was studied for its ability to grow at different temperatures (4°C, 10°C and 45°C, growth at pH 9.6, growth in the presence of 6.5% NaCl, 40% bile salt, and survival at 60°C for 30 min as well as growth in Bile esculin Azide agar.

1.3.10.3 **Biochemical characterization of the Lactic Acid Bacteria isolate**

Sugar fermentation patterns were evaluated using Analytical Profile Index (API) 50 CHL identification kits (Biomerieux, VITEK 2) as per the manufacture’s instruction.

1.3.10.4 **Molecular identification by species specific PCR**

1.3.10.4.1 **Isolation of genomic DNA (Sambrook et al., 1989)**

Bacterial genomic DNA isolation of the selected LAB isolate was performed according to the procedure given below.
1. Overnight culture (25 ml in MRS broth suspension) was taken and centrifuged at 10,000 rpm for 10 min (4°C).
2. The pellet was resuspended in 10 mM tris hydrochloride and 100 mM NaCl (2.5 to 3 ml each) and centrifuged at 10,000 rpm for 10 min (4°C).
3. The pellet was resuspended in 2.5 ml TE and 50 µl lysozyme from stock (50 mg/ml) and incubated at 37°C for 20 min.
4. 25 µl of RNAase from stock (100 mg/ml) was added and incubated at room temperature for 10 min.
5. 2.5 ml of SDS (2% in TE), was added and incubated at 50°C for 45 min.
6. Proteinase K (50 µl) from stock (20 mg/ml) was added and incubated at 50-55°C for 10 min.
7. Equal volume of phenol was added, mixed gently and centrifuged at 10,000 rpm for 10 min (4°C). Aqueous phase was transferred to a fresh tube.
8. Equal volume of phenol: chloroform (1:1) was added, mixed gently and centrifuged.
9. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently and centrifuged.
10. 1/10 volume of 3 M sodium acetate was added and kept in ice for 20 min.
11. Equal volume of isopropanol was added and left for 2-3 min, centrifuged and supernatant was decanted.
12. The pellet was washed in 70% alcohol, centrifuged for 3 min and supernatant was decanted.
13. The DNA was dried and dissolved in 25-30 µL TE, stored at 4°C until electrophoresis.
**1.3.10.4.2 Preparation and examination of agarose gel.** (Sambrook *et al.*, 1989)

Preparation and examinations of agarose gel of the sample was performed according to the procedure given below.

1. The edges of a clean, dry glass plate were sealed with tape so as to form a mold. The mold was set on a horizontal section of the bench.

2. Sufficient electrophoresis buffer was prepared 5X TBE buffer to fill the electrophoresis tank and to cast the gel. 0.8% agarose was prepared in electrophoresis buffer (1X TBE) in an Erlenmeyer flask. The slurry was heated in a boiling water bath until the agarose dissolved.

3. The molten agarose was allowed to cool and 2 µl of Ethidium Bromide was added and mixed.

4. The isolated DNA and DNA marker (Supermix DNA ladder-GeNei) was loaded in respective wells and Agarose gel electrophoresis was run.

**1.3.10.4.3 Primer** (Cheng *et al.*, 1997).

21-mer primers -

Forward primer - EM1A: TTG AGG CAG ACC AGATTG ACG

Reverse primer - EM1B: TAT GAC AGC GAC TCC GATTCC were used for the DNA amplification.

EMIA and EMIB code for the ATP dependent DNA helicase gene of *Enterococcus faecium* and would produce a 658 DNA base pair product characteristic of the species.
1.3.10.4.4 PCR mixture

- Template DNA: 1 µl (50ng)
- PCR buffer: 2.5 µl
- dNTPs: 2 µl
- 25 mM MgCl₂: 1.5 µl
- Forward primer: 1 µl
- Reverse primer: 1 µl
- Nuclease free water: 15.75 µl
- Taq DNA polymerase: 0.25 µl (0.5U)

1.3.10.4.5 PCR Cycle

The PCR cycle was performed in a final volume of 25 µl. The following PCR programme consisting of a predenaturation step of 95°C for 10 min, followed by 40 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min and a final extension step of 72°C for 10 min and 4°C for infinity was used.

The reaction was carried out in Agilent Technologies Sure Cycler 8800.

1.3.10.4.6 Agarose gel electrophoresis of amplified product

Amplified product was observed by Agarose gel electrophoresis with 1.2% agarose in 1X TBE buffer along with the marker DNA super ladder (Merck).
1.3.10.4.7. Sequencing of the amplified product

The sequence of the insert was determined using the automated DNA sequencing service provided at SciGenomics Laboratory, Cochin, India. The BLAST program (www.ncbi.nlm.nih.gov/blst) was employed in order to assess the degree of DNA similarity. The sequence was (see Appendix B) was deposited in the National Centre for Biotechnology Information (NCBI) gene bank data base.

1.3.10.5 16s rRNA sequencing

DNA isolation was performed as in 1.3.10.4.1

1.3.10.5.1 Primer

Forward primer- 27 F $5^1$ AGAGTTTGATCMTGGCTCAG $3^1$
Reverse primer- 1492 R $5^1$ AAGGAGGTGWTCCARCC3$^1$

1.3.10.5.2 PCR mixture

Template DNA - 1 µl
PCR Master Mix - 25 µl
Forward primer - 2 µl
Reverse primer - 2 µl
Nuclease free water - 20 µl

1.3.10.5.3 PCR Cycle

An initiation step at 94°C for 4 min followed by 30 cycles of 94°C for 1min, 55°C for 1min, 72°C for 2 min followed by final extension at 72°C for 1min and holding temperature at 4°C for 1 min.

