CHAPTER 1

REVIEW OF LITERATURE

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Review of literature

1.1. Introduction

Bacterial life inhabited earth billions of years before humans evolved, and occupy almost every terrestrial and aquatic niche on our planet. Humans provide residence to numerous microbial communities; the microbial world has played a role in influencing the structure and function of humans (Hickmann 2005; Lee and Mazmanian 2010). The surfaces of the human body inside and out, for example the skin, mouth and the intestines, are covered with millions of individual micro-organisms that do not do us any harm. In fact they help to protect us from becoming infected with harmful microbes. They are known as the normal body flora. In humans, the interaction between host and bacterial cells is especially important in the gastrointestinal tract where the bacteria live symbiotically within the human gastrointestinal tract (Gill *et al*. 2006; Lee and Mazmanian 2010; Geuking *et al*. 2011). Thus, the gut serves as a useful setting to understand the benefits of this symbiotic relationship. Together the bacteria play an important role in the protection of the organism against harmful microrganisms and also strengthen the host's immune system. The B-group vitamins necessary for normal homeostasis and vitamin K required for proper blood coagulation are both produced by the resident intestinal microflora (Hill 1997). Other products such as short chain fatty acids produced by intestinal microflora also provide additional energy source and support the growth of intestinal epithelial cells (Simon and Gorbach 1984). Intestinal bacteria are also required for development of gut-associated

lymphoid tissues (GALT), which perform a variety of host immune functions, such as mucosal immunity and oral tolerance. The gut commensal bacteria play an important role in the development of preimmune antibody (Ab) repertoire by promoting somatic diversification of Ig genes in B cells that have migrated to GALT (Rhee *et al*. 2004). Finally, intestinal epithelial layer permeability and major part of epithelial cell function are shaped by modulating expression of associated genes upon the exposure of bacteria in the early stages of life and weaning (Hooper *et al*. 2001). It is well established that the human immune system and GIT requires the presence of diverse and high numbers of microorganisms for proper maturation and function throughout the life of the host.

Probiotics are defined as "Live microorganisms that when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). They are usually consumed after antibiotic therapy (for some illnesses), which destroys the microbial flora present in the digestive tract (both the useful and the targeted harmful microbes). Probiotics can be found in dairy and non-dairy products. Regular consumption of food containing probiotic microorganisms is recommended to establish a positive balance of the population of useful or beneficial microbes in the intestinal flora. Indian probiotic market occupies <1% of the total global market and is now likely to grow due to efforts by some leading companies like Amul, Yakult, Nestle India and Mother Dairy gradually entering the scenario (Raja and Arunachalam 2011; Pandey *et al*. 2015). The probiotic market in India is projected to register a compounded annual growth rate (CAGR) of 19.8% during 2014-19. Probiotic products mainly in the area of gastrointestinal health form the core of Indian functional food market. More than 25 companies have established themselves in India.

Major pharmaceutical players who have invested in manufacturing probiotics or prebiotics in India include companies like Sun pharma, Dr. Reddy's Laboratories, USV (Union Square Ventures), Zydus Cadila and Unichem (Dixit *et al*. 2016).

Many of the probiotic bacteria belong to Lactic acid bacteria (LAB), a family of microorganisms which ferments various substrates primarily into lactic acid. The majority of LAB are Gram positive, anaerobic or facultative anaerobic, nonsporulating and acid tolerant. The probiotic microorganisms consist mostly of the strains of the genera *Lactobacillus* and *Bifidobacterium*, but strains of *Bacillus*, *Pediococcus* and some yeasts have also been found as suitable candidates. *Lactobacillus* is an important genus among LAB family and many *Lactobacillus* strains are widely used as probiotic bacteria (Nikolova *et al*. 2009; Xu *et al*. 2009; Sikorska and Smoragiewicz 2013; Li *et al*. 2015).

1.2. History of probiotics

The name probiotic comes from the Greek 'pro bios' which means 'for life'. Louis Pasteur identified the bacteria and yeasts responsible for the process of fermentation, but did not link these microbes to any apparent health effects (Barnett 2000). In 1905, Elie Metchnikoff, who had worked with Pasteur in the 1860s, was credited with making the association of longevity among Bulgarians, not to the yogurt they consumed, but rather to the lactobacilli used to ferment the yogurt and the presence of these lactobacilli in the colon (Metchnikoff 1907). Metchnikoff first introduced the probiotic concept in 1908, had observed that rural dwellers in Bulgaria lived to very old ages, despite extreme poverty and harsh climate. They had an average lifespan much greater than wealthier Europeans,

and he noted that they drank fermented milk products. Metchnikoff sumrised that the lactic acid bacteria associated with fermented milk products had anti-aging health benefits. He named the organism "*Lactobacillus bulgaricus*". Although there is reference to sour milk or fermented cultures as far back as the Bible and the sacred books of Hinduism, Elie Metchnikoff is regarded as the grandfather of modern probiotics. Metchnikoff went on to publish "The Prolongation of Life: Optimistic Studies", stating that "Ingesting microorganisms could have substantial health benefits in humans" (Metchnikoff 1907). In 1908 Metchnikoff received the Nobel Prize in medicine for his work demonstrating that harmful microbes can be replaced by beneficial microbes to treat intestinal illnesses. Lactobacilli and Bifidobacter are two kinds of lactic acid bacteria, which are found in the gut and are considered as probiotic, because of their beneficial effects on health (Wood 2012).

In 1899, another important discovery was made at the Pasteur Institute by Henri Tissier who isolated a Y-shaped bacteria from the gut flora of breast-fed infants. He called the organisms "bifidobacteria". In 1906, Tissier observed that these bacteria could lessen diarrhea in babies (Tissier 1906).

A German physician and scientist, Alfred Nissle, was intrigued by the potential uses and benefits of probiotics. Nissle isolated a new strain of *Eschericia coli* from the feces of a world war I soldier who was afflicted with shigella but did not develop the diarrheal illness, during an outbreak of shigellosis. The new bacterial strain was named "*Eschericia coli* Nissle 1917." Nissle used the strain to treat intestinal diseases (like gastrointestinal

infectious salmonellosis and shigellosis), caused by shigella and salmonella, with great success (Nissle 1918), since there were no antibiotics at that time. In 1965, the term 'probiotics' was first used by Lilly and Stillwell (1965) in a different context to represent 'substances secreted by one organism which stimulate the growth of another'. After nine years, Parker (1974) described probiotics as "organisms and substances which contribute to intestinal microbial balance". Fifteen years later, Fuller (1989) proposed that probiotics were 'live microbial supplements which beneficially affects the host animal by improving its microbial balance. This was followed by Salminen *et al*. (1998) who defined probiotics as 'foods containing live bacteria which are beneficial to health'. In 2001 the World Health Organization defined probiotics as "live microorganisms, which, when administered in adequate amounts, confer health benefits to the host" (FAO/WHO, 2001). This is now the widely used and accepted definition as it embraces all applications of live microbes, not just those for intestinal benefits.

At the end of the century, it became clear that intestinal microflora had several functions, including metabolic, trophic and protective ones (Guarner and Malagelada 2003). Metabolic functions are primarily characterized by the fermentation of non-digestible dietary residue and endogenous mucus, savings of energy as short-chain fatty acids, production of vitamin K, and absorption of ions. Trophic functions are based on the control of epithelial cell proliferation and differentiation, and development and homeostasis of the immune system (Canny and McCormick 2008). Finally, protective functions are connected with the barrier effect and protection against pathogens (Del Piano *et al*. 2006). The health benefits derived from the consumption of foods containing *Lactobacillus acidophilus*,

Bifidobacterium spp. (*B. bifidum*, *B. animalis*, and *B. infantis*) and *L. casei* are now well documented. *Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus* are yoghurt starter cultures, which offer some health benefits; however, they are not natural inhabitants of the intestine. *Lactobacillus* and *Bifidobacterium* are lactic acid-producing probiotic bacterial organisms, incorporated in preparation of fermented dairy products which has documented health benefits (Shah 2007).

1.3. Criteria for probiotics

Screening for viable probiotic candidates can be labor intensive, but certain properties have proven useful. These properties fall into 4 broad categories: (1) survival in the target organ, (2) interaction with host systems, (3) antipathogenic actions, and (4) safety. For probiotics to be successful, they must possess some characteristics:

Some of these properties are-

Safety concern:

- **Origin** - The origin of probiotics depends upon the application of probiotics. When selecting for human purposes probiotic strains should be isolated from a human microflora so that they are more likely to adhere to human intestinal wall than others and are more likely to be safe.

- **Non-pathogenic and Non-infectious** - The probiotic strains should be Generally Recognized as Safe (GRAS) so before selecting the probiotics, toxicological studies must be performed.

- **Virulence factors- toxicity, metabolic activity and antibiotic resistance** - Probiotic strains should be non-toxic, so the toxicological studies must be performed. Assessment of the side effects during previous human studies and assessment of certain metabolic activities (e.g. D-lactase production, bile salt deconjugation) are also most relevant determinations, in relation to the selecting of the probiotic strains intended for the human consumption.

  Probiotics might contribute to the transfer of antibiotic resistance genes to other commensal bacteria or pathogens present in the GIT. The dissemination of antibiotic resistance genes can reduce the therapeutic possibilities in infectious diseases. Therefore the determination of antibiotic resistance genes in probiotic bacteria is the one of the main target for a point of safety, for human consumption.

Functional aspects:

- **Tolerate low pH of gastric juice and resistant to bile acids** - For survivability in the gut, the probiotic organism must tolerate acidic pH and bile toxicity of the digestive tract.

- **Adherence to epithelial cells and intestinal mucosa** - In the context of effective colonization, the ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of bacteria used as probiotics. Probiotics inhibit the pathogens by adhering to the intestinal epithelial and mucosal surfaces by blocking the adhesion sites.

- **Validated and documented health beneficial effects -** The selection of the probiotic organisms depends upon health claims. These organisms must be able to exert their benefits on the host (such as favourably alter the intestinal microflora balance, promote intestinal integrity and mobility, inhibit the growth of pathogens and increase resistance to infection) through the growth and/or activity in the human body.

- **Persistence and multiplication in the host** - The survivability and colonization or multiplication in the host are considered critical to ensure optimal functionality and expression of health promoting physiological functions by probiotics.

Technological properties:

- **Genetically stable strains** - Probiotic strains should be stable enough to withstand a conventional industrial production process and have the capabilities for its survival in the food, feed and dietary supplements. Probiotic stability is affected by the high temperature, oxygen humidity and high water activity in the culture.

- **Desirable viability during processing and storage -** The viability of probiotics is a key parameter for developing probiotic foods. Storage of probiotic supplement at 4 to 5 °C is recommended to maintain the viability of the micro-organisms. Probiotic supplements must be kept in refrigerated conditions otherwise they have not stay viable.

- **Phage resistance** - Probiotic bacteria must be resistant in different phages including, during fermentation, industrial production process, and in commercial products.

- **Ease of large scale production** - Probiotic strains are commercially demanded for its health properties. Consequently, required new technologies that enable high cell yield at large scale.

Desirable physiological property includes:

- **Modulation of immune responses** - Stimulating of specific and nonspecific immunity may be one possible mechanism of probiotics to protect the host from intestinal disease. This mechanism is not well documented, but it is thought that specific cell wall components or cell layers may act as adjuvants and increase humoral immune response.

- **Secrete antimicrobial substance and exclusion or reduction colonization of pathogenic microorganisms** - The probiotic strain should be capable of producing antimicrobial substances is most important in developing the probiotic supplement and probiotic rich foods. They may express bacteriocin, lactic and acetic acid and other antibacterial like substances against pathogens, besides competition for adhesion sites and inhibit the binding of pathogens to the intestinal and mucosal surfaces.

- **Lactose metabolism (Promotion of intestinal lactose digestion)** - β-Galactosidase deficiency causes lactose intolerance and amelioration of this situation by β-galactosidase from LAB is a result of converting lactose into easily metabolisable glucose and galactose. The addition of LAB producing β-galactosidase as probiotic in dairy products, can thus be used for improvement of lactose digestion.

- **Reduce cholesterol level** - Probiotic produced "bile salt hydrolase (BSH)" plays a significant role in cholesterol removal by deconjugating the bile salts. BSH activity increases the rate of excretion of free bile acids. Such mechanism could be used in controlling serum cholesterol levels by colonic microbes.

- **Antimutagenic and anticarcinogenic** - Probiotics could reduce the risk of cancer by decreasing the levels of carcinogenetic enzymes produced by colonic flora through normalization of intestinal permeability and microflora balance as well as production of antimutagenic organic acids and enhancement of the host's immune system.

