Probiotic characteristics of *Lactobacillus acidophilus* and *Lactobacillus paracasei* and their effects on immune response and gene expression in mice

By

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Gunaranjan Paturi
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List of Publications

Journal Papers


2. Paturi G., Phillips, M., Kailasapathy, K. Effect of probiotic strains *Lactobacillus acidophilus* LAFTI L10 and *Lactobacillus paracasei* LAFTI L26 on systemic immune functions and bacterial translocation in mice. Journal of Food Protection (*Manuscript accepted*).


4. Paturi G., Phillips, M., Kailasapathy, K. Microarray analysis of *Lactobacillus acidophilus* LAFTI L10 induced gene expression in the small bowel of mice. (*Manuscript submitted*).

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List of Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally Regarded As Safe</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable Bowel Syndrome</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>MRS</td>
<td>de Man, Rogosa and Sharpe agar or broth</td>
</tr>
<tr>
<td>NGYC</td>
<td>Non-fat skim milk, glucose, yeast extract and cysteine medium</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer (Cell)</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>WC</td>
<td>Wilkins-Chalgren agar</td>
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ABSTRACT

Probiotic bacteria such as Lactobacillus and Bifidobacterium species are normal inhabitants of healthy gastrointestinal (GI) tract, which may promote beneficial effects on host through limiting the growth of undesirable microorganisms and modulating the immune system. In the present study, Lactobacillus and Bifidobacterium strains were screened for their in vitro acid and bile tolerance, autoaggregation, coaggregation and hydrophobic abilities to identify potential probiotic bacteria. Lactobacillus acidophilus LAFTI L10 and Lactobacillus paracasei LAFTI L26 were selected based on their overall tolerance to in vitro acidic conditions to further investigate their influence on various immune functions and gene expression in mice.

Immunofluorescent analysis of small intestine in mice fed with L. acidophilus or L. paracasei demonstrated an increase of immunoglobulin (Ig)-A, interleukin (IL)-10 and interferon (IFN)-γ producing cells compared to control mice. In systemic immune response, proliferative responses of splenocytes stimulated with mitogens, concanavalin A (ConA) and lipopolysaccharide (LPS) showed differences between L. acidophilus and L. paracasei strains. In comparison to control mice, IL-10 and IFN-γ levels in blood serum and splenocytes stimulated with ConA were enhanced in mice fed with either L. acidophilus or L. paracasei. Increased phagocytic activity of peritoneal macrophages against Candida albicans was determined in mice fed with L. acidophilus or L. paracasei. Translocation of Lactobacillus spp. and total anaerobes to Peyer’s patches as well as mesenteric lymph nodes were modulated in L. acidophilus or L. paracasei-fed mice. Furthermore, there was no bacterial
translocation to spleen, liver or blood in mice fed with *L. acidophilus* or *L. paracasei*.

In cholera toxin (CT) mice model, *L. acidophilus*-fed mice increased the nitric oxide (NO) levels in serum, whereas *L. paracasei*-fed mice enhanced the NO levels in serum and intestinal fluid. Mice fed with *L. acidophilus* or *L. paracasei* showed an increased IgA response to CT in intestinal fluid and serum compared to control mice. The analysis of cytokine producing cells in small intestine of *L. acidophilus* or *L. paracasei*-fed mice showed the regulation of Th1 and Th2 cytokines such as IFN-γ, tumor necrosis factor (TNF)-α, IL-4, IL-6 and IL-10.

Microarray analysis of *L. acidophilus* induced gene expression in small intestine of normal mice demonstrated an up- and down-regulation of 27 and 35 genes, which are involved in cell-cell signalling, cell growth and proliferation, cell death and various metabolic functions. In CT mice model, *L. acidophilus* influenced an up- and down-regulation of 28 and 32 genes in small intestine that are involved in cell functions, drug metabolism and immune responses.

In summary, *L. acidophilus* and *L. paracasei* showed tolerance to various gastric conditions and bile salts. *Lactobacillus acidophilus* and *L. paracasei* enhanced gut and systemic immune functions, particularly non-specific and specific immune responses in normal and CT mice. Moreover, *L. acidophilus* regulated the genes involved in various biological functions in small bowel of normal and CT mice, which provided a basis in understanding the pathways through which these bacteria are beneficial to the host.
CHAPTER 1

Introduction
1.1. GUT MICROFLORA

The gastrointestinal (GI) tract of human consists of diversified microflora ranging between 300 - 500 bacterial species. The microflora of GI tract is about 10 times higher than eukaryotic cells in the human body (Simon and Gorbach, 1986; Bengmark, 1998). In a healthy host, enteric bacteria that were acquired from birth colonise the alimentary tract and their composition remains mostly constant thereafter. Aerobic and facultative microorganisms such as coliforms, lactobacilli and streptococci are first to colonise the GI tract, which are considered to be important in reducing oxidation process in the intestine that lead to the colonisation of anaerobic bacteria such as *Bacteroides* and *Bifidobacterium* species.

The consumption of solid food gradually transforms the intestinal microflora into stable microbial communities in adults, where anaerobic microbes prevail (Fig. 1.1). The microflora in adult GI tract comprises of 99.9% obligate anaerobes, where gastric secretions and rapid motility in the upper small intestine restrict them to $10^3$ organisms per millilitre of luminal fluids at this site and between $10^{11} - 10^{12}$ organisms per gram or millilitre of colon contents (Xu and Gordon, 2003; Backhed et al., 2005; Fig. 1.2A).

Bacterial composition in GI tract differ based on anatomical properties of the interacting sites and other conditions such as pH, oxygen availability and transit time of food contents. Bacterial species belonging to genera *Bacteroides*, *Bifidobacterium*, *Fusobacterium*, *Butyrivibrio*, *Clostridium*, *Eubacterium* and *Lactobacillus* are some of the resident microflora in GI tract. Predominantly, bifidobacteria and lactobacilli
represents 90% of the bacterial population, whereas less than 0.01% consists of diversified bacterial population such as pathogenic microorganisms (Tournut, 1993).
Figure 1.1. Development of human and murine gastrointestinal ecosystems. Abbreviations in the figure: IELs - intraepithelial lymphocytes; LP - lamina propria; slgM - surface immunoglobulin M; slgA - surface immunoglobulin A; TCR - T-cell receptor. (Adapted from McCracken and Lorenz, 2001).
The intestinal microflora influence a wide variety of host functions such as synthesis of biotin, folate, vitamin K and conversion of unabsorbed dietary sugars into short-chain fatty acids, which can be used as an energy source by colonic mucosa (O’Hara and Shanahan, 2006; Fig. 1.2B). The ability of commensal microflora differs in promoting the development of gut-associated lymphoid tissues (GALT). However, commensal microflora play an important role in maintaining immune homeostasis in developing and adult gut. Fermentation processes of colonic microflora enable to salvage energy from food components that have not been digested in the upper GI tract. Gibson et al. (1999) suggested that colonic bacterial fermentation provides approximately 7% - 8% of total dietary energy that was required daily by the host. Backhed et al. (2004) demonstrated that germ-free mice require a higher uptake of calories to maintain normal body mass in comparison to normal mice.