The reaction was carried out in Agilent Technologies Sure Cycler 8800.
1.3.10.5.4 Agarose gel electrophoresis of amplified product

Amplified product was observed by Agarose gel electrophoresis with 1.2% agarose in 1X TBE buffer.

1.3.10.5.5 Sequencing

The sequence of the product was determined using the automated DNA sequencing service provided at Sci Genomics Laboratory, Cochin, India. The BLAST program (www.ncbi.nlm.nih.gov/blast) was employed in order to assess the degree of DNA similarity. The sequence was (see Appendix c) was deposited in the National Centre for Biotechnology Information (NCBI) gene bank database.

1.3.11 Phylogenetic tree

Sequences were aligned and edited manually by Bioedit Sequence alignment Editor. The phylogenetic tree was constructed by the tree building software Mega 5.05 Version. Phylogeny reconstruction analysis was conducted by maximum likelihood method. Test of phylogeny was done by Bootstrap using 1000 Bootstrap replication. Model used was Jukes Cantor. ML Heuristic method was used for inferring the tree by Nearest Neighbor Interchange. Initial trees were made automatically.
1.4 Results

In the present study for the isolation and identification of Lactic Acid Bacterial strains, infant feces was chosen as the source of sample since breast fed infants develop a probiotic rich gut microflora with less pathogenic bacteria moreover LAB and Bifidobacteria dominate the microbiota of the full term neonates. Out of the 25 infant fecal samples subjected for isolation in the presence of 5% CO₂, we obtained 25 isolates with distinct morphology on TPY medium. Among the 25 isolates, 21 isolates were Gram positive as presented in Table 1.3. They were further subjected for preliminary screening for Lactic Acid Bacteria. Based on the results of catalase and oxidase test, hanging drop preparation, spore formation and finally the ability to coagulate skim milk (summarized in Table 1.4), 14 among the 21 Gram positive strains were identified as belonging to the group LAB.

Table 1.3 Macroscopic and microscopic features of the twenty five bacterial isolates obtained from infant feces.

<table>
<thead>
<tr>
<th>No</th>
<th>Sample Name</th>
<th>Colony Characteristics</th>
<th>Gram’s Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CF1PP</td>
<td>Pinpoint round creamish butyrous colonies</td>
<td>Gram positive short bacilli</td>
</tr>
<tr>
<td>2</td>
<td>CF1A</td>
<td>Small round creamish opaque colonies with entire edge</td>
<td>Gram positive coccobacilli</td>
</tr>
<tr>
<td>3</td>
<td>CF1B</td>
<td>Very Small round smooth creamish opaque colonies with entire edge</td>
<td>Gram Positive cocci in pairs</td>
</tr>
<tr>
<td>4</td>
<td>CF2</td>
<td>Small round smooth butyrous opaque colonies with entire edge</td>
<td>Gram positive cocci</td>
</tr>
<tr>
<td>5</td>
<td>CF3</td>
<td>Small round smooth opaque white colonies with entire edge</td>
<td>Gram positive coccobacilli</td>
</tr>
<tr>
<td>6</td>
<td>CF4B</td>
<td>Small round smooth creamish colonies</td>
<td>Gram positive rods</td>
</tr>
<tr>
<td>7</td>
<td>CF5C</td>
<td>Medium sized round creamish colonies</td>
<td>Gram positive rods</td>
</tr>
<tr>
<td>CF</td>
<td>Description</td>
<td>Gram reaction</td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>-------------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>CF6A</td>
<td>Pinpoint round opaque white colonies</td>
<td>Gram positive coccobacilli</td>
<td></td>
</tr>
<tr>
<td>CF8</td>
<td>Small round smooth cream colonies with irregular margin</td>
<td>Gram negative cocci</td>
<td></td>
</tr>
<tr>
<td>CF9</td>
<td>Medium sized creamish smooth butyrous round nonpigmented colonies which are raised at the centre</td>
<td>Gram negative bacilli</td>
<td></td>
</tr>
<tr>
<td>CF10B</td>
<td>Medium sized round smooth white colonies</td>
<td>Gram positive thin rods</td>
<td></td>
</tr>
<tr>
<td>CF11</td>
<td>Medium sized mucoid round pale yellow opaque colonies</td>
<td>Gram negative bacilli</td>
<td></td>
</tr>
<tr>
<td>CF12</td>
<td>Large irregular creamish mucoid colonies with serrated margin</td>
<td>Gram positive bacilli</td>
<td></td>
</tr>
<tr>
<td>CF13C</td>
<td>Large round smooth creamish colonies</td>
<td>Gram positive short rods</td>
<td></td>
</tr>
<tr>
<td>CF14</td>
<td>Small round smooth cream colonies with regular margin</td>
<td>Gram positive cocci</td>
<td></td>
</tr>
<tr>
<td>CF15</td>
<td>Small white round smooth convex colonies with entire edge</td>
<td>Gram negative bacilli</td>
<td></td>
</tr>
<tr>
<td>CF16</td>
<td>Small round pigmented smooth colonies with entire edge</td>
<td>Gram negative bacilli</td>
<td></td>
</tr>
<tr>
<td>CF18</td>
<td>Small round butyrous convex opaque colonies with entire edge</td>
<td>Gram positive cocci</td>
<td></td>
</tr>
<tr>
<td>CF19</td>
<td>Medium sized smooth round white opaque colonies</td>
<td>Gram positive short rods</td>
<td></td>
</tr>
<tr>
<td>CF20</td>
<td>Small round creamish colonies raised colonies</td>
<td>Gram positive cocci in short chains</td>
<td></td>
</tr>
<tr>
<td>CF22</td>
<td>Small round mucoid creamish opaque colonies with entire margin</td>
<td>Gram negative coccobacilli</td>
<td></td>
</tr>
<tr>
<td>CF23</td>
<td>Medium sized white transparent colonies with irregular margin</td>
<td>Gram positive coccobacilli</td>
<td></td>
</tr>
<tr>
<td>CF24C</td>
<td>Medium sized smooth round white opaque colonies</td>
<td>Gram Positive short rods</td>
<td></td>
</tr>
<tr>
<td>CF25</td>
<td>Small round opaque smooth colonies</td>
<td>Gram positive cocci in pairs and short chains</td>
<td></td>
</tr>
<tr>
<td>CF25C</td>
<td>Very small round white opaque smooth colonies with entire edge</td>
<td>Gram positive large cocci</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.4 Microscopic and biochemical features of the selected Gram positive isolates for preliminary identification as Lactic Acid Bacteria