The probiotic potential of different bacterial strains, even within the same species, differs. Different strains of the same species are always unique, and may have differing areas of adherence (site-specific), specific immunological effects, and actions on a healthy *vs*. an inflamed mucosal milieu may be distinct from each other. Bacteria are prevalent in several regions of the body, including the mouth, nose, pharynx, intestinal tract, vaginal tract, and

skin (Willis *et al*. 1999). A number of health effects are associated with usage of probiotics like, stabilization of indigenous microbial population, protection against intestinal infection, alleviation of lactose intolerance, increased nutritional value of foods, reduction of serum cholesterol levels and nonspecific enhancement of the immune system (Hooper *et al*. 1999; Perdigon *et al*. 2002; Sullivan and Nord 2005; Kim *et al*. 2008). There are differing degrees of evidence supporting the verification of such effects. The steps involved in establishing a bacterial strain as a novel probiotic are given in Figure 1.1.

1.4. *Lactobacillus* strains

Lactobacillus was first isolated in 1900 by Moro and he named the strain as *Bacillus acidophilus*, which is the generic name for intestinal *Lactobacillus acidophilus* (Holland 1920). Lactobacilli are non-spore-forming, non-motile, Gram-positive rods or coccobacilli that are an important part of the normal human bacterial flora commonly found in the mouth, gastrointestinal (GI) tract and female genitourinary tract (Hammes and Vogel 1995; Cannon *et al*. 2005; Kononen and Wade 2007). Lactobacilli are either facultative anaerobes or obligate anaerobes. They are strictly fermentative; lactic acid is the major metabolic end product of lactobacilli during glucose fermentation. Glucose is converted predominantly to lactic acid in the homofermentative case, or equimolar amounts of lactic acid, CO_2 and ethanol (and/or acetic acid) in the heterofermentative pathway (Gomes and Malcata 1999). Lactobacilli are a rather diverse group of bacteria, as is illustrated by their large GC content, which ranges from 32% to 53% (Kononen and Wade 2007).

The GI tracts and genital tracts of various mammals are commonly colonized with *Lactobacillus* spp. (Madigan *et al*. 2006). The environmental factors such as oxygen availability, pH, presence of specific substrates and bacterial interactions majorly affect their distribution in a given niche. They are considered protective organisms and are thought to inhibit the growth of pathogenic organisms via the production of lactic acid and other metabolites. Lactobacilli are important organisms used also in industrial food production (Hammes and Tichaczek 1994). Over the last century, the food microbiology industry has extensively studied lactobacilli and deemed the bacteria safe for human consumption.

1.5. Source, isolation and identification of *Lactobacillus* strains

1.5.1. Source

The probiotic strains can be isolated from different sources: conventional sources and unconventional sources. Conventional sources includes dairy products, human and animal gut and/or breast milk and unconventional sources includes non-intestinal sources and non-dairy fermented food products, such as traditional fermented foods, traditional fermented drinks, vegetables, and fruit juices (Pundir *et al*. 2013; Ramirez-Chavarin *et al*. 2013; Siddiqee *et al*. 2013). The differences in the raw materials and ingredients used, are the main factors that lead to the different available species or strains of probiotics in these sources. Siddiqee *et al*. (2013) reported that the animal intestines harbour the most potential sources of LAB while other sources, like flesh, long grass, fruit juice, and vegetables, can also be screened to find LAB.

Probiotic bacteria may also be screened from various non-intestinal sources, like grains (Hamet *et al*. 2013), fruit pulp (Garcia *et al*. 2016), soil (Chen *et al.* 2005; Yanagida *et al.* 2006) and honey-comb (Tajabadi *et al*. 2013). Tajabadi *et al*. (2013) screened LAB from the honey of giant honey bees and found that most of these LAB isolates were *Lactobacillus* spp., mainly *Lactobacillus kunkeei*. This bacterial species have antagonistic effects against yeast growth and the spoilage-related effects of yeast in honey (Olofsson and Vasquez 2008). Potential probiotics were also isolated from other unconventional sources like environments around food products, such as the air of the working and storage rooms of a bakery and the air surrounding environments for preparing sourdoughs. These air samples have been found to contain *L. plantarum* and a similar species was also isolated from the dough (Scheirlinck *et al*. 2009).

The various traditional fermented foods that have been found to contain LAB are made from a variety of raw materials, including salted crab (Senthong *et al*. 2012), seafood (Nanasombat *et al*. 2012), pork (Siripornadulsil *et al*. 2014), soybeans (Cho *et al*. 2014), fish (Paludan-Müller *et al*. 1999) and vegetables (Pundir *et al*. 2013). LAB species have been isolated from fermented foods from several countries. Six genera of LAB species (isolated from fermented foods) have been identified in Thailand: *Enterococcus*, *Lactobacillus*, *Aerococcus*, *Weissella*, *Tetragenococcus* and *Pediococcus*. Most common LAB genera "*Enterococcus"*, was isolated from fermented fishes and crustaceans while *Lactobacillus* species is most commonly isolated from fermented meats and fermented plants. However, the main species isolated from fermented plant materials are *L. plantarum* and *L. fermentum*. Out of 306 isolates, four probiotic LAB species have been used as starter

cultures in Poo-Khem (Thai traditional salted crab) foods for humans because they showed probiotic properties including acid and bile tolerance, antagonistic effects against pathogenic bacteria and hydrophobic activity. These four species were identified as *Enterococcus thailandensis* and *L. plantarum* and two strains of *L. fermentum* (Senthong *et al*. 2012). In India, genera of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Vagococcus* and *Weissella*, have been isolated from a traditional fermented soybean food. These isolates, except *Weissella* sp. and *Vagococcus* sp., showed antibacterial activity against some pathogenic bacteria such as *Bacillus cereus*, *Escherichia coli* (*E. coli*) and *Salmonella paratyphi* (Thokchom and Joshi 2012). In addition, *L. plantarum* isolated from fermented idli batter has been tested successfully for co-aggregation with pathogens like *Listeria monocytogenes* and *E. coli* (Agaliya and Jeevaratnam 2012) in India. Furthermore, *L. plantarum* RYPR1 has been isolated from indigenous fermented beverage Raabadi, consumed during summers in Haryana and Rajasthan regions of India exhibited a good hypocholesterolemic effect (Yadav *et al*. 2016). Another study also showed antioxidant and cholesterol assimilation activities of Indian fermented food isolates *L. casei*, *L. acidophilus* and *Lactococcus lactis* (Jain *et al*. 2009). *L. helveticus* MTCC 5463 has been isolated from the vaginal tract of a healthy adult female in India, showed antimicrobial activity, hypocholesterolemic effect, as well as positive immunomodulating effects (Prajapati *et al*. 2011).

Probiotics that were selected for use in humans originated from the human body, including the human feces of both healthy adults (Choi *et al*. 2005) and breast-fed infants (Munoz *et al*. 2011), as well as human breast milk (Martin *et al*. 2006) or from human foods, which

were usually fermented dairy products. It is reported in earlier studies that the human breast milk isolated bacteria have usually been of the *Lactobacillus* genus (Yavuzdurmaz 2007; Shokryazdan *et al*. 2014), while bacteria from the feces of healthy human adults and breast-fed infants have been from *Lactobacillus* (Verdenelli *et al*. 2009; dos Santos Pozza *et al*. 2011) and *Bifidobacterium* genera (Munoz *et al*. 2011). A few studies have reported probiotic *Enterococcus faecalis* found in human feces (Nueno-Palop and Narbad 2011). *L. salivarius* was found in human milk and infant feces in individuals of a mother-child pair (Jimenez *et al*. 2010). *L. rhamnosus* and *L. casei* were isolated from human breast milk and showed resistance to low pH (pH 3), tolerance against a 0.3 % bile concentration and antimicrobial activity against *E. coli, B. cereus* and *Staphylococcus aureus* (Kavitha and Devasena 2013). Based on these evidences, it has recently become accepted that the human milk constitutes an important natural source for selection of probiotic LAB and bifidobacteria aimed for use in infant formulas (Sornplang and Piyadeatsoontorn 2016). Tulumoglu *et al*. (2013) isolated lactobacilli from the feces of 4–15 years aged children. One of the 20 lactobacilli isolated from these specimens, isolate *L. pentosus* had basic probiotic properties of acid and bile tolerance and antimicrobial activity. This isolate also had other probiotic properties, including the abilities to produce and aggregate exopolysaccharides (EPS) and to provide a cholesterol removal effect (Tulumoglu *et al*. 2013). Human GIT is the most promising source of probiotics isolation as more than 400 different bacterial species resides in human gut (Falk *et al.* 1998). When it is isolated from human gut, there is an increased chance of obtaining probiotic bacteria with acid tolerance and high bile as they are already exposed to detrimental effects in human body before colonizing gut. Currently many of lactobacilli strains used as probiotic are of human origin

such as *L. rhamnosus* GG, *L. plantarum* 299v, *L. gasseri* LA39, and *L. reuteri* (Kawai *et al*. 2001; Doron *et al*. 2005; Goossens *et al*. 2005).

Over the past 20 years, the probiotic strains widely used in animals, especially in Europe and Japan, are spore forming bacteria of the genus *Bacillus* (Elmabrok and Hussin 2012). Currently, most of the probiotics used in animal farming are lactic acid bacteria (LAB). Gastrointestinal (GI) tracts of various animal are used as probiotics sources, including pigs (Giang *et al*. 2010), ruminants (Ghorbani *et al*. 2002) and poultry (Ibourahema *et al*. 2008). These probiotics are also isolated from the feces of different animal species, including chickens (Belkacem *et al*. 2009), pigs (Petsuriyawong and Khunajakr 2011) and ruminants (Stein *et al*. 2006). It is reported that the most *Lactobacillus* strains used in humans are also used as probiotics in animals, but *Bifidobacterium* strains isolated from a human origin were used as probiotics only in humans. Doi *et al*. (2013) used *L. plantarum*, *L. pentosus*, *L. rhamnosus*, *L. buchneri*, *L. rapi*, *Pediococcus pentosaceus* and *P. lolii* as starter cultures in silages for ruminants.

Probiotic bacteria widely used in aquatic animals include LAB and *Bacillus* species, most of these are isolated from gastrointestinal tracts from aquatic animals. Several studies have reported isolation of the probiotics from both fresh water and sea water animals. Diaz *et al*. (2013) reported that *L. salivarius* from bottlenose dolphin can inhibit the growth of *Salmonalla enteritidis* strains that isolated from both marine animals and humans. Sofyan *et al*. (2010) reported that *L. paracasei* isolated from eyes shellfish (abalone) showed

resistance to acidic and bile conditions and had the ability to inhibit pathogenic bacteria such as *E. coli*, *B. cereus* and *S. aureus*.

1.5.2. Isolation

An initial isolation of a probiotic LAB is to preserve the sample in adequate condition before being incubated in selective medium. Most probiotics are strict anaerobic or facultative anaerobic or microaerophilic so the samples should be immediately placed under adequate condition and processed as soon as possible (within 3 h), in order to isolate bacteria successfully. The samples have to be homogenised completely and diluted and cultured in selective media. Depending on the source of the sample, selective inhibitory agents can be incorporated in selective medium to avoid the growth of particular group of microbes predominating in sample. For example, in lactobacilli isolation from faecal samples cycloheximide was added in selection medium in order to avoid the yeast overgrowth (Endo and Okada 2007).

Several media have been developed for selective isolation of lactobacilli and bifidobacteria (Rogosa *et al*. 1951; Munoa and Pares 1988; Dave and Shah 1996; Hartemink and Rombouts 1999). Rogosa *et al*. (1951) developed a selective medium for isolation and enumeration of oral and faecal lactobacilli and *Bifidobacterium*. This selective medium is still the widely used for lactobacilli isolation. This media contains a Columbia agar base supplemented with propionic acid. The low pH of this medium, is tolerated by lactobacilli and bifidobacteria but inhibits the growth of other predominating organisms in human faeces, such as *Bacteroides* and *Eubacterium* species.

The cultured plate is incubated at 37 °C for 48-72 h in microaerophilic or CO_2 rich atmosphere for the growth of lactobacilli and anaerobic condition for bifidobacteria. Subsequently, the colonies appearing on selective medium are picked up and transferred to a propagation broth medium or agar plate i.e. de Man Rogosa Sharpe (MRS) medium. Each colony is further subjected to microscopic and biochemical analysis.