Gut microflora also interacts with the host immune system in order to protect from pathogenic bacteria and for the overall development and function of the GI tract (Fig. 1.2B). Several gnotobiotic studies demonstrated the beneficial role of microflora in influencing the physiological functions of gut. In comparison to normal animals, reduction of digestive enzyme activity and local cytokine production were detected in germ-free animals. Earlier studies suggested that the absence of indigenous microflora modified the intestinal epithelial cells, which lead to changes in morphological and functional properties of germ-free animals (Alam et al., 1994; Wostmann, 1996). In germ-free animals, development of GALT, lamina propria cellularity and mucosal vascularity was also less effective. However, introducing intestinal microflora into germ-free mice helped the restoration of specific mucosal immune system (Umesaki et al., 1995). Furthermore, germ-free mice demonstrated
the inhibition of macrophage chemotaxis and phagocytic activity and less number of spleen-derived macrophage precursors (Wostmann, 1996; Nicaise et al., 1998).

Earlier studies reported that commensal bacterial ligands are important in the development and function of gut mucosal immune system (Rakoff-Nahoum et al., 2004; Mazmanian et al., 2005). These commensal bacteria are also capable of influencing the regulation of T-helper (Th) cell type-1 or type-2 cytokines (Cebra, 1999; Shanahan, 2002a). Cytokines such as interleukin (IL)-1β, IL-2, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β increased the proliferation of intestinal stromal cells (Fritsch et al., 1999).

Intestinal mucosal surfaces are vulnerable to various harmful microorganisms from outside environment that transit through the intestine, which may be harmful to the host. In order to protect the host from these harmful microorganisms, active secretory gut immune system has evolved. The release of secretory immunoglobulin (sIg)-A into the intestinal fluid occurs in healthy host, which is important in preventing the adherence of foreign antigens to epithelial cells. This process is known as immune exclusion, which provides protection to mucosal surfaces without causing inflammatory responses. The gut microflora also acts as a barrier in protecting the host from pathogenic bacteria by producing regulatory factors such as short-chain fatty acids and bacteriocins. Cebra, (1999) suggested the antagonistic activity of intestinal microflora in inhibiting the colonisation of pathogenic bacteria in gut. The innate immune defence mechanisms of intestinal epithelial cells include mucins and antimicrobial compounds such as lysozyme and defensins, which are important in
preventing the microbial adherence to epithelial cell surfaces (Magnusson and Stjernstorm, 1982; Ouellette, 1999).
Figure 1.2. Overview of gastrointestinal (GI) tract. (A) Microbial distribution (Anaerobic and aerobic genera) in various compartments of the GI tract. (B) Functional properties of intestinal microflora influencing intestinal mucosa. (Adapted from O’Hara and Shanahan, 2006).
1.2. GUT IMMUNE SYSTEM

In humans, gut is considered as a major immune organ. Gut mucosal surfaces are constantly exposed to antigens derived from both diet and intestinal microflora. Gut defence mechanisms have the capability of discriminating non-pathogenic commensal bacteria and antigens from enteropathogens. Specifically, gut immune responses towards antigens derived from commensal bacteria are non-harmful to the organism, which further induce systemic tolerance against intestinal antigens. This process can be expressed as immunological tolerance, which is important for gut integrity. Earlier studies reported the mechanisms that are involved in immunological tolerance (Schwartz, 1989; Garside et al., 1999). It was also suggested that the failure of immunological tolerance towards luminal bacteria could lead to inflammatory bowel disease (Duchmann et al., 1995).

The mucosal immune system is separated from gut lumen by a thin layer of intestinal epithelial cells that lines the mucosal intestinal surface. These intestinal epithelial cells are important in providing first line defence by preventing lumen antigens from accessing the host immune system. However, antigens constantly interact with immunosensory cells of the intestinal epithelium through different mechanisms (Fig. 1.3). The sampling of luminal antigens is performed through M-cell, which resides in specialised epithelium that overlies the Peyer’s patches (Neutra et al., 1996). The antigen presentation and affinity maturation of the transported luminal antigens occur in Peyer’s patches (McGhee et al., 1999; Owen, 1999). In Peyer’s patches, activated antigen-specific lymphocytes migrate to mesenteric lymph nodes and re-enter the circulation through thoracic duct. Perdue, (1999) suggested that the transport of antigen molecules can occur either through transcellular or paracellular pathways.
The other route of sampling antigens could be through the dendrites of intestinal dendritic cells (Rescigno et al., 2001). The intestinal dendritic cells are also important in inducing local immune responses towards commensal bacteria through ingestion and retaining live commensal bacteria, which are further transported to mesenteric lymph nodes (Macpherson and Uhr, 2004). This process suggests the role of mesenteric lymph nodes as potential gatekeepers by preventing the access of commensal bacteria to internal environment.