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Oxidase test</th>
<th>Catalase test</th>
<th>Motility</th>
<th>Spore formation</th>
<th>Coagulation of skim milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF1PP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF1A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CFIB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF4B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF5C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF6A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF10B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF12</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CF13C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CF15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF16</td>
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<td>CF18</td>
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<td>-</td>
</tr>
<tr>
<td>CF23</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CF24C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF25C</td>
<td>_</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1.4.1 Acid tolerance test

All the 14 LAB isolates were tested for their ability to survive in acidic conditions at pH 2, 3 and 4. The capability to survive in different pH
conditions of the gastrointestinal tract (GIT) is a characteristic feature of probiotic bacterial strains. The isolates varied in their ability to survive in acidic conditions suggesting that the property is strain dependent. 11 isolates among the 14 LAB showed satisfactory growth at pH 2, 3 and 4 after 6 h of incubation. The isolates CF2, CF1B, CF1A2 CF5C and CF1PP had more growth with increase in turbidity with increase in pH. They were viable after 24 h of incubation. Although the isolates CF10B, CF15, CF16 and CF25C showed growth at pH 4, the growth at pH 2 and 3 was very meager and the viability of the cells were found to have lost at pH 2 after 24 h. The other isolates CF3, CF6A, CF13C and CF24C, even though showed lesser growth at pH 2 and pH 3 than at pH 4, they were still viable at pH 2 and pH 3 after 24 h of incubation. Fig. 1.3 summarizes the results of tolerance to acid by the 14 LAB isolates.

![ACID TOLERANCE](image)

**Fig. 1.3** Growth profile at different pH conditions. Acid Tolerance of LAB isolates grown in MRS broth adjusted to pH 2, 3 and 4 as determined by the culture’s turbidity after 6 h. Bars represent the standard deviation of the mean values of the OD$_{650}$ measurements of three independent experiments (n=3).
1.4.2 Bile Tolerance test

The 14 LAB isolates were subjected to bile tolerance test in order to examine the ability to survive in the small intestine. The test was performed at three different concentrations of bile salt, 0.3%, 0.5%, and 1%. The LAB isolates CF1PP, CF1B, CF1A2, CF2, CF3, and CF5C showed good turbidity at the three different concentrations of bile salts with more growth at 0.3% bile salt than at 0.5 and 1%. Although the other strains grew well at 0.3 and 0.5% bile salt concentrations, the turbidity was less in the growth media supplemented with 1% bile salt. Least growth at 1% bile salt was observed for CF13C, CF16, CF6A and CF24C. Our findings revealed that the LAB isolates showed individual variation in their ability to grow in MRS medium supplemented with bile salt (Fig. 1.4).

![Bile Tolerance](image)

**Fig. 1.4** Growth profile in different bile salt concentrations. Bile Tolerance of LAB isolates grown in MRS broth with 0.3% 0.5% and 1% bile salt as determined by the culture’s turbidity after 6 h. Bars represent the standard deviation of the mean values of the OD$_{530}$ measurements of three independent experiments (n=3).
1.4.3 Resistance to artificial gastric fluid

This test helps to investigate the effect of enzymes like pepsin in addition to the acidic pH present in the GIT. Tolerance to artificial gastric condition was examined at pH2 and pH3 for 180 min of incubation at 37ºC. All the 14 isolates grew better at pH 3 than at pH 2 in the gastric juice. It was observed that the LAB isolates CF1PP, CF1A2, CF1B, CF2 and CF5C proliferated well at pH2 and at pH3 in the presence pepsin and electrolytes. (Fig.1.5). Least proliferation was observed for CF10B followed by CF25, CF4B and CF16. It was also observed that the growth of the other isolates CF6A, CF13C,CF15, CF16, CF24C was very less at both the pH after 180 min of incubation whereas CF3 showed similar proliferation at both the conditions and showed less variation in the growth rate when compared to the growth of the control at pH 6.5.

![Tolerance to artificial gastric juice](image)

**Fig. 1.5** Tolerance to artificial gastric juice. Tolerance of LAB isolates to artificial gastric juice at pH 2 and 4 grown in MRS broth with as determined by the culture’s turbidity after 6 h. Bars represent the standard deviation of the mean values of the OD<sub>560</sub> measurements of three independent experiments (n=3).
1.4.4  **Haemolytic property of the selected Lactic Acid Bacteria isolates**

No clear zones around the growth of the LAB were observed on MRS medium supplemented with blood after 24 h of incubation. The result indicates lack of β haemolytic property of the LAB. The five LAB isolates did not produce any effect on the blood agar indicating their non haemolytic nature (Table 1.5).