1.5.3. Identification

Probiotics in human gastrointestinal tract are identified by colony morphology, fermentation patterns, serotyping or some combination of these. According to the WHO/FAO, probiotics are strain specific so they must be identified at genus, species and strain level. For the nomenclature of the bacteria scientifically recognized names must be applied. The taxonomy for many years relied on phenotypic properties, on the type of sugar fermented and fermentation end product generated. Thus, the probiotics have been primarily classified as LAB. The taxonomic classification based on biochemical analysis has been used for many decades but many groups of bacteria cannot be differentiated at genus level with this approach and also lead to misidentification of bacteria. Several bacterial genera and yeasts have been proposed as a probiotic cultures, the most commonly used are *Lactobacillus* and *Bifidobacterium* species. Biological molecular tools have been increasingly used for identifying the probiotics, including a DNA base composition (mol % of guanine plus cytosine), a DNA homology accompanied with polymerase chain reaction (PCR) technique and DNA sequencing using 16S rRNA gene region. 16S rRNA gene sequencing is the single most powerful molecular technique presently used for bacterial species identification (Wilson 1995). 16S rRNA gene sequencing is developed to

simplify sequences using species-specific PCR primers that targeted some regions of lactobacilli genome such as the 16S–23S rRNA spacer region (Tannock 1999), This conserved fragment is used for phylogenetic classification and identification is based on relatedness with the sequences available in databases such as DDBJ (DNA Data Bank of Japan), ENA (European Nucleotide Archive), GenBank (Woese 1987; Winker and Woese 1991). WHO recommends all strains should be deposited in an internationally recognized culture collection. The 16S RNA gene analysis is combined with other methods to identify bacterial communities of the gut and other ecological sources. The amplified 16S rRNA gene sequence coupled with PAGE using temperature or chemical denaturation (Muyzer and Smalla 1998), is hybridised using fluorescent oligonucleotide probes that target specific 16S rRNA gene (fluorescence in situ hybridisation) (Langendijk *et al*. 1995) or digested with restriction enzymes terminal restriction fragment length polymorphis.

However, the 16S rRNA gene fragment is small (1500 bp) compared with whole genome of bacteria. Complementary information is necessary to differentiate strains of a given species. Alternatively, the intergenic spacer region between 16S and 23S rRNA gene exhibits a great deal of sequence and length variation (Leblond-Bourget *et al*. 1996). The variation in this region has been used for differentiating species of prokaryotes. Identification of lactobacilli (isolated from human feces, rodent gastrointestinal samples and porcine gastrointestinal contents) at species level was reported by Tannock *et al*. (1999) using intergenic region sequence analysis. Additionally, it can be used as a qualitative technique to confirm isolates as lactobacilli by simply comparing the electrophoretic mobility pattern of amplified products on agarose gel with that of any

standard lactobacilli strain. The analysis of the whole bacterial genome is the most useful tool to identify and characterize bacteria including probiotics.

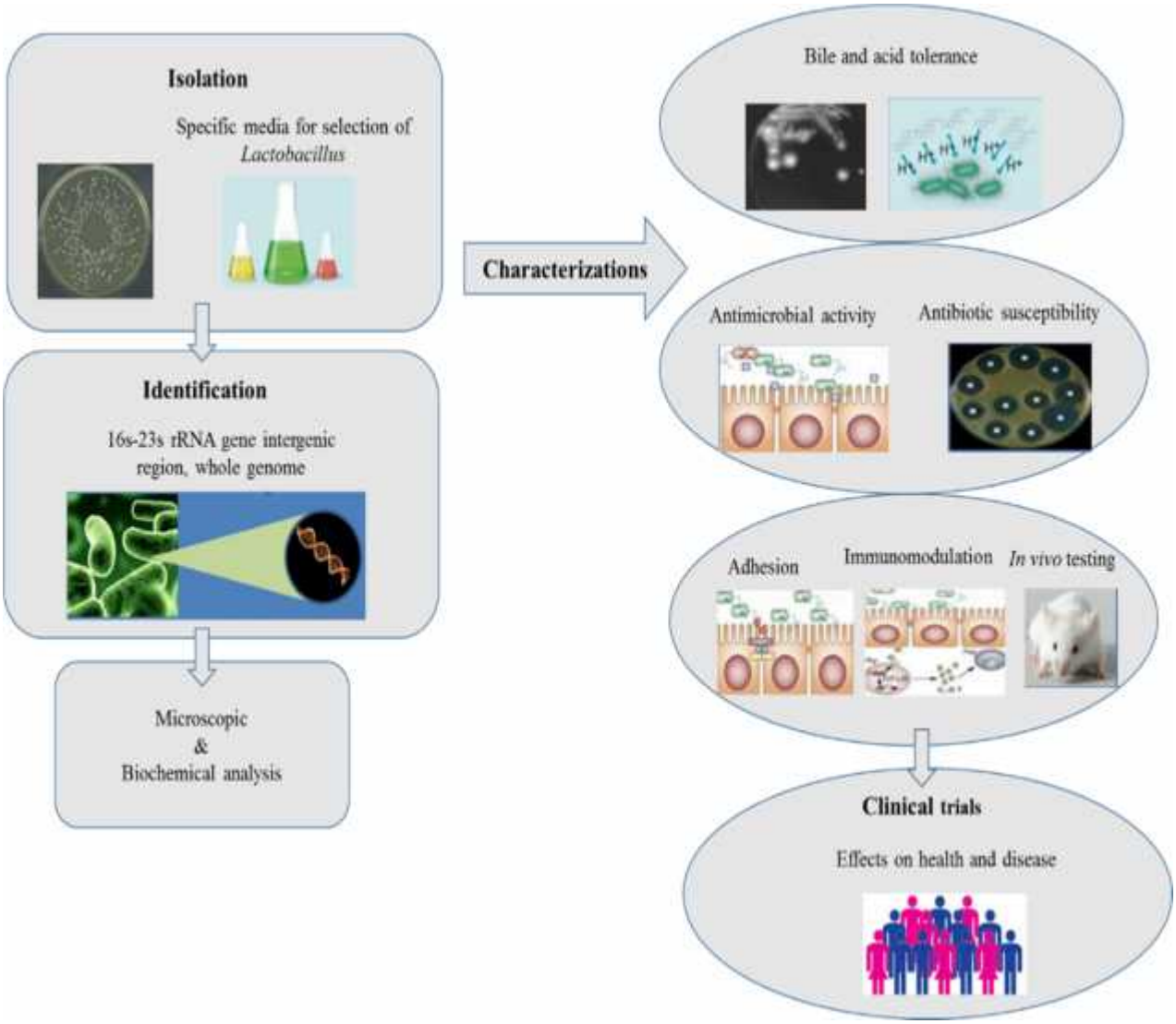

Figure 1.1. Flow chart indicating the various steps in order to isolate and characterize a novel probiotic strain.

1.6. Characterization of lactobacilli as probiotics

1.6.1. Acid and bile tolerance

One of the most important criteria for selection of probiotic strains is their tolerance to acid and bile salt. Bacteria used as probiotics join the food system and travel to the lower intestinal tract via the mouth. It goes to the stomach and enters the upper intestinal tract which contains bile. The strains should have the ability to resist the digestion process. Strains must be able to survive in the stressful conditions of the stomach (pH 1.5-3.0) and upper intestine which contain bile. To show probiotic property, they should reach to the lower intestinal tract and maintain themselves there.

Probiotic strains in the human gastric juice are more accurate indication of the ability of the strains to survive passage through the stomach. The survival of ingested probiotics in different parts of gastrointestinal tract varies with the strains. *L. acidophilus* and *Bifidibacterium* sp., pass through the whole gut at very high concentration while some strains are rapidly killed in the stomach (Lankaputhra and Shah 1995). The pH of the gastric environment is 1.2 to 2.5. Various *in vitro* and *in vivo* studies have demonstrated that probiotic organisms can survive in the gastric transit where the cells are exposed to pH values <2.0, the pH value of gastric acid varies in the range of about 1.5–4.5 in a period of 2 h, depending on the entering time and the type of gastric contents (Dunne *et al*. 2001; Verdenelli *et al*. 2009). Survival of lactobacilli in the acidic condition has also been enhanced in presence of the metabolized sugar that allow the cell membrane proton pumps to operate and prevent the lowering of intracellular pH (Binns 2013). Amachi *et al*. (1998)

reported the action of cell membrane bound H^+-ATPase for acid tolerance of *Lactococcus lactis*. It was demonstrated that it is important for the bacteria to increase H^+-ATPase activity quickly and pump out H^+ in order to maintain intracellular pH. The acid sensitive bacteria are damaged when exposed to acidic condition since their H^+-ATPase activity cannot be increased (Matsumoto *et al*. 2004). The bifidobacteria however proved less acid resistant than the lactobacilli, particularly when exposed to the human gastric juice (von Wright and Salminen 1998; Robinson 2002).

Bile is a viscous alkaline fluid consisting of bile salts, bile pigments, bile acids, cholesterol and phospholipids. Bile salts are water soluble and synthesized in liver from cholesterol and secreted from the gall bladder into the duodenum in the conjugated form (500-700 mL/day). These acids are conjugated as an N-acyl amidate with glycine or taurine to form amphipathic molecules capable of fat emulsification. In this form, bile may also act as an antimicrobial detergent capable of dissolving bacterial membranes (Begley *et al*. 2005).

In *in vivo* condition, Bacteria that tolerate bile have ability to hydrolyze the amide bond between a conjugated bile acid or bile salt and its corresponding amino acid moiety to release the amino acid and an unconjugated bile acid or bile salt. Bile-salt hydrolase (BSH) enzymes of these bacteria catalyze these biotransformation reactions (Begley *et al*. 2006). Unconjugated bile-acids are themselves potent inhibitors of microbial proliferation, thus intestinal BSH positive bacteria usually exploit further enzymatic, transport or precipitation methods (Whitehead *et al*. 2008; Fang *et al*. 2009) to eliminate the inhibitor from their gastrointestinal environment.

1.6.2. The antimicrobial properties

Antimicrobial activity is one of the most important selection criteria for probiotics and is most important in developing the probiotic supplement and probiotic rich foods. Several metabolic compounds produced by lactic acid bacteria which showed antimicrobial effects, include organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl. However, low molecular weight proteinaceous substances or bacteriocins with specific inhibitory activity against closely related species are most studied (Ouwehand and Vesterlund 2004). Organic acids and hydrogen peroxide are nondiscriminatory incidental antimicrobial products of standard *Lactobacillus* metabolism that can be inhibitory or detrimental to both Gram- positive and Gram-negative species (Strus *et al*. 2006; Pridmore *et al*. 2008). For example, the strong anti- *Helicobacter pylori* activity exhibited by *L. salivarius* UCC119 was due to high accumulation of lactic acid (Ryan *et al*. 2008). *L. rhamnosus* GG has been shown to inhibit *S. enterica serovar Typhimurium* growth by secretion of lactic acid (de Keersmaecker *et al*. 2006). Other comprehensive study of 20 different *Lactobacillus* species revealed that the inhibition of *Staphylococcus aureus* was due to *Lactobacillus* acidogenesis (Elkins *et al*. 2008). Although the mode of action of lactic acid as an antimicrobial substance is still not clearly understood, but it is believed that lactic acid cooperates with other agents to permeabilize the outer membranes of Gram-negative bacteria (Alakomi *et al*. 2000). It may chelate essential nutrients such as iron to inhibit growth of pathogens (Shelef 1994).

Production of H_2O_2 by lactobacilli is also suggested to be an important property in terms of antimicrobial effect. In general, lactobacilli reduce oxygen to H_2O_2 during growth in the

presence of oxygen (Marty-Teysset 2000). Especially, vaginal *Lactobacillus* isolates are efficient producers of hydrogen peroxide. Otero and Nader-Macias (2006) demonstrated role of H_2O_2 in the anti-*S. aureus* activity by bovine-derived vaginal *Lactobacillus gasseri* strains *in vitro.* Hydrogen Peroxide producing eight *Lactobacillus johnsonii* strains and a *L. gasseri* ATCC33323T strain, exhibited anti- Salmonella activity *in vitro* (Pridmore *et al*. 2008). Hydrogen peroxide production has also been reported for several *L. salivarius* strains isolated from adult and infant feces (Song *et al*. 1999).

Antimicrobial compounds, termed bacteriocins, produced by probiotic *Lactobacillus* are ribosomally synthesized, small, heat-stable peptides (Cotter *et al*. 2005). The antimicrobial activity spectra of *Lactobacillus* bacteriocins are most studied. Two broad classes of bacteriocins have been proposed. Class I bacteriocins, known as 'lantibiotics', are characterized by presence of unusual amino acids, such as lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine. Class I bacteriocin 'nisin' was discovered in 1928 (Hurst 1967) which was produced by the *Lactobacillus lactis* subsp. Nisin is the only purified bacteriocin approved for use in the product proposed for human consumption. *Staphylococcus*, *Micrococcus*, and *Listeria* species mainly inhibited by nisin. In 1948, subtilin, a nisin analogue differing by 12 amino acid residues was discovered by Hansen (1993). A number of subclasses of this group exist: Class Ia (cationic and hydrophobic peptides) and Class Ib (globular peptides and no net charge) (Altena *et al*. 2000). Class II bacteriocins or 'nonlanthionine containing bacteriocins' are small heat-stable heterogenous group of bacteriocins that can be further divided into four sub-classes: pediocin-like anti *Listeria* peptides, two-peptide, cyclic, and non-pediocin single linear peptides (Cotter

2005). This class of bacteriocins are often found and characterized in lactobacilli. *Lactobacillus plantarum* produces a class II bacteriocin called plantaricin S. Class III bacteriocin such as 'acidophilucin A' is produced by *L. acidophilus* (Ouwehand and Vesterlund 2004). Other probiotic strains also produce various kinds of bacteriocins which showed adverse effect on various pathogenic organisms. *Lactococcus lactis* produces Lactococcin; *Streptococcus salavarius* produces Salivarcin; *L. acidophilus* produces Acidocin 8912; *L. plantarum* produces Plantarcin; *L. delbrueckii* produces Lacticin A (Mishra and Prasad 2000; Dash 2009).