Lamina propria and intraepithelial spaces are considered as main effector sites in the intestine. The lamina propria mainly consists of over 60% immune cells, which include immunoglobulin (Ig)-A producing B-cells, T-cells and macrophages (McGhee et al., 1999). In the lamina propria, B-cells undergo differentiation into plasmacytes in order to secrete dimeric IgA. The intestinal epithelial cells are capable of producing polymeric Ig receptors, which transport dimeric IgA into the intestinal lumen (Underdown and Schiff, 1986). It is often described that plasma cells on the mucosal surfaces as well as in the lamina propria are capable of secreting upto 40 mg of IgA per day, which is an important characteristic for an active gut immune system. In lamina propria, T-cells have the capability of interacting with B-cell immunoglobulin isotype (Lebman et al., 1990; Shockett and Stavnezer, 1991). The T-cells in lamina propria are mainly CD4⁺ T-helper lymphocytes, which express CD40 ligands and release cytokines such as IL-4, IL-5 and IL-10 (Liu and Arpin, 1997). Furthermore, cytokines released from CD4⁺ T-cells and intestinal epithelial cells modulate IgA producing B-cells, which are known to be important in mucosal defence mechanism.
**Figure 1.3.** Schematic representation of gut immune system. (Adapted from McCracken and Lorenz, 2001).
1.3. PROBIOTICS

The term probiotic, meaning ‘for life’ was first coined in 1960s in order to describe “substances secreted by one microorganism that stimulate the growth of another” (Lilly and Stillwell, 1965). However, the role of these beneficial bacteria in promoting positive health effects upon host has been fully recognised only in the last two decades. Fuller, (1989) redefined the word probiotics as “viable microorganisms that contribute to intestinal microbial balance and have the potential to improve the health of their human host”. Recently, probiotics have been defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Reid et al., 2003).

Probiotics are live microbial food supplements that have been used for many years in animal feed industry. However, probiotics are now widely used in the manufacturing of fermented dairy products. Of the numerous microbes that are beneficial inhabitants of GI tract, microorganisms that have been considered as probiotics include yeast (Guslandi et al., 2000), *Escherichia coli* (Tromm et al., 2004) and enterococci (Lund and Edlund, 2001). The most commonly used have been bifidobacteria and lactic acid bacteria (LAB) such as lactobacilli, lactococci and streptococci (Madsen, 2001; Isolauri et al., 2002; Table 1.1). Lactobacilli are rod-shaped bacteria, which are capable of producing short-chain fatty acids, acetate and lactate through carbohydrate fermentation process. Several *Lactobacillus* species are known to inhabit the intestinal tracts of humans and animals. In large intestine, bifidobacteria constitute in large numbers among other beneficial microorganisms, which are capable of synthesising vitamins and digestive enzymes (Gibson et al., 1995). Generally, *Lactobacillus* and *Bifidobacterium* strains are used in the
development of probiotic products intended for human consumption due to the belief that these bacteria are members of intestinal microflora and also considered as “generally regarded as safe” (GRAS) (Berg, 1998; Klein et al., 1998).

The probiotic effects are strain-specific, therefore it is difficult to generalise the beneficial effects of a probiotic strain even comparing to strains of the same species. Recent Food and Agriculture Organisation (FAO) and World Health Organisation (WHO) guidelines suggested that probiotic bacteria incorporated in food products must demonstrate their tolerance to gastric juices and bile salts in order to survive during their transit in GI tract (FAO/WHO, 2001; 2002). Additionally, probiotic bacteria must be capable of colonising the GI tract and have the potential in maintaining their efficacy during shelf-life of the product. Potential probiotic bacteria possess some or all of the functional properties such as: (i) tolerance to acid, bile, enzyme and oxygen; (ii) ability to adhere host epithelial cells; (iii) colonisation in the GI tract; (iv) pathogen exclusion; (v) production of antimicrobial substances towards pathogens; (vi) non-pathogenic and non-carcinogenic and (vii) favourable towards well balanced microbial gut ecosystem. Several studies reported the influence of probiotic bacteria on synthesis of various energy sources such as folic acid production (Deeth and Tamime, 1981; Alm, 1982) and short-chain fatty acids (Mallett et al., 1989). Furthermore, studies also showed that probiotic bacteria are also capable of influencing various immune responses such as humoral, cellular and non-specific immunity (Matsuzaki et al., 1998; Chiang et al., 2000; Cross et al., 2002; Elahi et al., 2005; Takahashi et al., 2006).
### Table 1.1. Microorganisms that are commonly considered as probiotics.

<table>
<thead>
<tr>
<th><em>Lactobacillus</em> spp.</th>
<th><em>Bifidobacterium</em> spp.</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. acidophilus</em></td>
<td><em>B. adolescentis</em></td>
<td><em>Enterococcus faecalis</em></td>
</tr>
<tr>
<td><em>L. brevis</em></td>
<td><em>B. animalis</em></td>
<td><em>Enterococcus faecium</em></td>
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<tr>
<td><em>L. casei</em></td>
<td><em>B. breve</em></td>
<td><em>Escherichia coli Nissle</em></td>
</tr>
<tr>
<td><em>L. crispatus</em></td>
<td><em>B. bifidum</em></td>
<td><em>Saccharomyces boulardii</em></td>
</tr>
<tr>
<td><em>L. curvatus</em></td>
<td><em>B. infantis</em></td>
<td><em>Streptococcus cremoris</em></td>
</tr>
<tr>
<td><em>L. delbrueckii subsp. bulgaricus</em></td>
<td><em>B. lactis</em></td>
<td><em>Streptococcus diacetylactis</em></td>
</tr>
<tr>
<td><em>L. fermentum</em></td>
<td><em>B. longum</em></td>
<td><em>Streptococcus intermedius</em></td>
</tr>
<tr>
<td><em>L. gasseri</em></td>
<td><em>B. thermophilum</em></td>
<td><em>Streptococcus salivarius</em></td>
</tr>
<tr>
<td><em>L. johnsonii</em></td>
<td></td>
<td><em>Streptococcus thermophilus</em></td>
</tr>
<tr>
<td><em>L. lactis</em></td>
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<tr>
<td><em>L. paracasei</em></td>
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<tr>
<td><em>L. plantarum</em></td>
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<tr>
<td><em>L. reuteri</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. rhamnosus</em></td>
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</table>
1.4. IMMUNE RESPONSES OF LACTIC ACID BACTERIA

Certain strains of LAB have the potential in contributing towards host defence mechanisms of GI tract (Deplancke and Gaskins, 2001) through interactions with the immune system (Lu and Walker, 2001). Several factors such as survival, adherence, colonisation and sites of interaction in GI tract influence the ability of immune enhancing properties of LAB. Earlier studies reported that probiotic bacteria are capable of influencing host cell functions such as immune responses and intestinal barrier integrity (Rosenfeldt et al., 2004; Sartor, 2005). The capability of dendritic cells in recognising bacterial antigens also helps in regulating T-cell responses. *In vitro* studies demonstrated the differential cytokine expression of murine dendritic cells influenced by LAB and probiotic cocktail VSL#3, which favoured Th1, Th2 or Th3 immune responses (Christensen et al., 2002; Hart et al., 2004). Particularly, *Lactobacillus* strains demonstrated the regulation of IL-10 producing T-cells through interaction with dendritic cell-specific intracellular adhesion molecule 3-grabbing nonintegrin (Smits et al., 2005).