1.4.5  **Antibacterial activity of the selected Lactic Acid Bacteria isolates**

Antibacterial activity against five selected strains of pathogenic microorganisms including the Typhoid fever pathogens was tested. The supernatant of all the LAB isolates at its initial pH (between 2.5 and 3.5) showed inhibition of all five pathogens. However it is clear from the results in Table 1.5 that the degree of inhibition varied with each pathogen and LAB isolates. The low pH of the culture supernatant between 2.5 and 3.5 might account for this observation. However, when the test was repeated after adjusting the pH of the supernatant to 4, the antibacterial activity was observed only for the isolate CF1B. When the pH of the culture supernatant was adjusted to 6.5, none of the selected LAB isolates including CF1B inhibited the pathogens.

*Salmonella typhi* and *Salmonella paratyphi* A are two main causative agents of the typhoid fever in India and are also the major food borne pathogens. Hence focus was mainly given to those LAB isolates which could inhibit the growth of *Salmonella typhi* and *Salmonella paratyphi* A. In the present study the LAB isolates CF1B and CF5C exhibited more antibacterial activity towards the above pathogens. However the activity of CF5C was lost when the pH of the culture supernatant was increased to pH 4 (Table 1.5). On the other hand, inhibition of enteric fever by CF1B was found to be
stable below pH 5. To analyse the effect of pH on the growth of the pathogens, they were inoculated to nutrient broth adjusted to pH 4 and 5 and incubated overnight. Remarkably, there was increase in turbidity of the broth and both the pathogens were found to be viable at pH 4 and 5 (Table 1.6). When the above results are summarized it can be proved that the inhibition of the pathogens by the LAB isolate CF1B towards the enteric fever pathogens was not merely due to production of acid but also may be due to an inhibitory agent whose activity is optimum below pH 5. Fig. 1.6a and 1.6b represents the inhibition of *Salmonella typhi* and *Salmonella paratyphi A* by the cell free supernatant of CF1B at pH 4.

**Table 1.5** Antibacterial activity and haemolytic property of cell free supernatant of the five selected LAB isolates at pH 4, 5 and 6.5.

<table>
<thead>
<tr>
<th>LAB Isolates</th>
<th>V.cholerae</th>
<th>E.coli</th>
<th>Klebsiella.sp</th>
<th>S.typhi</th>
<th>S.paratyphi A</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF1PP</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CF1A2</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF1B</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CF2</td>
<td>++</td>
<td>+</td>
<td>_</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CF5C</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

(+++ represents a zone size >=10mm, +represents a zone size <10mm and – represents absence of inhibitory action)

**Table 1.6** Growth of the Enteric fever pathogens at pH 4 and 5

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Growth at pH 4</th>
<th>Growth at pH 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhi</em></td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Salmonella paratyphi A</em></td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

+++ indicates growth.
When we summarize the above results, it can be learned that out of the 14 LAB isolates, CF1PP, CF1A2, CF1B, CF2 and CF5C have better tolerance to acidic pH, better growth in the presence of bile salts, as well as better resistance to simulated gastric juice. These capabilities determine their transit tolerance capability in the gastrointestinal tract. The isolates CF1B and CF5C showed higher inhibition towards the enteric fever pathogens than the other three isolates. But the inhibition of CF5C was limited to the initial pH of the culture supernatant. On the other hand, CF1B presents better chance to resist the adverse conditions prevailing in the gastrointestinal tract as evident from the results of probiotic characteristics (Fig. 1.3, Fig.1.4 and Fig. 1.5). CF1B showed more growth at pH 2 and 3, more growth in the presence of even 1% bile salts as well in artificial gastric juice. When we consider results of probiotic characteristics and antibacterial activity together we find CF1B as a better candidate for the study. Hence CF1B was selected as the candidate strain to explore the probiotic and health promoting capabilities in the subsequent chapters.
1.4.6 Mixed culture method

When inoculated on to MRS and Mc Conkey agar after co culture, only a single type of colony, characteristic of the LAB isolate was observed on MRS medium whereas no growth characteristic of Salmonella sp was observed on Mc Conkey agar. Complete inhibition of the pathogens Salmonella typhi and Salmonella paratyphi A was found when they were co-cultured with the LAB isolate, CF1B (Table 1.7). Lactic Acid Bacteria have been shown to inhibit in vitro growth of many enteric pathogens and have been used in both humans and animals to treat a broad range of disorders.

Table 1.7 Growth on MRS and McConkey agar after mixed culture of the selected LAB isolate with Salmonella typhi and Salmonella paratyphi A.

<table>
<thead>
<tr>
<th>Media Used</th>
<th>Colony characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS agar</td>
<td>Small round opaque slightly creamish smooth butyrous colonies with entire edge.</td>
</tr>
<tr>
<td>McConkey agar</td>
<td>No growth observed</td>
</tr>
</tbody>
</table>

1.4.7 Identification of the selected Lactic Acid Bacteria isolate from infant gut flora

To identify possible isolates belonging to genus Bifidobacterium F6PPK test was performed. The result showed no formation of red colour characteristic of Bifidobacterium in both the tubes (Fig. 1.7) indicating that the isolates does not belong to this group.