1.6.3. Antibiotic susceptibility

The extreme use of antibiotics has played a significant role in the outspread/emergence of antibiotic resistant bacteria. Many food products which are estimated to involve microbial fermentation processes using lactic acid bacterial (LAB) (FAO/WHO 2001) strains have to be systematically examined for antibiotic susceptibility in order to avoid the spread of antibiotic-resistant determinants through the food chain (Teuber *et al*. 1999). When probiotic strains enter the gut, they interact with the native microbiota and gene transfer can occur (Mathur and Singh 2005; Ammor *et al*. 2007). Probiotics might contribute to the transfer of antibiotic resistance genes to other commensal bacteria or pathogens present in the GIT. The dissemination of antibiotic resistance genes can reduce the therapeutic possibilities in infectious diseases. Therefore the determination of antibiotic resistance genes in lactobacilli is the one of the main target for a point of safety, used as probiotic strains for human consumption or as starter cultures of fermented food or feed products. The presence of several resistance genes in many LAB from the human gastrointestinal

tract (GIT) has been reported (Axelsson *et al*. 1988; Scott *et al*. 2000). Antibiotic resistance profiles have recently been reported for several lactobailli. Bayer *et al*. (1978) demonstrated among 40 strains of *Lactobacillus* spp., 95% were tolerant to ampicillin, 78% to penicillin, and 85% to cephalothin. In lactobacilli, resistance against neomycin, kanamycin, streptomycin and gentamicin has been observed more frequently. (Danielsen 2002; Coppola *et al*. 2005; Zhou *et al*. 2005). Tetracycline resistance gene *tet* (M) and erythromycin resistance gene *erm* (B), are two of the most commonly observed resistance genes in lactobacilli followed by *cat* genes for chloramphenicol resistance (Lin *et al*. 1996; Danielsen 2002; Gevers *et al*. 2003; Cataloluk and Gogebakan 2004). Several species of *Lactobacillus* including *L. rhamnosus* and *L. casei* are intrinsically resistant to vancomycin. Klein *et al*. (2000) also established vancomycin resistance probiotic *Lactobacillus* strains. In recent years, several studies have been undertaken for the antibiotic susceptibility of intestinal lactobacilli species from humans (Charteris *et al*. 1998; Mandar *et al*. 2001; Duskova and karpiskova 2013). The phenotypic studies of microbial resistance of the strains according to the EFSA (European Food Safety Authority) breakpoints (minimum inhibitory concentration (MIC) of the antibiotic) indicated they are sensitive to the antibiotics evaluated (Maldonado and Nader-Macías 2015). However, due to the multiplicity of methods used and the unrelatedness of the strains, there is still a lack of agreement in the resistance-susceptibility breakpoints for most antibiotics in LAB (Charteris *et al*. 1998; Danielsen and Wind 2003).

1.6.4. Adhesion of *Lactobacillus* strains

Adhesion of probiotic bacteria to intestinal epithelial cells and mucus layer are also one of the most important selection criteria for probiotic microorganisms. Adhesion to the intestinal mucosa and epithelial cells may prevent the probiotic cells being washed out and therefore, allowing temporary colonization, improve barrier function, immune modulation and competitive exclusions of pathogens. In support of this concept, several researchers have developed and utilized *in vitro* cell culture models, and *in vivo* animal systems. Human gut isolated probiotic strain adheres and colonizes better than other such as animal (Nemcova 1997; Klaenhammer and Kullen 1999) and food (Bezkorovainy 2001) origin probiotic strain.

In vitro adhesion models

In vitro model systems have proved efficient for providing a good measure on the adhesive ability of a potential probiotic. Tissue culture cell lines and mucus preparations are the most frequent *in vitro* models utilized.

The intestinal epithelial cell lines

Methods of culturing epithelial tissues were not developed until mid-1970s and the main developments in this field of research took place in the 1980–1990s. Initially, two approaches were suggested: (1) *in vitro* culturing of normal enterocytes like HT-29 (Quaroni *et al*. 1979) and (2) differentiating of malignant tumor cells of the large intestine into cells similar to enterocytes like Caco-2 (Dexter and Hager 1980; Pinto 1983). The human epithelial cell line Caco-2 has been widely used as a model of the intestinal

epithelial barrier. Caco2 cell line is heterogeneous and originally derived from a colon carcinoma. When cultured under specific conditions the cells become differentiated, with full confluence, makes up a monolayer of highly polarized cells whose structure is typical of enterocytes, with the nucleus situated in the base part, with dense mitochondria and the brush border in the apical part (Hidalgo *et al*. 1989). Due to this type of structure, it is used as a model enterocyte line in *in vitro* cultures. Caco-2 cells express tight junctions and a number of enzymes such as peptidases, esterases, P-glycoprotein and transporters (for amino acids, bile acids, carboxylic acids, etc.) that are characteristic of such enterocytes.

HT-29 cell line also showed typical characteristics of enterocyte differentiation and has been used for *in vitro* adhesion assays (Gopal *et al*. 2001). The HT-29 cell line was isolated from a large intestine adenocarcinoma tissue, like Caco-2. Its morphology is typical of epithelial cells in *in vitro* cultures, but it does not differentiate to brush border-creating forms. A large portion of the HT-29 cell line population is made up of goblet-like cells; hence this line produces large amounts of mucin. Under standard culture conditions, these cells grow as a nonpolarized, undifferentiated multilayer. Two distinct sub-populations of HT-29 cell line: 5-fluorouracil (HT-29-FU) or methotrexate (HT-29-MTX), are mucus-secreting cells. HT-29-MTX cells are polarized goblet cells that secrete mucins, similar to mucins of the human colon (Leteurtre *et al*. 2004). These mucin-secreting subclones are used as *in vitro* models for studying the adhesion of bacteria with intestinal cell as well as mucus. Mack *et al*. (2003) reported the ability of *Lactobacillus* strains to inhibit the adherence of enteric pathogen to intestinal epithelial cells by increasing the extracellular secretion of MUC3 mucin. Incubation of *L. plantarum* 299v and *L. rhamnosus* GG with

HT-29 cells increased expression of MUC2 and MUC3 intestinal mucins, and both the mucins were able to inhibit the adherence of pathogenic *E. coli* to HT-29 intestinal epithelial cells (Mack *et al*. 1999). *Lactobacillus* species also increased MUC2 mucin expression in Caco-2 intestinal cell line, thus blocking pathogenic *E. coli* invasion and adherence (Mattar *et al*. 2002). MUC1 and MUC3 expression were also elevated with probiotic stimulation, but to a lesser extent (Caballero-Franco *et al*. 2007). Lactobacilli can also displace bound pathogens, such as *Salmonella* species, from human mucins or Caco-2 cell surfaces (Lee *et al*. 2003). Lipotechoic acid (LTA), a molecule associated with the surface of *L. johnsonii* La1 participated in the attachment to Caco-2 intestinal cells and further demonstrated an immunomodulatory effect on gut homeostasis (Granato *et al*. 1999).

HeLa cells have also been used to study the adhesion of commensal and probiotic bacteria, other than intestinal cell lines. It is a cell type in an immortal cell line and was derived from human cervical carcinoma cells. This is a very aggressive cell line that can easily overwhelm other cell lines. Kaewnopparat *et al.* (2013) demonstrated the adhesion ability of *L. fermentum* SK5 and its inhibition of *E. coli* and *Gardnerella vaginalis* to HeLa cells. HeLa cells were also used by others for bacterial adhesion studies (Fourniat *et al*. 1992; Mastromarino *et al*. 2002). Intestine 407 (Int 407), derived from a malignant small intestine of human embryo. Lewandowska *et al*. (2005) reported that Int 407 possesses some fragments of HeLa's DNA and have been used occasionally in bacterial adhesion experiments (Kapczynski *et al*. 2000; Avall-Jaaskelainen *et al*. 2003; Ingrassia *et al*. 2005).

The Mucus layer

The mucus layer covering the epithelial cells serves as an important site for colonization. It is the first point of contact between the intestinal microbiota and the host, thus the use of mucus as an *in vitro* adhesion model has been an important development. Mucin is mainly synthesized by goblet cells but other cells such as enterocytes also produce in little amounts. Immobilized human intestinal mucus glycoproteins have been used as substrata for lactobacilli and GI pathogen adherence (Ouwehand *et al*. 2001). Mucin can also be isolated from faeces (Miller and Hoskins 1981). It is supposed that mucin contains receptors resembling those of enterocytes, which causes it to be the first layer to which bacteria adhere. Of the 18 mucin-type glycoproteins expressed by humans, MUC2 is the predominant glycoprotein found in the small and large bowel mucus. The NH- and COOH-termini are not glycosylated to the same extent, but are rich in cysteine residues that form intra- and inter-molecular disulfide bonds. These glycan groups confer proteolytic resistance and hydrophilicity to the mucins, whereas the disulfide linkages form a matrix of glycoproteins that is the backbone of the mucus layer (Lievin-Le Moal and Servin 2006; Caballero-Franco *et al*. 2007; Ohland and MacNaughton 2010). The interaction of lactobacilli with mucus has been reported *in vitro* by many researchers (Tuomola *et al*. 2000; Jonsson *et al*. 2001; Martin *et al*. 2010). Several reports also suggested that lactobacilli can also promote mucus secretion as one mechanism to improve barrier function and exclusion of pathogens (Mack *et al*. 1999; Mattar *et al*. 2002).

Beneath the mucus layer, the host extracellular matrix (ECM) composed of various secreted proteins such as laminin, fibrin, heparin, fibronectin and collagen can also serve

as adhesion sites in the intestinal mucosa. Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) is a term used for a group of bacterial surface adhesins that binds to ECM molecules (Patti *et al*. 1994). Two of such adhesins, fibronectin-binding protein and collagen-binding protein, have been well characterized in lactobacilli (Toba *et al*. 1995; Buck *et al*. 2005; Munoz-Provencio *et al*. 2010). Lactobacilli adhesins can be classified according to their adhesion targets in intestinal mucosa (i.e. mucus and ECM components) (mucus-binding protein (Mub), their localization on the bacterial surface (i.e. surface layer proteins), and/or according to the way they are anchored in the bacterial surface (i.e. sortase dependent proteins). Some data is available at the genetic level as regards proteins secreted by lactobacilli having ability to adhere to mucus (Vesterlund *et al*. 2005).

The most widely used *in vivo* models for study of bacterial interaction are germ-free mice and antibiotic-treated mice. Although the bacterial species in the human GI tract are different from those in mice, it is likely that the principals involved in bacterial competition within the mouse gut hold for interactions in the human gut as well (Hao and Lee 2004).

1.7. Physiological attributes of lactobacilli

1.7.1. BSHs and lowering cholesterol levels

Hypercholesterolemia is the most important risk factor for cardiovascular diseases and is considered one of the major causes of death and disability in many countries. Previous studies have shown that a 1% reduction in serum cholesterol concentration may reduce the

risk of coronary heart disease by 2–3% (Manson *et al*. 1992). Lactic acid bacteria like lactobacilli are able to produce bile salt hydrolase (BSH), the enzyme responsible for bile salt deconjugation in the enterohepatic circulation, and liberation of free primary bile acids. Researchers found that BSH activity was able to hydrolyze conjugated glycodeoxycholic acid and taurodeoxycholic acid, leading to the deconjugation of glyco- and tauro-bile acids (Begley *et al*. 2005). Additionally, BSH activity benefits the bacterium by enhancing its resistance to conjugated bile salts and increasing its survival in the gastrointestinal tract and thus its ability to colonize it (Ridlon *et al*. 2006; Jones *et al*. 2008). BSH has been purified and biochemically characterised from several bacterial strains (Grill *et al*. 1995; Corzo and Gilliland 1999) and genes have been cloned and sequenced (Oh *et al*. 2008). In recent years, interest has arisen in the possibility of using bile salt deconjugation by *Lactobacillus* strains as a biological hypocholesterolaemic agent (Nguyen *et al*. 2007; Park *et al*. 2007). Several mechanisms for cholesterol removal by probiotics have been proposed. They are mainly: 1) Assimilation: When cultivated in the presence of bile salts, bacteria intake cholesterol from the culture (Tomaro-Duchesneau *et al*. 2014); 2) Coprecipitation: Under acidic conditions, the deconjugated bile salts co-sediment with cholesterol (Ahn *et al*. 2003); 3) Adsorption and incorporation: In this case, cholesterol is adsorbed by or incorporated by the growing cells surface (Zhao and Yang 2005); 4) microbial transformation of cholesterol to coprostanol (Lye *et al*. 2010); 5) production of short-chain fatty acids (SCFAs) during the growth of bacteria (de Preter *et al*. 2007); 6) Cholesterol oxidase activity: Cholesterol oxidase (3-β hydroxysterol oxidase, EC 1.1.3.6) catalyses the oxidation of cholesterol to 4-cholestene-3-one with the reduction of oxygen to hydrogen peroxides. Cholesterol oxidase are secreted bacterial enzymes that catalyze

the first step in the degradation of cholesterol (Ahire *et al*. 2012; Kumari and Shamsher 2015) and 7) reduction of the cholesterol absorption by host (Begley *et al*. 2006). In host, deconjugated bile salts are less efficiently reabsorbed than their conjugated counterparts, which results in the excretion of larger amounts of free bile acids in feces. Also, free bile salts are less efficient in the solubilization and absorption of lipids in the gut. Therefore, deconjugation of bile salts could lead to a reduction in serum cholesterol either by increasing the demand for cholesterol for *de novo* synthesis of bile acids to replace those lost in feces or by reducing cholesterol solubility and thereby absorption of cholesterol through the intestinal lumen.