Epithelial cells of the intestine and colon also play an important role in immune mechanisms. Zhang et al. (2005) reported that Caco-2 cells cultured with *L. rhamnosus* Goldin-Gorbach (GG) regulated NF-κB signalling pathway to reduce TNF-α induced IL-8 production. *Lactobacillus reuteri* and VSL#3 also demonstrated their influence on NF-κB signalling pathway (Ma et al., 2004; Petrof et al., 2004). Probiotic bacteria are also capable of strengthening the intestinal barrier. For example, probiotic *E. coli* Nissle 1917 influenced the regulation of NF-κB signalling pathway by inducing the expression of antimicrobial peptide β-defensin-2 (Wehkamp
et al., 2004), which could be important in enhancing intestinal barrier functions such as inhibiting the growth of pathogenic bacteria.

Several reports have suggested that early exposure of live microorganisms with dietary antigens could be helpful in developing gut barrier functions (Helgeland et al., 1996; Sudo et al., 1997). Moreau et al. (1978) reported the development of gut barrier through increased duodenal IgA plasmocyte cells. Peyer’s patches are known to be important in sampling luminal bacterial antigens where they interact with antigen-presenting cells carrying major histocompatibility complex (MHC) class II (Weiner et al., 1994). The antigens that are transported through Peyer’s patches are considered important towards the overall development of local secretory immune responses. Subsequently, immune responses initiated in GALT can influence immune responses at other mucosal sites. In vitro cell culture models have been used to demonstrate the immune responses induced by non-pathogenic bacteria. As an example, Haller et al. (2000) reported the effects of non-pathogenic bacteria in inducing cytokines such as IL-8, IL-10 and TNF-α by Caco-2 cells.

Many investigators reported the influence of LAB in inducing sIgA responses in GALT. Orally administered L. acidophilus and L. casei increased the sIgA levels in small intestine of mice (Perdigon et al., 1995). In mice, L. acidophilus and Bifidobacterium species modulated the mucosal and systemic immunity through increased IgA responses to cholera toxin, which was used as an oral immunogen (Tejada-Simon et al., 1999a). Lactobacillus GG also showed the capability in increasing IgA response in children with Crohn’s disease (Malin et al., 1996) and acute rotavirus diarrhoea (Kaila et al., 1992). Moreover, several studies also reported
the capability of probiotic bacteria in modulating IgE production. For example, decrease in the production of IgE in serum was detected in mice orally fed with *L. casei* Shirota (Matsuzaki et al., 1998). Similar results related to the reduction of IgE production was also observed in IL-10 deficient mice fed with *L. plantarum* 229v (Schultz et al., 2002).

The regulation of both innate and acquired immune responses was mediated through cytokines. Several studies reported the influence of LAB in regulating T-cell functions. As an example, cell wall and cytoplasmic components of LAB demonstrated the capability of stimulating cytokines such as IL-6 and TNF-α and nitric oxide (NO) (Tejada-Simon and Pestka, 1999b). Maassen et al. (2000) also reported an increase of TNF-α, IL-2 and IL-1β in gut villi of mice fed with *L. reuteri* or *L. brevi*. Furthermore, *Lactobacillus* strains demonstrated the enhancement of TNF-α and IFN-γ producing cells in the lamina propria of gut (Perdigon et al., 2002).

The cytokine, IFN-γ is capable of enhancing nitric oxide production, activating macrophages and NK cells. The phagocytic activity of macrophages plays an important role in preventing microbial infections, whereas NK cells are pivotal in defence against viral infections and cancer. Schiffrin et al. (1995) demonstrated the supplementation of *L. johnsonii* La1 or *B. lactis* Bb12 for 3 weeks, which increased the phagocytic activity of peripheral blood leukocytes in humans. Interestingly, the influence of probiotic bacteria differ in the modulation of phagocytic activity in healthy and allergic subjects (Pelto et al., 1998). Furthermore, Donnet-Hughes et al. (1999) also reported the potential of LAB on phagocytic activity of the peripheral blood leukocytes in healthy adult volunteers.
In general, probiotic bacteria can be regarded as a dietary adjuvant, which beneficially affect the host physiology through modulating mucosal and systemic immune functions. Lactic acid bacteria have been considered as potential candidates for oral vaccination purposes due to their various functional properties and long history of usage as safe food grade organisms. Maassen et al. (2000) demonstrated the differences between *Lactobacillus* strains in inducing cytokines and their adjuvant properties in gut. Although several studies reported the adjuvant effects of LAB with various antigens, adjuvant characteristics of *Lactobacillus* strains often vary due to the differences in bacterial strains, experimental designs, animals and antigens (Tejada-Simon et al., 1999a; Cano et al., 2002; Plant and Conway, 2002; He et al., 2005; Kim et al., 2005).

1.5. THERAPEUTIC EFFECTS OF LACTIC ACID BACTERIA

Lactic acid bacteria have the potential to induce several beneficial effects on human health, which are listed in Table 1.2. The insights into the therapeutic effects of LAB in GI tract and their effects on host immune functions are discussed below.

1.5.1. Diarrhoeal diseases

Certain LAB are proven to be beneficial in the prevention or amelioration of various diarrhoeal diseases. Several human intervention studies reported the probiotic effects of *L. rhamnosus* GG against rotavirus diarrhoea in infants (Isolauri et al., 1991; Kaila et al., 1992; Majamaa et al., 1995). Szajewska et al. (2001) demonstrated the capability of *L. rhamnosus* GG in reducing the duration of diarrhoea in comparison to placebo. Additionally, *L. rhamnosus* GG prevented the urease-producing bacterial growth during rotavirus diarrhoea (Isolauri et al., 1994). Recently, Paton et al. (2005)
demonstrated the treatment and prevention of *E. coli* diarrhoea through a toxin binding recombinant probiotic. Probiotic bacteria also showed the capability of producing bacteriocins such as nisin in order to inhibit the growth of pathogenic bacteria (Dodd and Gasson, 1994; Miraglia del Giudice and De Luca, 2004).