As summarized in the Table 1.8, physiological characterization of the selected LAB isolate from infant gut flora showed good growth in the presence 6.5% NaCl and at 9.6 pH. Appreciable growth was observed at 4, 10 and 45°C and was able to withstand 60°C for 30 min. Black coloration of Bile esculin Azide agar was observed after 12 h of incubation (Fig. 1.8).
The result indicated hydrolysis of esculin by the LAB isolate. The test is characteristic of the genus *Enterococcus*.

![Fig. 1.7 F6PPK Test of the LAB isolate and *Escherichia coli*](image)

Phenotypically, on the basis of its morphological and physiological characteristics, the LAB isolate was identified as belonging to the genus *Enterococcus*. Finally, according to the fermentation pattern on AP1-Strep, the isolate was assigned to the species *Enterococcus faecium* (Fig 1.9). Identification was genotypically confirmed by using species specific PCR amplification using the EM1A and EM1B. Amplified product with 658 base pairs characteristic of *Enterococcus faecium* was obtained in the agarose gel electrophoresis (Fig. 1.10). The amplified product was sequenced with the forward primer EM1A and a 602 bp trimmed product was obtained. The sequence showed 100% identity with the gene coding for the protein, ATP dependent DNA helicase (*Rec G* gene) of *Enterococcus faecium*. The gene accession number was obtained as KF745072 for the sequence of the gene coding for *Rec G* gene. The gene
accession number was obtained as KF745071 for 16s rRNA gene (Table 1.9). The strain was named as *Enterococcus faecium* MBTU-P1F1.

**Table: 1.8** Physiological characterization of the selected LAB isolate CF1B.

<table>
<thead>
<tr>
<th>Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 45°C</td>
<td>+ ve</td>
</tr>
<tr>
<td>(a) 10°C</td>
<td>+ ve</td>
</tr>
<tr>
<td>(c) pH 9.6</td>
<td>+ ve</td>
</tr>
<tr>
<td>Growth in 6.5% NaCl</td>
<td>+ ve</td>
</tr>
<tr>
<td>(b) 40% Bile Salt</td>
<td>-ve</td>
</tr>
<tr>
<td>Growth in Bile esculin Azide agar</td>
<td>+ ve Black colouration</td>
</tr>
<tr>
<td>Survival at 60°C for 30 min.</td>
<td>+ ve</td>
</tr>
</tbody>
</table>

**Fig. 1.8** Hydrolysis of bile esculin by the selected Lactic Acid Bacteria isolate
Isolation, screening and selection of potential Lactic Acid Bacteria strain from infant feces

Fig. 1.9  Biochemical Profile and identification of the selected LAB isolate by API 50 CHL

Fig. 1.10  PCR amplified product of 658 base pair size obtained using the primers EM1A and EM1B (M – marker, S - Sample)

Amplification of the 16S rRNA gene of the test strain formed a product of 928 bp size (Fig. 1.11). The trimmed sequence obtained after sequencing of the PCR product with the forward primer 27 F produced 776 base pairs.
Agarose gel electrophoresis of the PCR product obtained after the amplification of the 16S rRNA genome of *Enterococcus faecium* isolated in the study from infant feces

**Phylogenetic tree and sequence similarities of the isolated strain**

The phylogenetic tree was constructed by the tree building software Mega 5.05 and is displayed in Fig. 1.12. The test strain clusters with 100% Bootstrap support with the strain *Enterococcus faecium* H2 (Gene accession number EU 887814).

**Table 1.9** Molecular characterization of the selected LAB isolate

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Amplified gene region</th>
<th>Gene Bank accession number</th>
<th>Bacterial genus</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBTU-P1F1</td>
<td><em>Rec G</em> gene</td>
<td>KF745072</td>
<td>Enterococcus</td>
<td><em>Enterococcus faecium</em> MBTU-P1F1</td>
</tr>
<tr>
<td></td>
<td>16s rRNA gene</td>
<td>KF745071</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.12** Phylogenetic tree of *Enterococcus faecium* MBTU-P1F1
1.5 Discussion

Recent studies have evoked an increased awareness towards the incorporation of probiotics in food due to their benefits in various health aspects such as enhancement of immune system, action against pathogens etc. The most extensively studied and widely used probiotics are LAB, particularly the *Lactobacillus* and *Bifidobacterium* (Parvez et al., 2006). Even though India is one among the leading countries with dairy industries, the awareness among the common man regarding the role of probiotics and related functional foods in health is limited. However there is significant increase in research undertaken in this area during the past 5 years especially in developing countries including India and much still remains to be done to standardize the meaning of the term probiotic and which strains actually fulfill the criteria for true probiotic microorganisms. Factors such as the increasing levels of multidrug resistance among pathogenic organisms, the increasing demands of consumers for natural substitutes for drugs, and the emergence of scientific and clinical evidence showing the efficacy and effectiveness of some probiotic strains are now leading physicians to examine probiotics and other alternatives to pharmaceutical remedies (Reid et al., 2003). Over the world, the research of novel probiotic strains is important in order to satisfy the increasing request of the market and to obtain new functional products. These new functional products must contain probiotic cultures more active and with better probiotic characteristics comparing to those already present on the market. Hence in the present study we have tried to isolate a potential strain of Lactic Acid Bacteria or *Bifidobacterium* with essential probiotic properties and to explore the health promoting properties associated with it for incorporating in probiotic formulations. The first chapter deals with isolation and selection of a suitable probiotic strain.
Selection criteria were primarily based on fulfillment of essential capabilities to resist the biological barriers which the probiotic strains come across before they reach the ecological niche where they need to exert their beneficial effects. The ability to curtail infections is the most important function of a probiotic strain and probiotics could be a safe, cost effective approach that can add as a barrier against or resistance to microbial infections. The endemic nature of enteric fever especially in developing countries such as India coupled with the emergence and of antibiotic resistant strains has posed a continual threat in the battle against the infection. Such conditions suggest an important role for effective probiotics in the mitigation of such illness and improvement of health. Taken into consideration the above situation, we had considered antagonism towards *Salmonella typhi* and *Salmonella paratyphi* A as an important criterion while screening and selecting a suitable probiotic candidate.