There are many experiments that have been conducted *in vitro* or *in vivo* to investigate the cholesterol-lowering effect of lactic acid bacteria, especially strains of *Lactobacillus* and *Bifidobacterium* (Wang *et al*. 2012; Oner *et al*. 2014). For this study, lactobacilli have mostly been carried out on strains isolated from humans (Pereira and Gibson 2002), swine (Ahn *et al*. 2003), fermented milk preparations (Tanaka *et al*. 1999), and nondairy fermented product (Kumar *et al*. 2013). Ramasamy *et al*. (2010) demonstrated the deconjugation of bile salts and removal of cholesterol *in vitro* by 12 *Lactobacillus* strains isolated from chickens. Other studies have also reported the ability of *Lactobacillus* strains to assimilate cholesterol from laboratory media (Gilliland *et al*. 1985; Hosono 1999). In the 1970s, fermented milk containing a wild *Lactobacillus* strain was reported to have a hypocholesterolemic effect in humans (Mann and Spoerry 1974). Tomaro-Duchesneau *et al*. (2014) investigated 11 *Lactobacillus* strains for their potential to assimilate cholesterol in both bacterial media and under simulated gastrointestinal conditions. Sanders (1999)

proposed that the BSH activity increases the rate of excretion of free bile acids. Such mechanism could be used in controlling serum cholesterol levels by colonic microbes.

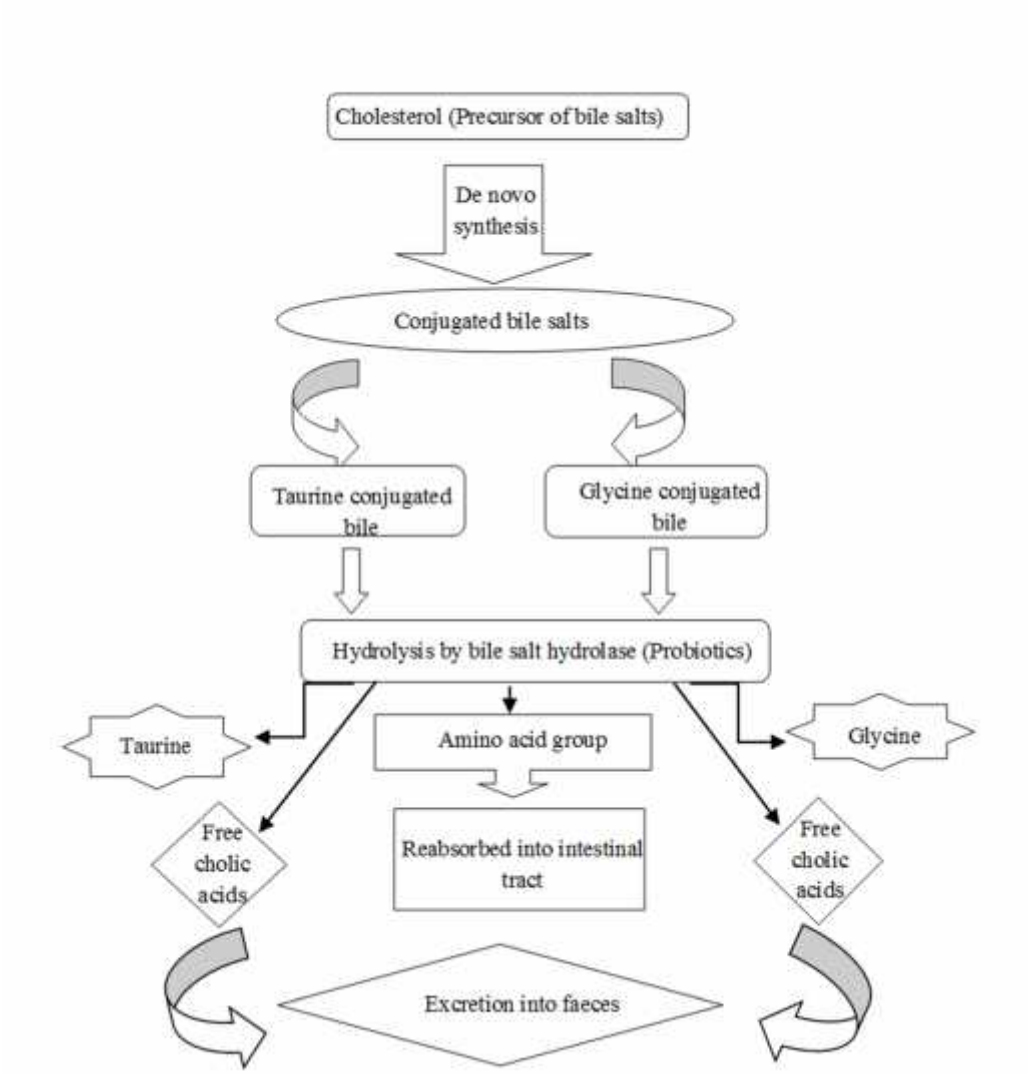

Figure 1.2. Cholesterol as the precursor for the synthesis of new bile acids and the hypocholesterolemic role of bile salt hydrolase (BSH) (Modified image, from Ooi and Liong 2010)

1.7.2. β-Galactosidase production and Lactose intolerance

The enzyme β-galactosidase, most commonly known as lactase, catalyses the breaking of β-galactosidic bond present in lactose to form glucose and galactose (Vasiljevic and Jelen 2001; He *et al*. 2008; Sharma and Singh 2014). This enzyme is widely distributed in

animals, plants and numerous microorganisms i.e., bacteria, fungi, archaea and yeast (Wallenfels and Weil 1972). β-Galactosidases work in a relatively broad pH range: enzymes from fungi act between pH 2.5–5.4, yeast and bacterial enzymes act between pH 6.0–7.0. Depending on the natural source where lactose is present, pH values range between ~ 3.5 or 5.6 of acid whey to 6.5 of milk. Among all microorganisms a large number of lactic acid bacteria are most suitable because they are Generally Regard as Safe (GRAS), and have been considered to be excellent sources of β-galactosidases. This enzyme has potential applications in food industry such as digestibility of dairy products (treatment of lactose intolerance individuals), involved to improve sweetness, solubility, and flavour (Richmond *et al*. 1981; Husain 2010). Lactose is the main source of calories from milk of all mammals. Intestinal absorption of lactose requires hydrolysis to its component monosaccharides glucose and galactose by the brush-border enzyme lactase and transported into the bloodstream. In humans, lactase expression is at its peak by birth and following the first few months of life, lactase activity starts to decrease. There is a decrease in lactase activity following weaning to undetectable levels as an outcome of the normal maturational down-regulation of lactase expression (Vesa *et al*. 2000).

Lactose intolerance is a genetically determined β-galactosidase deficiency resulting in the inability to hydrolyse lactose into the glucose and galactose. In lactose intolerance individual, undigested lactose consequently enters the colon where it is fermented by the resident microflora, resulting in symptoms including abdominal pain, bloating, diarrhoea, and flatulence (Marcon 1997). The consumption of probiotics has many beneficial effects on improving the digestion ability of humans and animals. Especially in humans, the use

of probiotics has been explored to compensate lactase in lactose intolerant individuals (Fioramonti *et al*. 2003). Among LAB, yoghurt bacteria *Lactobacillus delbrueckii*, *Lactococcus lactis* and *Streptococcus thermophilus* are the highest β-galactosidase producers (Vasiljevic and Jelen 2001; Trevan *et al*. 2004). *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842, used in the production of yogurt is capable of producing relatively high levels of intracellular β-galactosidase in comparison to other dairy cultures (Bury and Jelen 2000). Lactobacilli strains are commonly used in the industry as a probiotic. It is well known that β-galactosidase from lactic acid bacteria is an intracellular enzyme, and it is not released to the outside of cells under conventional fermentation conditions (Bury *et al*. 2001). Thermophilic sources have been found to produce thermo stable β-galactosidase. *S. thermophilus* β-galactosidase has an optimum temperature of 55 °C (Greenberg and Mahoney 1982). Fuller *et al.* (1991) suggested that *L. acidophilus* containing yogurt improves the lactose intolerance. Kinova *et al*. (2008) described the beneficial effects of *Lactobacillus* present in fermented milk products. In 1993, Schaafsmais described that consumption of yogurt containing *L. bulgaricus* and *S. thermophiles* alleviate the lactose intolerance through their enzyme β-galactosidase when the product reaches the intestinal tract (Schaafsma 1993). Masood *et al*. (2011) also suggested that lactose intolerance which can be reduced by regularly consuming fermented dairy products is due to the production of β-galactosidase enzyme by lactic acid bacteria present in them. The effectiveness of probiotic on improved utilization of lactose in lactose intolerant patients is usually monitored by hydrogen breath analysis method; a measure of hydrogen excretion in the breath is correlated with colonic fermentation and lactose maldigestion (Levitt and

Donaldson 1970). Small intestinal and/or colonic metabolism of lactose is represented in Figure 1.3.

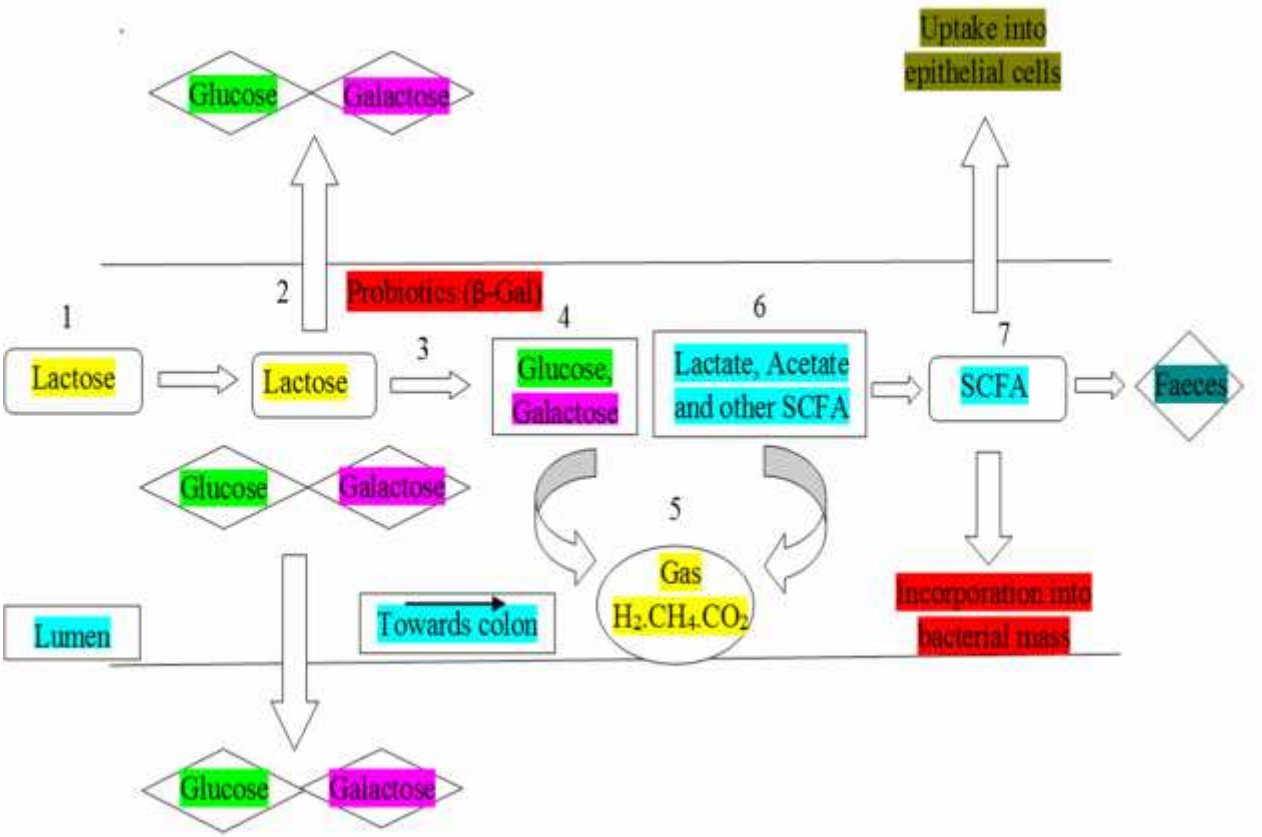

Figure 1.3. Schematic representation of small intestinal and colonic metabolism of lactose (Modified image, from Vonk *et al*. 2012). 1) Lactose enters the small intestine, 2) Lactose is then hydrolyzed by lactase/β-gal from the host or by probiotics, 3) Excess amounts of lactose spill over into the colon, 4) Lactose enters the colon and is fermented by the microbiota into glucose and galactose, 5) Gasses such as hydrogen, methane and carbondioxide are formed, 6) Lactate is also formed and converted into short chain fatty acids (SCFA), also in this stage other gasses are formed, 7) These SCFAs can be taken up by epithelial cells or can be used by the microbiota or excreted in the faeces.