1.5.2. **Inflammatory bowel disease**

Inflammatory bowel disease (IBD) has a complex etiology, which was characterised by chronic or recurrent intestinal inflammation. Ulcerative colitis and Crohn’s disease are the best known forms of IBD. The mechanisms responsible for the cause of IBD still remain unclear. Several studies suggested that certain probiotic bacteria are capable of treating mild or moderately active IBD (Fedorak and Madsen, 2004; Sartor, 2004). Particularly, Pathmakanthan et al. (1999) reported the decrease of lactobacilli in the inflamed mucosal tissues of ulcerative colitis patients. Therefore, restoration of microflora in GI tract through selected probiotic bacteria could be considered as a therapeutic strategy for ameliorating intestinal inflammation.

1.5.3. **Irritable bowel syndrome**

Irritable bowel syndrome (IBS) is associated with manifestation of abdominal pain, bloating, flatulence and diarrhoea. Several studies suggested the role of colonic microflora in pathogenesis of IBS (Bradley et al., 1987; Madden et al., 2001). However, the cause of IBS is still unknown. In double blind and placebo controlled studies, orally administered *L. plantarum* reduced flatulence and abdominal pain (Nobaek et al., 2000; Niedzielin et al., 2001). Brigidi et al. (2001) also reported the beneficial effects of VSL#3 by improving the clinical picture and altering the faecal
microflora composition with an increase of lactobacilli, bifidobacteria and \textit{S. thermophilus} in patients having IBS. In another study, \textit{L. plantarum} did not either modify the colonic fermentation process or improve symptoms in patients with IBS (Sen et al., 2002). Recently, O’Mahony et al. (2005) suggested the immunomodulatory role of \textit{B. infantis} by improving the symptoms in patients with IBS. However, further studies are needed to define the mechanisms of probiotic bacterial influence on IBS.

1.5.4. \textit{Helicobacter pylori} infections

\textit{Helicobacter pylori} are responsible for causing chronic gastritis and idiopathic peptic ulcer disease. Previous studies showed the potential of probiotic bacteria in inhibiting the growth of \textit{H. pylori} (Midolo et al., 1995; Kabir et al., 1997). Aiba et al. (1998) showed the capability of \textit{L. salivarius} in inhibiting the growth of \textit{H. pylori} by producing high amounts of lactic acid. It was postulated that higher levels of lactic acid production could be helpful in reducing the urease activity of \textit{H. pylori}. It has also been reported that the combination of probiotic cultures such as \textit{L. acidophilus} La5 and \textit{B. latics} Bb12 showed significant decrease in the urease activity after 6 weeks of oral administration in humans with \textit{H. pylori} (Wang et al., 2004). In contrast, a probiotic combination of \textit{L. rhamnosus} GG, \textit{L. rhamnosus} LC705, \textit{B. breve} Bb99 and \textit{Propionibacterium freudenreichii} subsp. shermanii JS were not effective in improving intestinal inflammation symptoms in humans with \textit{H. pylori} (Myllyluoma et al., 2005). Further studies are needed to determine the etiology of the disease and well defined experimental methodologies could possibly substantiate the efficacy of probiotic bacteria.
The mechanisms of probiotic bacterial interaction with the host need to be investigated to elucidate their potential role especially in promoting human health and well-being. Earlier studies attempted to define the cellular targets of probiotic bacteria, whereas the molecular basis of the probiotic functions remains unknown. The emerging demand of probiotic bacteria in functional food applications require fundamental understanding of the molecular structures that are involved in microbial-host interactions. Moreover, genomic approaches are considered necessary in identifying the key gene targets of probiotic functions.
Table 1.2. Therapeutic properties of lactic acid bacteria. (Adapted from Saxelin et al., 2005; Reid et al., 2006).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Probiotic effect</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><em>L. acidophilus</em> La5</td>
<td>Suppression of <em>Helicobacter pylori</em> with use of La5 and <em>B. lactis</em> BB12.</td>
<td>Wang et al., 2004.</td>
</tr>
<tr>
<td><em>L. brevis</em> CD2</td>
<td>Decreases <em>H. pylori</em> colonisation, thus reducing polyamine biosynthesis.</td>
<td>Linsalata et al., 2004.</td>
</tr>
<tr>
<td><em>L. casei</em> Shirota</td>
<td>Improvement in treatment of constipation.</td>
<td>Koebnick et al., 2003.</td>
</tr>
<tr>
<td><em>L. gasseri</em> OLL 2716</td>
<td>Suppressed <em>H. pylori</em> and reduced gastric mucosal inflammation.</td>
<td>Sakamoto et al., 2001.</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GR-1 and <em>L. fermentum</em> RC-14</td>
<td>Reduction in, and better treatment of, urogenital infections in women taking oral lactobacilli daily for 2 months.</td>
<td>Reid et al., 2004.</td>
</tr>
<tr>
<td><em>L. johnsonii</em> La1</td>
<td>A moderate but significant difference in <em>H. pylori</em> colonisation was detected in children.</td>
<td>Cruchet et al., 2003.</td>
</tr>
<tr>
<td><em>L. plantarum</em> 299v</td>
<td>Decreased incidence of infections in liver transplant patients.</td>
<td>Rayes et al., 2002.</td>
</tr>
<tr>
<td><em>L. plantarum</em> 299v</td>
<td>Reduction of the recurrence of <em>Clostridium difficile</em> enterocolitis.</td>
<td>Wullt et al., 2003; Plummer et al., 2004.</td>
</tr>
<tr>
<td>Strain</td>
<td>Probiotic effect</td>
<td>Reference</td>
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<td>----------------------</td>
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</tr>
<tr>
<td><em>L. reuteri</em> ATCC 55730</td>
<td>For treatment of diarrhoea and to produce CD4-positive T-lymphocytes in the ileal epithelium.</td>
<td>Valeur et al., 2004.</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td>Improved treatment of diarrhoea and management of atopy.</td>
<td>Szajewska et al., 2001; Kirjavainen et al., 2003.</td>
</tr>
<tr>
<td>VSL#3</td>
<td>Effective for the management of remission of pouchitis and colitis.</td>
<td>Mimura et al., 2004.</td>
</tr>
</tbody>
</table>
1.6. FUNCTIONAL GENOMICS AND LACTIC ACID BACTERIA

The word “functional genomics” constitute genomics, transcriptomics, proteomics, metabolomics and bioinformatics, which can also be termed as systems biology. The incorporation of functional genomics in understanding the complex intestinal ecosystem is gaining momentum to elucidate the relationship between diet and human health. However, understanding the role of nutrients in modulating gene and protein expression and subsequently their influence on cellular and metabolic functions are necessary in preventing diet-related diseases.