For the isolation and identification of potential strains infant feces was chosen as the source of sample since breast fed infants develop a probiotic rich gut microflora with less pathogenic bacteria (Weizman *et al.*, 2005) and LAB and *Bifidobacterium* dominate the microbiota of full term neonates. Moreover LAB of human and animal microbiota is regarded safe and is believed to be beneficial to the host (Shea Beasely, PhD thesis). Only host specific microbial strains are able to compete with the indigenous microflora and to colonize the niches. The probiotic strain must be tolerated by the immune system and not provoke the formation of antibodies against the probiotic strain. Many of the bacteria used for probiotic preparations have been isolated from human fecal samples to maximize the likelihood of compatibility with the human gut microflora and hence enhance their chances of survival (Burns and Rowland, 2000). Strains isolated from the
human intestinal tract are generally recommended suitable for probiotic use in humans (Shortt, 1999) because some health promoting benefits may be species specific, and microorganisms may perform optimally in the species from which they were isolated. Medium of choice for culturing was TPY because it is an enriched media containing all the essential vitamins and growth factors required for the growth of fastidious microbes and is the recommended media for Bifidobacterium sp. as well as favourable for growth of LAB strains. Isolation was performed in CO₂ incubator in the presence of 5% CO₂. Bifidobacterium is a stringent anaerobe whose growth can be enhanced in the presence of CO₂ (Bergey’s manual of Systemic Bacteriology). Out of the 25 isolates with distinct morphology obtained on TPY agar, the 21 Gram positive strains were subjected for preliminary screening for LAB. From the 21 Gram positive isolates, those which was non-motile, non-spore-former, negative for catalase and oxidase tests and which coagulated skimmed milk were chosen. These primary tests are employed for the preliminary screening of LAB and based on the above tests we identified 14 isolates as LAB for examination of probiotic properties.

Resistance to gastric acidity and bile salts are the frequently suggested in vitro tests for the evaluation of probiotic potential of bacterial strains according to the guidelines for the evaluation of probiotics in food (FAO/WHO, 2002). The stomach and the surroundings of the human gastrointestinal tract have the highest acidity and the pH of these areas may fall to as low as 1.5. In most in vitro assays pH 3.0 has been chosen to evaluate resistance to gastric transit due to substantial decrease in the viability of strains at pH 2.0 or lower (Vinderola and Reinheimer, 2003). We examined the ability of the 14 LAB isolates from infant feces to survive in acidic conditions at pH 2, 3 and 4. Even though all the LAB isolates were
obtained from infant gut flora, they varied in their resistance to acidic pH indicating that the property is strain specific.

Meira et al. (2012) reported that acid tolerance is strain specific and is a trait of individual strains strongly affected by experimental conditions. It has been suggested that the variation in the acid tolerance might be related to the difference in H\(^+\)-ATPase activity, which controls the intracellular H\(^+\) concentration thereby maintaining pH homeostasis and cell viability (Meira et al., 2012; Guo et al., 2009). Satisfactory growth was observed in the case of 5 (CF1PP, CF1A2, CF1B, CF12 and CF5C) isolates. These isolates showed increase in turbidity with increase in pH. The isolates, CF4B, CF10B, CF16 and CF25 showed poor growth at pH 2, but not at pH 3 and 4. These isolates were not viable at pH2 after 24 h of incubation. There are reports that *Lactobacillus acidophilus* (BG2FO4) showed rapid decline in numbers at pH 2.0 but at pH 4.0 there is no significant decrease. According to Kailaspathy and Chin, (2000) many species of *Lactobacillus acidophilus* and *Bifidobacterium* spp intrinsically lack the ability to survive adverse conditions in the gut. It has been reported that *Lactobacillus acidophilus* has high cytoplasmic buffering capacity allowing it to resist changes in cytoplasmic pH and gain stability under acidic conditions (Kailaspathy and Chin, 2000) Ability to survive in low pH is an essential property required for bacterial strains to be characterised as human probiotic as they get exposed to similar conditions in the gastrointestinal tract. Our findings show that the isolates CF1PP, CF1A2, CF1B, CF2 and CF5C survive the best under low pH condition ranging between 2 and 4 for more than 24 h indicating that they are capable of surviving in low pH conditions that prevail in the human gastrointestinal tract much longer than the period during which they would have to remain there with the buffering action of food. It has been shown that
bacteria are exposed to this condition in only for several dozen minutes (Ganong, 1995).