1.7.3. α-Galactosidase production

Soy products have an excellent status based on their high protein content and all the essential amino acids to meet biological requirements when consumed at the recommended level of protein intake (Naganagouda and Mulimani 2006). However, soybeans as well as

other legumes characteristically contain high concentrations of the α-galacto oligosaccharides (α-GOS) such as stachyose, melibiose and raffinose as well as branched polysaccharides such as galactomannans and galactoglucomannans (Leske *et al*. 1993; Naumoff 2004). Because of intestinal disturbances caused by α-GOS, the consumption of soybean and other legumes are limited (Naumoff 2004), since mammals are deficient in the enzyme α-galactosidase (α-Gal), which hydrolyses the α-1, 6 linkages found in these sugars (Scalabrini *et al*. 1998). Alpha GOS are not digested in the upper gastrointestinal tract and reach the large intestine where they are fermented by the resident microbiota. The resulting production of fermentative gases can induce abdominal pain as well as flatulence (Messina 1999; Smiricky-Tjardes *et al*. 2003). Such negative aspects reduce the acceptability of soy products as a major human food source (Suarez *et al*. 1999).

Enzyme treatment with microbial α-galactosidase would be promising for the elimination of these oligosaccharides (Thananunkul *et al*. 1976). *Lactobacillus* species have long been used in food processing, and some of these are able to produce α-Gal (Silvestroni *et al*. 2002; LeBlanc *et al*. 2004; Carrera-Silva *et al*. 2006). Previous studies also have shown a reduction in gastrointestinal discomfort due to gas, after addition of lactobacilli to pulse and soybean meal containing diets (Kidd *et al*. 2001; Hsieh and Chou 2006; LeBlanc *et al*. 2008). LeBlanc *et al*. (2004) reported that *L. fermentum* CRL722 is able to grow on soymilk and to remove or degrade raffinose and stachyose due to its high α-Gal activity. Additionally, Farzadi *et al*. (2011) also claimed that α-galactosidase of *L. acidophilus* has ability to degrade raffinose family of oligosaccharides.

α-Galactosidase enzyme therefore has great potential in various applications like clearance of α-GOS and upgrade the nutrition of legume food (Thananunkul *et al*. 1976), production of sugars (Linden 1982). Fabry's disease of human is due to a deficiency of thermolabile lysosomal α-galactosidase A, and consuming this enzyme prevents the disease (Ulezlo *et al*. 1981). Type B erythrocytes, which contain 3-o-α-Dgalactopyranoside, can be transformed into type O erythrocytes by exposure to α-galactosidase (Tzortzis *et al*. 2003). Alpha galactosidase may be used in the future for such additional medical purposes as enzymotherapy.

1.8. Immunomodulation

Intestinal microflora and probiotics, accordingly, may influence the immune mechanisms of the host by effects on mucosal barrier mechanisms and on the functional maturation of the immune system. The primary effector arm of the immune system is the so-called innate immune system, which includes non-specific immune protection mediated by monocytes, macrophages, neutrophils and dendritic cells. The cells of the innate immune system have an important role as antigen presenting cells. The innate immune system further regulates the function of the antigen-specific adaptive immune system, such as the functional balance of immune response related to cytokine and chemokine profiles. Defective maturation of immune competence in association with poor microbial stimuli may thus be lead to dysregulation of both innate and adaptive immune systems.

Lactobacilli can elicit innate and adaptive immune responses in the host via binding to pattern recognition receptors (PRR) expressed on immune cells and intestinal epithelium.

Microbe-associated molecular patterns (MAMPs) are conserved molecular structures which is recognized by PRR and signal to induce the production of cytokines, chemokines and other innate effectors (Abreu 2010; Kawai and Akira 2010; Wells *et al*. 2010). PRRs can be divided into three families: i) Membrane bound PRRs includes Toll-like receptors (TLRs) and C-type lectin receptors (CLR) such as mannose and scavenger receptors, ii) Cytoplasmic PRRs includes retinoic acid inducible gene I (RIG-I)-like receptors and nucleotide oligomerization domain-like (NOD) receptors (NLR) and iii) Secreted PRRs includes Complement receptors, collectins, C-reactive protein, etc. TLRs are the best characterized family and around 10 TLRs have been identified in humans (Kawai and Akira 2010). TLR and NLR both have been shown that on MAMP binding they initiate the signalling cascade like mitogen-activated protein kinase (MAPK) pathway and the nuclear factor κB (NF-κB) pathway (Janssens and Beyaert 2002). Each PRR recognizes a specific molecular pattern and can be expressed on the cell surface, in intracellular compartments or in the cytosol.

PRR signalling pathways play a key role in both the innate and adaptive immune responses, by influencing the skewing of naïve T cells, the regulation of regulatory T cells and activation of antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages. DCs are specialized APCs, found throughout the lamina propria of the intestine as well as in the gut-associated lymphoid tissues such as the Peyer's patches and regulate both innate and adaptive immunity (Rescigno 2010). Activated DCs express high levels of MHC, co-stimulatory molecules and cytokines required for antigen presentation and T cell activation, clonal expansion and differentiation (Kapsenberg 2003). Different

strains and species of lactobacilli can differentially modulate the immune response through the stimuli passed (Wells 2011).

Intestinal epithelial cells also express a range of PRR to sense the presence of microbes. Upon activation, they produce a broad range of cytokines and chemokines including interleukins (ILs), tumour necrosis factor (TNF), growth factors and inducible small antimicrobial peptides called beta-defensins (BDs) (Cario and Podolsky 2000). Goblet and enterocytes of the intestinal epithelium cells (IEC) also express a range of PRR to sense the presence of microbes.

The human innate immune system is also comprised of various immune cells (i.e. monocytes/macrophages, neutrophils, and dendritic cells (DCs)). *Lactobacillus* can also be able to reverse an immunological responses by activating several signalling pathways and polarizing M1 macrophages to M2 macrophages (Jang *et al*. 2013). Monocytes and macrophages (MΦ) play a crucial role in innate and adaptative immunity in response to microorganisms and are major mediators of the inflammatory response. Monocytes/macrophages (MΦ), differentiate into either a pro-inflammatory (M1) subtype, also known as a classically activated subtype, or an anti-inflammatory alternatively activated subtype (M2) depend on their microenvironment, especially in relation to growth factors and cytokines (Jaguin *et al*. 2013; Wang *et al*. 2014). Among these factors, granulocyte–macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) are major ones, notably implicated in the differentiation of MΦ from monocytes. GM-CSF and M-CSF-generated MΦ share some common recognition

patterns, but also differently express some gene markers (Akagawa 2002; Akagawa *et al*. 2006). Another level of heterogeneity between MΦ is related to their activation/polarization status. Classical M1 and alternative M2 activation of macrophages, show a reflection of Th1–Th2 polarization of T cells (Wang *et al*. 2014). M1 and M2-type responses describe the opposing activities, M1-type macrophages release cytokines that inhibit the proliferation of surrounding cells and damage contiguous tissue, and M2-type macrophages release cytokines that promote the proliferation of contiguous cells and tissue repair. M1 phenotype is stimulated by microbial products or pro-inflammatory cytokines (IFN-g, TNF-α, IL-1, IL-6, IL-12, CXCL1-3, CXCL-5, and CXCL8-10 or Toll-like receptor (TLR) ligands) (Sica and Mantovani 2012), and the typical characteristics of M1 macrophages include high antigen presentation, high production of IL-12 and IL-23, and high production of nitric oxide (NO) and reactive oxygen intermediates (ROI) (Verreck *et al*. 2004). In contrast, M2-type responses are the "resting" phenotype, such responses can also be further amplified by IL4, IL-10, or IL-13. M2 macrophages are characterized by the upregulation of DC, mannose receptor, scavenger receptor A, scavenger receptor B-1, CD163, CCR2, CXCR1, and CXCR2 (Martinez *et al*. 2009). M2 macrophages produce ornithine and polyamines through the arginase pathway (Gordon and Martinez 2010), instead of generating NO or ROI.

IRF/STAT signaling is a central pathway in controlling macrophage M1–M2 polarization. Activation of IRF/STAT signaling pathways by IFNs and TLR signaling will skew macrophage function toward the M1 phenotype and M2 phenotype (Sica and Mantovani 2012). A predominance of NF-kB and STAT1 activation promotes M1 macrophage

polarization, resulting in cytotoxic and tissue-damage pro inflammatory functions. However, a predominance of STAT3 and STAT6 activation by IL-4/13 and IL-10 increases M2 macrophage polarization, associated with immune tolerance and tissue repairing (Nelms *et al*. 1999). IL-10 promotes M2 polarization through the induction of p50 NF-kB homodimer, c-Maf, and STAT3 activities (Park-Min *et al*. 2005). STAT mediated activation of macrophages is regulated by members of the suppressor of cytokine signaling (SOCS) family. SOCS family members are inducible inhibitors of cytokine signals and thus play a critical role in limiting inflammation responses. For example, IL-4 and IFN-g, the latter in concert with TLR stimulation, upregulate SOCS1 (Whyte *et al*. 2011) and SOCS3 (Liu *et al*. 2008), which in turn, inhibit the action of STAT1 and STAT3, respectively. M1– M2 polarization of macrophage is a tightly controlled process involving a set of signaling pathways, transcriptional and posttranscriptional regulatory networks. An imbalance of macrophage M1-M2 polarization is often associated with various diseases or inflammatory conditions. Polarization of M1 and M2 macrophages given in figure 1.4.

Polymorphonuclear neutrophils (PMNs) are highly motile phagocytic cells that also constitute the first line of defense of the innate immune system. Neutrophils express a vast repertoire of PRRs, including all members of the Toll-like receptor (TLR) family with the exception of TLR3; the C-type lectin receptors dectin 1 (CLEC7A) and CLEC2 (CLEC1B); and cytoplasmic sensors of ribonucleic acids (RIG-I and MDA5) (Hayashi *et al*. 2003; Tamassia *et al*. 2008; Greenblatt *et al*. 2010). In addition, neutrophils also express NOD1, although the expression and function of the NOD-like receptors (NLRs). Earlier study reported that lipopolysaccharide (LPS) and serum amyloid A have been Induce high

levels of IL-10 by human neutrophils (de Santo *et al*. 2010). Interestingly, the crosstalk between human neutrophils and NK cells is reciprocal, as culture of neutrophils with NK cells or NK cell-derived soluble factors (such as GM-CSF and IFNγ) promotes neutrophil survival, expression of activation markers, ROI production and cytokine synthesis (Costantini and Cassatella 2011). Human neutrophils can also crosstalk with B cells and T cell. Thus, in response to different signals neutrophils express a vast and diverse repertoire of cytokines that are crucial to the role of neutrophils in innate and adaptive immune responses and to their role in defense and pathology.