Molecular approaches have demonstrated that lactobacilli was able to survive through its intestinal passage and altered the microbial population and diversity of *Lactobacillus* species, but did not induce any adverse effects on dominant microflora (de Vos et al., 2004; Zoetendal et al., 2004). Similarly, molecular techniques also showed that *L. casei* and *L. plantarum* are active during the transit from stomach to colon in mice and played an important role in protein synthesis (Bron et al., 2004; Oozeer et al., 2004). Mechanisms such as autoaggregation and adhesion were considered as potential properties of LAB in colonising the GI tract. Roos et al. (1999) reported that genes coding for aggregation helicase and maltose-binding proteins are necessary in increasing the autoaggregation ability of *L. reuteri* 1063. Earlier studies demonstrated that lipoteichoic acid was involved in the adherence of *L. johnsonii* La1 to intestinal Caco-2 cells (Granato et al., 1999). The genome sequencing of *L. johnsonii* La1 uncovered the genes that were involved in lipoteichoic acid synthesis and cell surface molecules (Vaughan and Mollet, 1999). Furthermore, Mack et al. (1999) hypothesised the role of *L. plantarum* 299v and *L. rhamnosus* GG in inhibiting the adherence of pathogenic *E.*
coli to HT-29 intestinal cells through increased expression of intestinal mucin genes such as MUC2 and MUC3.

Several *in vivo* studies reported the influence of diet on gene expression. As an example, Rao et al. (2001) showed that mice fed with low diet in selenium decreased the expression of genes involved in detoxification and increased the expression of genes that were involved in DNA damage processing, oxidative stress and cell-cycle control. Similarly, magnesium deficiency up-regulated the genes involved in protection and repair of oxidative stress in thymocytes of rat (Petrault et al., 2002). Blanchard et al. (2001) demonstrated the potential of cDNA microarray analysis in revealing the intestinal gene expression changes that occurred in early stages of zinc deficiency in rats. Furthermore, Hooper et al. (2001) demonstrated the capability of commensal bacteria in modulating gene expression that are involved in various intestinal functions such as nutrient absorption, mucosal barrier fortification and xenobiotic metabolism.

The use of conventional methods restricted the understanding to a particular gene function, whereas the DNA microarray technique was capable of unravelling the overall gene expression patterns of the cell (Brown and Botstein, 1999; Khan et al., 1999). Whole genome analysis of gene expression in GI tract could further elucidate the microbial-host interactions. Earlier, several studies reported the beneficial effects of LAB on various biological functions of host, however applications of novel molecular approaches such as microarrays in combination with functional assays is necessary to unravel new target’s that were influenced by the probiotic bacteria.
1.7. AIMS

The overall aim of this study was to characterise the *in vitro* probiotic properties of *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 and evaluate their influence on mucosal and systemic immune responses and gene expression in mice.

The specific aims of this study were:

1. Characterisation of *Lactobacillus* and *Bifidobacterium* species for potential probiotic strains (Chapter 2).

2. Examination of *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 influence on mucosal and systemic immunity and bacterial translocation in mice (Chapter 3).

3. Determination of *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 effects on mucosal and systemic immune responses in cholera toxin mice model (Chapter 4).

4. Investigation of *L. acidophilus* LAFTI L10 influence on gene expression in the small bowel of normal and cholera toxin mice (Chapter 5).
CHAPTER 2

Selection and characterisation of *Lactobacillus* and *Bifidobacterium* species for potential probiotic strains
2.1. ABSTRACT

The present study aimed to investigate the in vitro probiotic properties of *Lactobacillus* and *Bifidobacterium* strains. A total of 12 strains were screened towards their tolerance to various acidic conditions. Following an initial screening, 6 strains were selected to further characterise their probiotic properties such as tolerance to bile salts, hydrophobicity, autoaggregation and coaggregation. The overall reduction in cell survivability of *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 in different pH conditions was less compared to other bacterial strains. Particularly, these strains showed high tolerance to pH 2.0 with more than $10^5$ CFU/ml in viability. *Lactobacillus acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 demonstrated high survivability especially in 1.0% bile salts when compared with other bacterial strains. A correlation between hydrophobicity and autoaggregation ability was observed among the bacterial strains. In contrast, *B. lactis* Bb12, demonstrated high degree of hydrophobicity, whereas autoaggregation ability was lower compared to other bacterial strains. Furthermore, coaggregation of *L. acidophilus* LAFTI L10 with other bacterial strains showed significant differences in their aggregation capabilities compared to *L. paracasei* LAFTI L26. Overall, *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 showed better tolerance to acid and bile conditions and also their surface adherence characteristics could be considered for their development as potential probiotic strains.
2.2. INTRODUCTION

Probiotic bacteria are commonly referred to as beneficial microbes, which are frequently used as active ingredients in fermented milk products. The use of probiotic bacteria in food products has been recently increased due to their potential attributes such as contributing to host defence mechanisms and improving the microbial balance of the GI tract. In order to exert beneficial effects on the host, probiotic bacteria are required to survive in enough numbers while transiting through constantly changing gastric and bile conditions of GI tract. The general acidity of stomach ranges from pH 2.5 to 3.5 (Holzapfel et al., 1998), however the pH of stomach could also be as low as pH 1.5 (Lankaputhra and Shah, 1995) or as high as pH 6.0 or higher after the intake of food (Johnson, 1977). There were no specific pH conditions or in vitro methods set to screen potential probiotic strains, however a wide range of pH conditions and in vitro methods were used to screen the acid tolerance of Lactobacillus and Bifidobacterium strains (Conway et al., 1987; Lankaputhra and Shah, 1995; Charteris et al., 1998; Chou and Weimer, 1999; Chung et al., 1999; Zarate et al., 2000).

Several studies reported the screening of Lactobacillus and Bifidobacterium strains tolerance to bile salts (Lankaputhra and Shah, 1995; Prasad et al., 1998; Chung et al., 1999; Truelstrup Hansen et al., 2002). In the small intestine, transit of food may take between 1 – 4 h (Smith, 1995), which can have a pH of around 8.0 (Keele and Neil, 1965). Bile concentrations ranging from 0.3% – 1.0% were used for in vitro screening to identify potential probiotic strains that were resistant to bile salts (Prasad et al., 1998; Jacobsen et al., 1999; Truelstrup Hansen et al., 2002). Goldin and Gorbach (1992)
suggested that bile salts of 0.15% – 0.3% concentrations were suitable for screening *Lactobacillus* and *Bifidobacterium* strains for human consumption.