Similarly, tolerance to bile is another basic criterion required from probiotic bacteria, a property which enables them to survive in the small intestine in the presence of bile salts. We evaluated the capability of all the 14 LAB isolates to grow in the presence of bile salts at three different (0.3, 0.5, and 1%) concentrations. Gastrointestinal systems have varying concentrations of bile and according to Dunne et al. (1999) the relevant physiological concentrations of human bile range from 0.3% to 0.5%. The rate of secretion of bile acid and its concentration depend on the type of food consumed. Bile concentrations may range from 0.5% to 2.0% in the first hour of digestion and the levels may decrease during the second hour. Again we found individual variation in the resistance of bile salts by the LAB isolates. In the present study the four isolates CF1PP, CF1A2, CF1B and CF2 out of the 14 LAB isolates showed the highest survival in the presence of bile salts in growth media followed by CF5C and CF4B with better survival than the others. It is generally considered necessary to evaluate the ability of potentially probiotic bacteria to resist the effects of bile acids, not only because it is a selection criterion, but also because Lactobacilli and Bifidobacteria have been shown to exhibit a strain variation in their tolerance to bile salts (Vinderola and Reihmer, 2003).

There are reports of recent investigations revealing survival of Lactic acid strains in up to 0.4% bile salts (Goderska and Czarnecki, 2007) and the ability to resist bile salts has been related to the ability to remove cholesterol from the intestinal environment (Begley et al., 2005; Vinderola et al., 2008).
Among the variables that affect survival of bacteria during passage through the gastrointestinal tract, the action of enzymes like pepsin has to be considered. Such an attempt was done by performing the test for resistance to artificial gastric fluid and is a frequently suggested *in vitro* test for the evaluation of probiotic potential of a bacterial strain Charteris *et al.* (1998). About 2.5 ml of gastric juice at a pH of approximately 2.0 is secreted each day in our stomach. Here we examined the tolerance of the 14 LAB isolates to artificial gastric condition at pH2 and pH3 for 180 min of incubation at 37ºC. All the 14 isolates grew better at pH 3 than at pH 2 in the gastric juice. Among the LAB isolates we obtained from infant feces appreciable proliferation was observed for CF1PP, CF1A2, CF1B, CF2 and CF5C at pH2 and at pH3 in artificial gastric juice. Least proliferation was observed for CF10B followed by CF25, CF4B and CF16. It was also observed that the growth of the other isolates CF6A, CF13C, CF15, CF16, and CF24C was very less at both the pH after 180 min of incubation.

The ability of probiotic bacteria to survive the passage through the stomach was reported to be variable and strain dependent by many researchers (Vinderola and Reiheimer, 2003). According to the study conducted by Schillinger *et al.* (2005) *Lactobacillus* strains from dairy products differed considerably in their resistance to acid in simulated gastric juice He reported that strains of *Lactobacillus casei* group including *Lactobacillus rhamnosus* GG were more susceptible to gastric acidity than strains of *Lactobacillus acidophilus* group. On the other hand, protective matrix provided by food can enhance the survival of probiotic bacteria when they are applied in dairy products.

Therapeutic benefits of probiotic bacteria can be achieved only if they can persist and survive in the upper regions of the gastrointestinal tract.
Viability and activity of probiotic bacteria are important considerations, as bacteria must survive during transit through the acidic conditions of the stomach and resist degradation by hydrolytic enzymes and and bile salts in the small intestine (Playne, 1994). Our study clearly indicates that the properties such as tolerance to acid, bile salts as well simulated gastric juice are strain dependent and that result obtained from one strain cannot be directly extrapolated to others. It is evident from the results of probiotic characterization that the 5 isolates CF1PP, CF1B, CF1A2, CF2 and CF5C have better ability to survive the barriers (gastric acidity, presence of bile salts and hydrolytic enzymes) during passage through the upper gastrointestinal tract. Hence we chose these 5 isolates for further evaluation of probiotic characters.

Determination of hemolytic activity is considered a safety aspect for selection of probiotic strains. None of the five isolate showed hemolytic property. Absence of hemolytic activity indicates the non virulent trait of bacteria and is an important selection criterion for probiotic strains (De Vuyst et al., 2003; Sieladie et al., 2011).

Antibacterial activity: The protective role of probiotic bacteria against gastrointestinal pathogens and the underlying mechanisms has received special attention recently. Pathogen inhibition by LAB might provide significant human health protection against infections as a natural barrier against pathogen (Collado et al., 2008). According to Rolfe et al., (2000), Lactic Acid Bacteria have been shown to inhibit the in vitro growth of many enteric pathogens and have been used in both humans and animals to treat a broad range of gastrointestinal disorders. We tested anti bacterial activity of the five selected strains which showed superior probiotic characteristics, against the pathogens, Vibrio cholera, Escherichia coli,
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*Klebsiella* sp, *Salmonella typhi* and *Salmonella paratyphi* A. The culture supernatant (pH between 2.5 and 3.5) of all isolates were capable of inhibiting pathogens including *Salmonella typhi* and *Salmonella paratyphi* A by agar diffusion assay. The degree of inhibition varied with each pathogen and isolate with CF1B and CF5B having more activity than the others. The inhibitory activity could be attributed to the low pH of the culture supernatant owing to the production of organic acids especially lactic acid by the LAB isolates. Noticeably, the same effect was also observed at higher pH (pH 4 and 5 but not at 7) in the case of the isolate CF1B. But the pathogens showed appreciable growth under identical pH conditions implying that the inhibition was not merely due to the extremely low pH of the culture supernatant but may also be due to an inhibitory agent whose activity is optimum below pH 5. It has been learned that probiotic bacteria can antagonize pathogenic bacteria by production of antimicrobial compounds, such as bacteriocins and siderophores (Gram *et al.*, 1999), reducing luminal pH, inhibiting bacterial adherence and translocation, or producing antibacterial substances and defensins. The gut flora resists colonization by pathogenic bacteria is by the production of a physiologically restrictive environment, with respect to pH, redox potential, and hydrogen peroxide production.