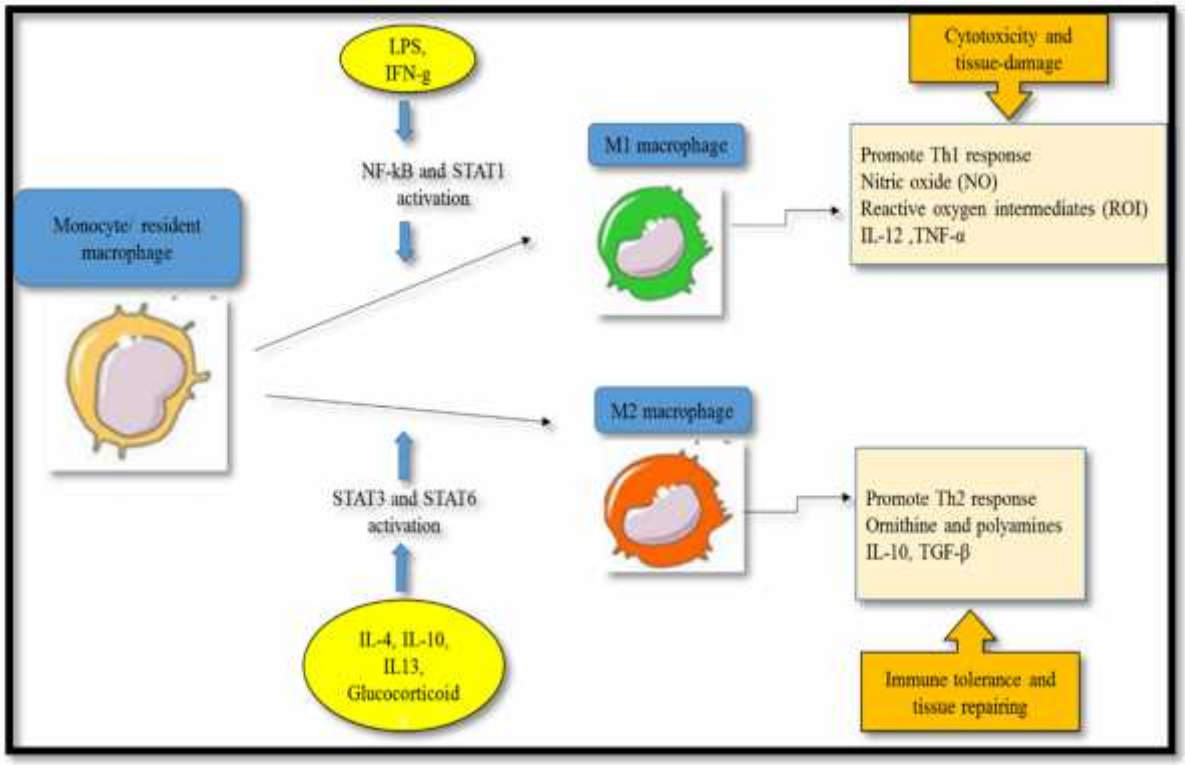

Figure 1.4. Diagram showing differentiation of monocytes to M1 and M2 macrophages.

Lactobacilli factors involved in immunomodulation

Cell wall components of the lactobacilli can potentially bind to TLR2 in combination with TLR6 and initiate signal. Bacterial lipoproteins were shown to recognize TLR2 (Aliprantis *et al*. 1999; Brightbill *et al*. 1999). Anionic properties of the wall teichoic acids (WTA) and lipo teichoic acids (LTA) may also generate immune signalling through their binding to scavenger receptors such as SRA which has been shown to bind purified LTA (Dunne *et al*. 1994). In cytoplasmic membrane of gram positive bacteria, lipid chain of LTA also binds to TLR2. However, the WTA are covalently attached to the peptidoglycan and presumably cannot signal through TLR2/6 as they lack the lipid anchor.

Both the techoeic acids, LTAs and WTAs are anionic through the D-ala substitution by the enzymes of dlt operon. A *Dlt*- mutant of *L. plantarum* strain NCIMB8826 showed much less incorporation of D-Ala in its TAs than the wild type strain. This defect significantly affected the immunomodulatory properties of the bacterial strain, by reduction in the secretion of proinflammatory cytokines by peripheral blood mononuclear cells and monocytes compared with the wild type strain (Grangette *et al*. 2005). In contrast, *dltD* mutation in *L. rhamnosus* did not show altered immunomodulation in epithelial cells and PBMCs in comparison to the wild type strain. The reasons for this differences are not clear but it may be related to species and strain dependent differences in LTA and WTA composition. For example the cell wall of *L. rhamnosus* and *L. casei* appears to contain only LTA in contrast to other lactobacilli which also contain WTA as well (Velez *et al*. 2007).

The innate immune system of human has evolved specific mechanisms to recognize bacterial DNA like *Lactobacillus* genomic DNA. TLR9 binds bacterial Cytosine-phosphate-guanine (CpG motifs) DNA (Stacey *et al*. 2003). In polarized epithelial cells basolateral TLR9 activate the NF-κB pathway, after stimulation with synthetic unmethylated CpG oligonucleotide mimics (CpG-ODN) while apical stimulation prevented NF-κB activation (Lee *et al*. 2006). *Lactobacillus* cell wall peptidoglycan (PGNpolymer) can activate NF-κB through human Toll-like receptors 2 (TLR2) (Volz *et al*. 2010). Peptidoglycan fragments are recognized by the NOD1 and NOD2 receptors of the NLR family of PRR. (Girardin *et al*. 2003a; 2003b). In addition to PGN and teichoic acids, exopolysaccharides (EPSs) are also commonly found within the cell wall of lactobacilli. EPSs and other cell wall polysaccharides are recognized by C-type lectin receptors (CLRs) that are involved in the recognition and capture of antigens by antigen presenting cells such as dendritic cells and macrophages.

As whole bacteria will most likely not enter the blood stream in large numbers. Instead, bacterial metabolites have been shown to cross the epithelial barrier, retain their bioactive properties, and affect peripheral immunity *in vitro* and *in vivo* (Ménard *et al*. 2004; Arpaia *et al*. 2013; Ashraf *et al*. 2014). Fong *et al*. (2016) suggests that LGG soluble factors exert similar immunomodulatory effects as the intact cells on PBMCs. Additionally, Johansson *et al*. (2016) demonstrate that soluble factors, including enterotoxins of two lactobacilli strains, *L. rhamnosus* GG (LGG) and *L.* reuteri DSM 17938, were also able to directly dampen *S. aureus* induced lymphocyte-activation *in vitro*.

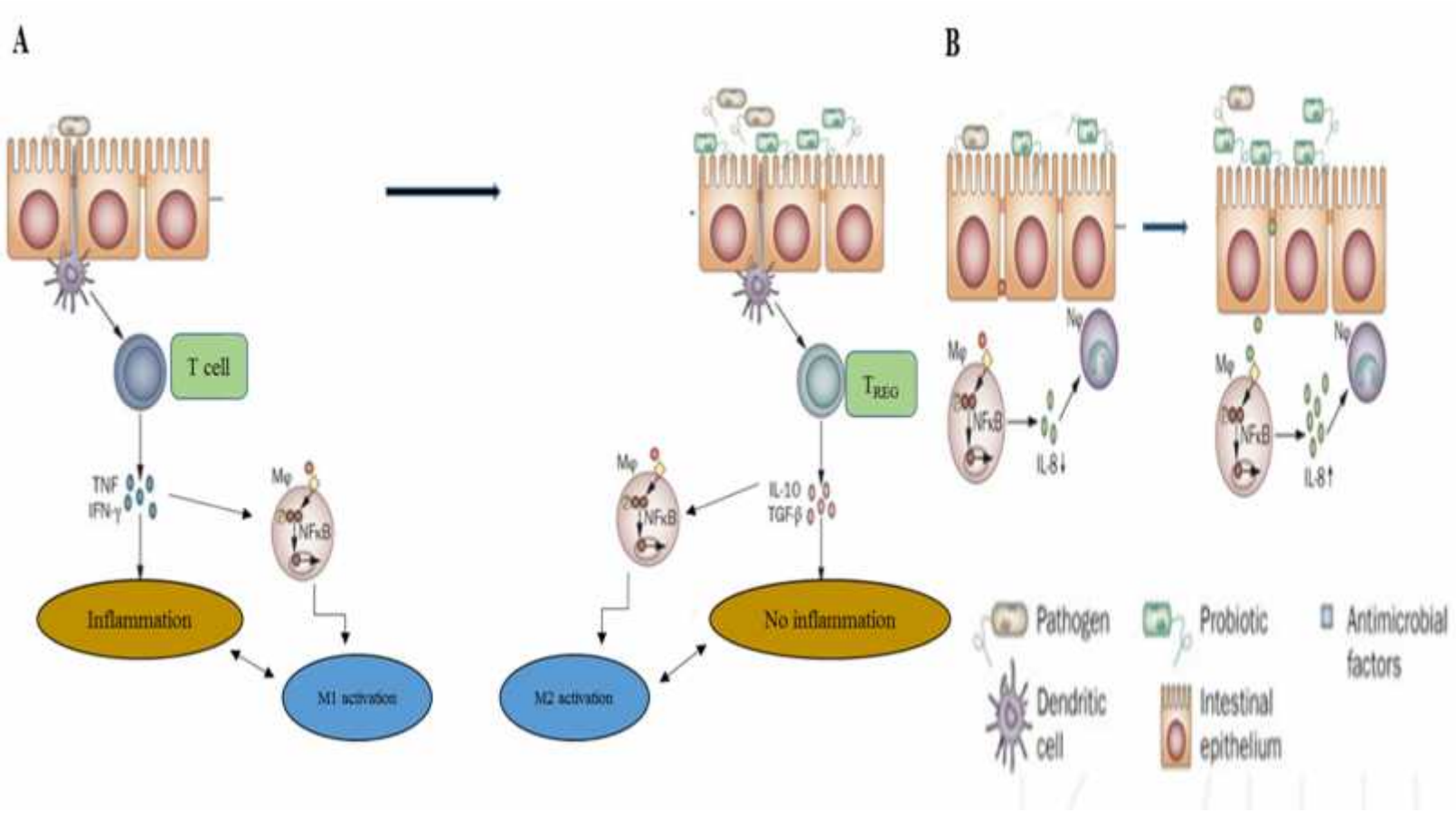

Figure 1.5. Schematic representation of potential mechanisms of action of probiotic lactobacilli (Modified image, from Harzallah and Belhadj 2013). A) Lactobacilli secrete antimicrobial peptides, which then travel to mesenteric lymph nodes and stimulate the immune system by signaling dendritic cells, and lead to the induction of T_{REG} cells and the production of anti-inflammatory cytokines, including IL-10 and TGF-β, these cytokine increases M2 macrophage polarization, while pathogen leads secretion of pro-inflammatory cytokines including TNF-α and INF-γ which increases M1 macrophage polarization. B) Lactobacilli may also trigger an innate immune response by initiating TNF production by epithelial cells and inhibiting (or activitating) NF-κB in Mφ and dampening (or priming) the host immune response by influencing the production of IL-8 and subsequent recruitment of Nφ to sites of intestinal injury. Abbreviations: Mφ, macrophage; Nφ, neutrophil; T_{REG} cell, regulatory T cell.

In vitro immunomodulation by lactobacilli

Lactobacilli mediate their immune-modulatory effects through the induction of regulatory cytokines, such as IL-10 (de Moreno de Leblanc *et al*. 2011), induction of T regulatory cells (Smelt *et al*. 2012; Liu *et al*. 2013), modulation of APC (Haileselassie *et al*. 2016), promotion of epithelial function and development (Yan *et al*. 2017) and by inhibition of pro-inflammatory cytokines (Kim *et al*. 2006). Lactobacilli and different types of immune cells such as human monocytic cell line THP-1, human monocyte derived DCs, human PBMCs and mouse bone marrow derived DCs have been used to evaluate the immunomodulatory potential under different *in vitro* co-culture systems (Meijerink and Wells 2010). IL-10 expression is typically measured in most reports because it is an anti-inflammatory cytokine that suppresses IL-12 production and subsequently IFN-g production, thus favouring a T-helper 2 (Th2) or a regulatory T cell (Treg) response. In addition, IL10 down-regulates antigen presentation and inhibits the activation of proinflammatory cytokines and chemokines. Three studies have shown that the ability of different lactobacilli to induce a high ratio of IL-10/IL-12 or IL-10/TNF-α production from immune cells correlates with their capacity to protect TNBS induced colitis in mice and rats (Peran *et al*. 2005; Foligne *et al*. 2007; Zoumpopoulou *et al*. 2009). Several studies have also shown that, differential immunomodulation such as cytokine responses of human PBMC and DC by lactobacilli depends on both the species and the strain (Miettinen *et al*. 1998; Christensen *et al*. 2002; Meijerink *et al*. 2010; van Hemert *et al*. 2010). Peña and Versalovic (2003) reported that secretory molecules of *L. rhamnosus* GG decreases TNF-α production in LPS-activated murine macrophages cell line RAW 264.7. Several lactobacilli strains can modulate stimulated responses by peripheral blood mononuclear

cells (PBMCs) *in vitro* (Pochard *et al*. 2002; Ghadimi *et al.* 2008). *Lactobacillus* species were also shown to exert immuno enhancing effects on the phagocytic activity of monocytes and PMN cells through the induction of chemokine and cytokine production (Vaarala 2003).