Microbial adhesion to intestinal cells is considered as a potential characteristic of probiotic bacteria, which could enable them to colonise the GI tract and induce potential health effects (Pedersen and Tannock, 1989; Alander et al., 1997). In order to demonstrate a potential probiotic strain, adhesion ability is considered as one of the screening criteria for selecting probiotic bacteria (Fogh et al., 1977). The *in vivo* bacterial adhesion studies involve difficulties to characterise the interactions especially in humans, which led to the development of several *in vitro* methods as model systems for screening potential adherent probiotic bacteria (Mayra-Makinan et al., 1983; Conway and Kjellberg, 1989; Ouwehand et al., 2001). Earlier studies showed the *in vitro* adherence capability of probiotic bacteria to Caco-2 cells (Kimoto et al., 1999; Todoriki et al., 2001; Fernandez et al., 2003; Lee et al., 2003). However, several studies suggested indirect *in vitro* methods to characterise the adhering properties of probiotic bacteria that demonstrated the correlation between cell adhesion ability, autoaggregation and cell surface hydrophobicity (Wadstrom et al., 1987; Marin et al., 1997; Del Re et al., 1998; Perez et al., 1998; Del Re et al., 2000; Kos et al., 2003; Pan et al., 2006).

The adhesion of bacterial cells is a multi-step process that involves physical contact with cell surface, which could be influenced by structural composition of the cell wall membrane and interacting surfaces. The overall adherence of microorganisms also depends upon non-specific physical interactions between the two surfaces, which enable them to interact specifically between adhesins and complementary receptors (Freter,
previous studies suggested that several factors contribute the adhesion of probiotic bacteria to host epithelial cells, such as cell surface hydrophobicity and autoaggregation (Cesena et al., 2001; Ehrmann et al., 2002; Ventura et al., 2002; Kos et al., 2003), lipoteichoic acids (Sherman and Savage, 1986; Granato et al., 1999) and cell wall proteins (Walter et al., 2005). Autoaggregation demonstrated its importance in adhesion to intestinal cell wall, whereas coaggregation was crucial to form as a barrier to prevent the colonisation of pathogenic microorganisms (Boris et al., 1997; Reid et al., 1988; Del Re et al., 2000). Autoaggregation and coaggregation properties were considered to be important for probiotic bacteria to persist in different locations of mucosal surfaces (Jankovic et al., 2003). Castagliuolo et al. (2005) reported the importance of aggregation characteristics of *L. crispatus* in exerting protective effects in colitis mice model.

It was reported previously, that cell surface properties such as hydrophobicity may affect autoaggregation and adhesion ability of the bacteria (Wadstrom et al., 1987; Perez et al., 1998; Del Re et al., 2000). It is well known that attractive forces between non-polar surfaces in water solutes control the hydrophobic interactions. However, cell surface structures of bacteria may have the potential to undergo physiochemical changes to alter the hydrophobic ability in response to environmental conditions such as pH, bile salts or ionic strength.

In the present study, tolerance to low pH and high bile conditions, hydrophobicity, autoaggregation and coaggregation ability were considered as potential probiotic characteristics (Prasad et al., 1998; Del Re et al., 2000; Kos et al., 2003; Castagliuolo et
al., 2005; Otero et al., 2006; Bujalance et al., 2007). Initially, 12 strains of *Lactobacillus* and *Bifidobacterium* species were screened for their tolerance to simulated gastric juice at various pH levels (pH 2.0, 3.0, 4.0 and 6.5). Based on acid tolerance, 6 strains were selected to further investigate their probiotic properties such as tolerance to bile salts (0.5% and 1.0%), hydrophobicity, autoaggregation and coaggregation.
2.3. MATERIALS AND METHODS

2.3.1. Microorganisms and growth conditions

The bacterial strains used in this study were obtained from various suppliers in Australia. *Lactobacillus acidophilus* ASCC 2400, *Lactobacillus casei* subsp. *casei* ASCC 2603, *Lactobacillus paracasei* subsp. *paracasei* ASCC 5437, *Lactobacillus rhamnosus* ASCC 2625, *Bifidobacterium breve* ASCC 1900, *Bifidobacterium bifidum* ASCC 1903, *Bifidobacterium infantis* ASCC 1912 and *Bifidobacterium longum* ASCC 5188 were obtained from Australian Starter Culture Research Centre (Werribee, VIC, Australia). DSM Food Specialties (Moorebank, NSW, Australia) provided *Lactobacillus acidophilus* LAFTI L10, *Lactobacillus paracasei* LAFTI L26 and *Bifidobacterium animalis* subsp. *lactis* LAFTI B94. *Bifidobacterium animalis* subsp. *lactis* Bb12 was supplied by Chr. Hansen, Bayswater, VIC, Australia.

All bacterial strains were grown anaerobically in gas jars using GasPak System (Oxoid, Adelaide, Australia) for 24 h at 37°C in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Adelaide, Australia). The bacterial cells were harvested at 4000 x g for 15 min at 4°C and washed twice with sterile 0.01 M phosphate buffered saline (PBS) solution. The cell viability of the bacterial cultures was determined by spread plate count on MRS agar, which was incubated under anaerobic conditions at 37°C for 48 h.

2.3.2. Acid tolerance

The acid tolerance of *Lactobacillus* and *Bifidobacterium* strains was determined by using milk-based medium (NGYC medium: 12% non-fat skim milk, 2% glucose, 1%
yeast extract and 0.05% L-cysteine) as described by Lankaputhra and Shah, (1995). The NGYC medium was adjusted to pH 2.0, 3.0, 4.0 or 6.5 (Control) with 5 M HCL or 1 M NaOH. The bacterial cell suspensions were added to the pH adjusted NGYC medium, which were then incubated anaerobically for 3 h at 37°C. The bacterial survivability was determined by spread plate count on MRS agar after incubated anaerobically at 37°C for 48 h. The average and standard error were estimated from 6 independent experiments. The overall reduction in the viability of bacterial strains in different pH conditions was calculated using the formula:

\[
\text{Overall reduction of viability} = (\text{pH 6.5} - \text{pH 4.0}) + (\text{pH 6.5} - \text{pH 3.0}) + (\text{pH 6.5} - \text{pH 2.0})
\]

### 2.3.3. Bile tolerance

The bile tolerance of *Lactobacillus* and *Bifidobacterium* strains was determined by using milk-yeast extract medium as described by Truelstrup Hansen et al. (2002). The milk-yeast extract medium (pH 6.9) consists of 10% non-fat skim milk powder, 0.5% yeast extract, 0.05% L-cysteine and 0% (Control), 0.5% or 1.0% (w/v) bile salts (Oxgall, Sigma, Australia). The bacterial cell suspensions were inoculated into the milk-yeast extract medium and incubated anaerobically for 6 h at 37°C.