It has been shown that pH plays a crucial role for the production of active bacteriocins (Christina *et al.*, 1995). Similar to our results Balasubramayam and Varadaraj, (1998) demonstrated that the stability of antibacterial activity of culture filtrate of certain *Lactobacillus* species remained constant at pH 4 and 4.5 and decreased with increasing pH levels and was completely lost at pH 6-6.5.
On account of the better probiotic characteristics as well as the antibacterial action towards *Salmonella typhi* and *Salmonella paratyphi A*, among the 14 LAB isolates, CF1B was selected as the candidate strain of choice.

Detection of Fructose 6 phosphate phosphoketolase (F6PPK) is the most reliable biochemical test for the identification of *Bifidobacterium* sp. On account of the dominance of *Bifidobacterium* sp in the gut flora of breastfed infants the selected LAB isolate was tested for the presence of F6PPK. Result of the test (absence of red colour) clearly indicated that the isolate does not belong to the genus *Bifidobacterium*. Physiological characteristics such growth at 4, 10 and 45°C, growth at pH 9.6, resistance to 40% bile, hydrolysis of bile esculin as well as survivability at 60°C for 30 min identified the isolate as belonging to the genus *Enterococcus*. The above features are typical of *Enterococcus* genus and are widely used for differentiation of LAB. *Enterococcus* will grow in a range of temperature and pH and can survive heating at 60°C for 30 min. (Foulquie *et al.*, 2006). Biochemical characterization patterns (bioMerieux VITEK2 Systems) were useful to give identification of the intestinal LAB isolate as *Enterococcus faecium*. Characterization of the LAB isolates based on its physiological properties and the biochemical profile are commonly employed in the identification of Lactic Acid Bacteria. The result was confirmed genotypically by species specific PCR using the primers EM1A and EM1B described by Cheng *et al.* (1997). Recombinant plasmids of several clones of the *Enterococcus faecium* genomic library were found to contain inserts which were specific for *Enterococcus faecium*. No significant homologies were found between the sequences of the recombinant plasmid pEM1225 (referring to the number of base pairs found in its insert) and those of other
genes found in the GenBank database. Primers EM1A and EM1B, selected from the 1,225-bp fragment, generate a 658-bp DNA product upon PCR amplification of DNA from Enterococcus faecium ATCC 19434 and ATCC 35667. No amplification product was found by PCRs of other Enterococcus species and the PCR-based diagnostic assay described Cheng et al. (1997) targets a nucleotide sequence that is strongly conserved in Enterococcus faecium (Cheng et al., 1997). Also Achemchem et al. (2005) reported this method as a very accurate assay for identifying strains of Enterococcus faecium.

Further molecular characterization based on 16s rRNA sequencing has shown 99% similarity with Enterococcus faecium. The strain has been designated as Enterococcus faecium MBTU-P1F1 and the gene accession number has been obtained. According to the guidelines of FAO and WHO for the evaluation of probiotics in food, the first consideration is to identify and characterize the organism to the genus and species level with internationally accepted methods, such as sequencing of DNA encoding 16S rRNA (Wang et al., 2002) The second consideration for particular strains that are being targeted for probiotic use is to have clear and consistent strain designation. This will allow physicians and consumers to track publications associated with that strain has probiotic effects (Reid et al., 2003). Enterococcus faecium is a species of bacteria that has been characterized as part of the normal gastrointestinal microbial flora in animals and human constituting a major population in the gut. Enterococcus faecium have been used as a human probiotic for more than 25 years. On account of its ability to inhibit the growth of the food borne pathogen Leroy et al. (2003) studied the effect of adding a strain of Enterococcus faecium as a coculture for the production of cheddar cheese. Carefully selected and researched strains of
Enterococcus faecium are well documented as safe and effective probiotics. They have been proven safe and effective in humans and livestock and have been recognized as such in the European Union, the United States, by the FDA, AAFCO and other countries as well. Enterococcus faecium is one of a large number of lactic acid producing bacteria that are used intensively in functional foods for humans and in food supplements for animals.

Conclusion

The growing awareness of relationship between diet and health has led to an increasing demand for food products that support health above and beyond providing nutrition. Research suggests that probiotic bacteria may mediate a variety of health effects through numerous proposed mechanisms. In the present study we tried to isolate Lactic Acid Bacteria with essential probiotic properties and to explore their antibacterial activity against the causative agents of enteric fever which is endemic in India. The chapter comprises of isolation of bacterial strains from infant gut microflora and screening for bacterial isolates belonging to the genera LAB and Bifidobacteria. Further screening identified a suitable candidate based on ability to resist biological barrier such as tolerance to acidic condition at pH 2,3 and 4, resistance to bile salts at 0.3, 0.5 and 1 g% as well as viability in simulated gastric juice at pH 2 and 3 and anti bacterial activity against selected pathogens. Here focus was given to the enteric fever pathogens Salmonella typhi and Salmonella paratyphi A, taking into consideration the endemic nature of the disease (typhoid fever) in India. The LAB isolate CFIB which possessed resistance to biological barrier (important criteria for selection of probiotic strains) and produced inhibitory agent against the enteric fever pathogens was selected for further studies. Examination of the physiological characteristics and carbohydrate fermentation pattern identified
the LAB as *Enterococcus faecium*. The isolate was identified to the strain level by species specific PCR assay and molecular characterization of the strain was performed by amplification of 16s rRNA. It was designated as *Enterococcus faecium* MBTU-P1F1 and the gene accession numbers for the amplified regions are KF745071 and KF745072.