1.9. Beneficial role of probiotics in human health and disease

First antibiotic was discovered in 1950s to treat infections/diseases or sometimes as a growth stimulants (for animals). But later on the use of antibiotics has revealed that many of them have side effects on one or more organs and hence frequent use can be fatal. On the other development of multidrug resistant pathogens have lead people to think an alternate for antibiotics. The search for an alternative is completed by the discovery of probiotics, which provides an effective and attractive alternate to above mentioned problems (Fuller 1989). Fioramonti *et al.* (2003) reported that secreted antimicrobial molecules from probiotics eliminate the pathogens, thus reducing the chances of the emergence of pathogens resistant. Re-establishment of the normal gut microflora is required post antibiotic therapy, however the use of probiotics eliminated this problem (Fuller 1989; Rolfe 2000). There are number of scientific literatures available which suggests the benefits of probiotics on the human health, including improvement of intestinal health, enhancement of the immune response, reduction of serum cholesterol, production of antimicrobial compound and cancer prevention. In fact, there is substantial evidence to support probiotic use in the treatment of acute diarrhoeal diseases, prevention of antibiotic-associated diarrhoea, and improvement of lactose metabolism. These health properties are strain specific.

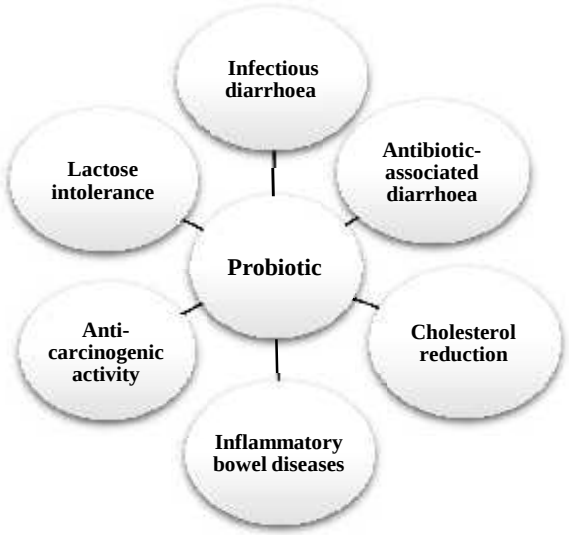

Figure 1.6. Prospective health attributes of probiotics.

Infectious diarrhoea

Intestinal infectious diseases caused by the pathogens like *Escherichia coli, Vibrio cholerae, Shigella* spp., *Campylobacter* spp., *Clostridium difficile* and rotavirus are the main cause of death in most developing countries (Nomoto 2005). On the other hand Glass *et al.* (1991) reported that around 16.5 million children of less than 5 years of age have approximately 21 to 37 million episodes of diarrhoea annually in countries like the USA. Rota viral diarrhoea is more prevalent amongst children aged between 6 to 24 months and this is characterized by vomiting and watery stool. It can be treated by fluid replacements which counteract dehydration and nutritional deficiency. Different researchers have reported the efficacy of different probiotics to treat rotaviral diarrhoea (Isolauri *et al.* 1991; Shornikova *et al.* 1997). Isolauri *et al.* (1991) reported that *L. rhamnosus* GG when administered in the form of fermented milk or freeze-dried powder can effectively shorten the course of acute rotavirus diarrhoea in children aged 4-45 months compared to the placebo group. Similar results were reported by Shornikova *et al.* (1997), when *L. reuteri*

was consumed by patients aged between 6-36 months. *Shigella dysenteriae* 1 is a causative agent for shigellosis which is a highly contagious infection and is characterized by diarrhoea, fever, vomiting, blood, pus or mucus in stools (Lindberg and Pal 1993). Animal studies provided the valuable information to understand the pathogenesis of the disease and also the effect of probiotics on it. Moorthy *et al.* (2007) reported the pre-treatment with probiotics like *L. rhamnosus* and *L. acidophilus* exhibited a protective role with reduced inflammation along with protection from *S. dysenteriae* 1 infection in rats.

Another gram-negative pathogenic bacterium which is frequently isolated from animal faeces is i.e. *Campylobacter jejuni.* It causes gut infection which results into gastroenteritis and characterized by abdominal pain, diarrhoea and fever. Food contaminated with *C. jejuni* is commonest causes of diarrhoea for human. *Bifidobacterium breves* was evaluated for the treatment of *Campylobacter* enteritis in 133 patients aged between 6 months to 15 years. Tojo *et al.* (1987) reported that this strain did not shorten the duration of diarrhoea but its administration successfully eradicated *C. jejuni* from faeces and restored the normal intestinal flora. Later on Chang and Chen (2000) reported that the mixtures of four lactobacilli (*L. acidophilus*, *L. fermenentum*, *L. crispatus*, and *L. brevis*) completely eradicated *C. jejuni* in simulated chicken digestive system. An antagonistic effect of lactobacilli on *E. coli* is routinely used as indicator of antimicrobial activity for selection of potential probiotic strains (Cole and Fuller 1984). They have also reported that the administration of fermented milk with *L. salivarius* decreased the number of coliforms including *E. coli* in the gut of new born rats. Similarly other researchers also reported same results with strains of *L. acidophilus* and *L. lactis* in new born pigs (Kohler and Bohl 1964;

Muralidhara *et al.* 1977). People travelling from developed countries to developing countries and generally acquired by traveller's diarrhoea which occurred due to ingestion of contaminated food or water. 80-85% of these cases have shown the infection with bacterial pathogens such as enterotoxigenic *E. coli* (ETEC) and *C. jejuni* (Hill 2000; Yates 2005). Furthermore, numerous animal studies have indicated an inhibitory effect of probiotics against enteropathogens mainly through the production of bacteriocins (Moslehi-Jenabian *et al*. 2011).

Antibiotic-associated diarrhoea

Antibiotics are used to kill off harmful bacteria that cause infections in the human body. Unfortunately, the good bacteria in the gut are often also killed as well, which can lead to antibiotic-associated diarrhoea. *Clostridium difficile* is a Gram-positive bacterium which produces toxin that causes colitis. This can be cured using antibiotics like metronidazole and vancomycin but it can reoccur in some cases (Surawicz 2003). The reason behind reoccurrence is still not clearly known but the production of *Clostridium* spores are considered to be key factor behind it (McFarland *et al*. 2002). *Lactobacillus* species like *L. delbrueckii* ssp. *bulgaricus* B-30892 showed elimination of *C. difficile* mediated cytotoxicity, using Caco-2 cells as a model (Banerjee *et al*. 2009). Earlier clinical studies showed that *L. rhamnosus* GG cured patients from *C. difficile* associated diarrhoea when used as adjunct therapy (Gorbach *et al*. 1987; Biller *et al*. 1995). Nomoto (2005) observed that prolonged treatment with antibiotics like clindamycin, cephalosporin and penicillin disturbed the endogenous gut microflora and in turn facilitates abnormal proliferation of opportunistic enteropathogens. This imbalance causes diarrhoea which is commonly

known as antibiotic associated diarrhoea and occurs in about 20% of treated patients (Nomoto 2005). This can be treated by administration of *L. rhamnosus* GG, *B. longum* and *E. faecium* SF68 in patients on antibiotic treatment. A significant decrease in the incidence of antibiotic associated diarrhoea was observed in the double-blind placebo controlled trials with probiotic cultures (Colombel *et al.* 1987; Buydens and Debeuckelaere 1996; Pant *et al.* 1996). When *S. boulardii* was concomitantly consumed with standard antibiotic therapy using vancomycin, the double blind placebo controlled clinical studies showed that the recurrence of the disease was considerably reduced compared to the placebo group (McFarland *et al*. 1995). The mechanism of action of probiotic is not well understood but it was observed that it produces proteolytic enzymes which digests toxin A or B of the pathogen and hence prevents adsorption of the toxin to receptors on the intestinal mucosa (McFarland *et al*. 1995). McFarland (2007) reported a supplement containing probiotic strains *S. boulardii* and a mixture of *L. acidophilus* and *B. bifidum* prevented the disease in the 12 controlled clinical trials.

Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are chronic conditions that are characterized by abdominal pain and other gastrointestinal symptoms including bloating and flatulence in the absence of structural abnormalities in the intestine. Human gastrointestinal barrier is mainly made up of several physical components (epithelial layer with mucus) and functional components (gut associated immune cells) (Fioramonti *et al.* 2003) which plays a role in restricting colonization of pathogens, systemic translocation of antigens and in eliciting antigen specific immune response (Sanderson and Walker 1993). However this

may get disturbed in certain conditions and leads to an inflammation due to reduction in gut microbial diversity and lost in positive host-microbial interaction. This can be healed/counteract by several lactobacilli by stabilization of normal microbiota during infection, degradation of antigens, re-establish these interactions via restoring the gut microbiota and reduction in production of inflammatory mediators (Isolauri *et al.* 2002). Due to this reasons, lactobacilli are being used as a therapeutic agent for the treatment of inflammatory bowel diseases (IBD) such as irritable bowel syndrome (IBS), Crohn's disease and ulcerative colitis (Schultz and Sartor 2000; Isolauri *et al.* 2002). However the reasons behind these diseases is not completely known, but studies indicate that the genetic predisposition and gut microbiota are thought to play an important role. Research showed that not only lactobacilli *L. salivarius* UCC118 and *L. rhamnosus* GG, but also *S. Cerevisiae* (*boulardii*) and a strain of *E. coli* (Nissle) were effective in alleviating the symptoms of IBD (Mattila-Sandholm *et al.* 1999; Gupta *et al.* 2000; Guslandi *et al.* 2000; Jonkers *et al.* 2012). Lactic acid bacteria may improve intestinal mobility and relieve constipation possibly through a reduction in gut pH (Mallett *et al*. 1989; Leopold and Eikeler 2000).

Anti-carcinogenic activity

Enteric bacteria sometimes produces certain enzymes like β-glucuronidase, β-glucosidase, azoreductase and nitroreductase, which are involved in the production of colon carcinogens from innocuous complexes. Whereas many probiotic bacteria exhibited the anti-carcinogenic properties, which by suppressing the bacteria that produce these enzymes and also they degrade the produced carcinogens in the gastrointestinal tract (Rafter 1995).

Additionally, *L. acidophilus* and *L. casei* supplementation in humans helped to decrease the levels of these enzymes (Lidbeck *et al*. 1991). While in mice, *Lactobacillus* GG suppressed these bacterial enzymes (Drisko *et al*. 2003). Goldin *et al.* (1992) and Bouhnik *et al.* (1996) reported the reduction in incidence and number of tumours in rate, supplemented with *L. rhamnosus* GG and *Bifidobacterium longum,* respectively. Bogdanov *et al.* (1962) reported the anti-carcinogenic properties of *L. bulgaricus* for the first time. N-nitrosamine is carcinogen, produced as a result of enzyme nitroreductase and is readily degraded by intestinal bacteria including *Lactobacillus* spp. (Rowland and Grasso 1975). Goldin and Gorbach (1984) suggested that diet and antibiotics can lower the generation of carcinogens in the colon and reduce chemically induced tumors. Another study revealed that there was decrease in production of enzyme in human subjects post regular consumption of fermented milk product prepared from *L. acidophilus.* Oral administration of *L. casei* has been shown to effectively reduce DNA damage, induced by chemical carcinogens, in gastric and colonic mucosa in rats (Li and Li 2003). Aso *et al*. (1992; 1994) suggested that the consumption of *L. casei* might delay the recurrence of bladder tumors. Pool-Zobel *et al*. (1993) observed that when *L. acidophilus* was heated, it resulted in loss of their anti-carcinogenic activity and this indicated the role of heat-labile molecules in anti-carcinogenic activity.

Under the present study, the isolation of lactobacilli was aimed from human gut origin in search of identifying novel probiotic with significant probiotic attributes for human consumption. In order to establish as probiotic, the isolates must possess probiotic properties such as acid and bile salts tolerance, antimicrobial activity towards pathogens,

resistance to antibiotics, adhesion ability and antagonistic effect on adhesion of pathogens to intestinal epithelial cells. To mimic *in vivo* conditions, the different adhesion assays were designed with lactobacilli and an enteropathogen. Additionally, *Lactobacillus* isolates have been studied for their possible application in human health and disease with regard to their ability to combat lactose intolerance, to remove cholesterol and potential to improve legume oligosaccharide digestibility. The most probiotic effects are achieved by immunomodulation ability of lactobacilli. Thus, the study of lactobacilli isolates to modulate the array of cytokines and chemokines was undertaken. In order to achieve this, the objectives of this study were the following:

Objectives:

1. Isolation, identification and characterization of *Lactobacillus* strains from human sources.
2. Study production of β-galactosidase and α-galactosidase and cholesterol removal by *Lactobacillus* strains.
3. Study immunomodulating potential of *Lactobacillus* strains on PBMCs, PMNs and macrophages.