The survival of the bacterial cells was determined by spread plate count on MRS agar after anaerobically incubated at 37°C for 48 h. The average and standard error were
estimated from 6 independent experiments. The overall reduction in the viability of the bacterial strains in different bile concentrations was calculated using the formula:

\[
\text{Overall reduction of viability} = (0\% - 0.5\%) + (0\% - 1.0\%)
\]

2.3.4. Hydrophobicity assay

The adhesion of bacterial strains to hydrocarbons was determined according to Perez et al. (1998). Briefly, bacterial cultures were grown anaerobically for 24 h at 37°C in MRS broth and harvested at 4000 x g for 15 min at 4°C followed by washing twice with sterile 0.01 M PBS solution. Bacterial cells were resuspended in 0.01 M PBS and absorbance \( (A_0) \) was read at 600 nm. A volume of 0.6 ml of \( n \)-hexadecane (Merck, Melbourne, Australia) was added to 3-ml aliquots of bacterial cell suspension. The samples were mixed thoroughly by vortexing for 2 min. The 2-phase separation was observed by incubating the samples at 37°C for 1 h. Aqueous phase was removed and absorbance \( (A) \) was measured at 600 nm. Hydrophobicity assay was repeated 6 times to estimate the average and standard error. The percentage of cell surface hydrophobicity is expressed as:

\[
\text{Hydrophobicity (H\%)} = \left\{ \frac{(A_0 - A)}{A_0} \right\} \times 100
\]

Where \( A_0 \) and \( A \) represents the absorbance of aqueous phase before and after contact with \( n \)-hexadecane.
2.3.5. Autoaggregation and coaggregation assays

The bacterial strains were grown anaerobically for 24 h at 37°C in MRS broth and harvested at 4000 x g for 15 min at 4°C followed by washing twice with sterile 0.01 M PBS solution. The bacterial cells were resuspended in 0.01 M PBS, which were used to perform autoaggregation and coaggregation assays.

Autoaggregation assay was performed according to Kos et al. (2003). The bacterial cell suspensions of 4-ml aliquots were mixed thoroughly for 10 s and incubated at 37°C for 6 h. The absorbance ($A_t$) of the upper suspension (0.1 ml) was measured at 600 nm. Autoaggregation assay was repeated 6 times to estimate the average and standard error. The autoaggregation percentage is expressed as:

$$\text{Autoaggregation} \ (\%) = 1 - \frac{A_t}{A_o} \times 100$$

Where $A_t$ represents the absorbance at 6 h and $A_o$ represents the absorbance at 0 h.

Coaggregation assay was performed according to Handley et al. (1987). Briefly, equal volumes (2-ml aliquots) of different bacterial cell suspensions were mixed together by vortexing for 10 s, while the control tubes consist of 4-ml aliquots of individual bacterial cell suspensions. The mixed bacterial cell suspensions and the control tubes were incubated at 37°C for 6 h and absorbance ($A$) of the upper suspensions (0.1 ml) was measured at 600 nm. Coaggregation assay was repeated 6 times to estimate the average and standard error.
The coaggregation percentage is expressed as:

\[
\text{Coaggregation (} \% \text{)} = \frac{(Ax + Ay)/2 - A(x + y)}{(Ax + Ay)/2} \times 100
\]

Where \( Ax \) and \( Ay \) represent individual bacterial strains in the control tubes and \( A(x + y) \) represents the mixture of bacterial strains.

2.3.6. Statistical analysis

The results were expressed as mean ± standard error of mean (SEM). The data were analysed using the one-way analysis of variance procedure of SPSS (Version 12.0.1). Significant differences between groups were identified by Duncan’s multiple range test (SPSS, Version 12.0.1).
2.4. RESULTS

2.4.1. Tolerance to simulated gastric juice

The effect of various gastric conditions on *Lactobacillus* and *Bifidobacterium* strains is shown in Table 2.1. All the bacterial strains showed differences in cell viability in various pH conditions for 3 h. *Lactobacillus acidophilus* LAFTI L10, *L. paracasei* LAFTI L26 and *B. lactis* LAFTI B94 strains were tolerant to acid at pH 2.0 with a reduction of 2.95 to 3.89 log colony-forming units (CFU)/ml. Particularly, *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 were tolerant to pH 2.0 with high survival at $10^5$ CFU/ml, whereas *B. lactis* LAFTI B94, *B. infantis* ASCC 1912, *B. lactis* Bb12 and *L. acidophilus* ASCC 2400 showed the capability of surviving at $10^4$ CFU/ml after 3 h of incubation. At pH 2.0, *L. rhamnosus* ASCC 2625, *B. bifidum* ASCC 1903, *B. longum* ASCC 5188, *L. paracasei* ASCC 5437 and *B. breve* ASCC 1900 showed less survival ranging from $10^1$ to $10^3$ CFU/ml. Furthermore, there was no survival of *L. casei* ASCC 2603 after incubating for 3 h at pH 2.0.

The ability of *L. acidophilus* LAFTI L10, *L. paracasei* LAFTI L26, *L. lactis* LAFTI B94, *B. infantis* ASCC 1912, *B. lactis* Bb12 and *L. acidophilus* ASCC 2400 to survive at pH 3.0 was more than $10^6$ CFU/ml after incubation for 3 h. All other *Lactobacillus* and *Bifidobacterium* strains also showed tolerance to pH 3.0 with survival ranging from $10^3$ to $10^5$ CFU/ml. Particularly at pH 3.0, *L. acidophilus* LAFTI L10 demonstrated greater tolerance with less reduction in cell viability by 1.95 CFU/ml, which was followed by *L. paracasei* LAFTI L26 and *B. infantis* ASCC 1912 by a decrease of 2.5 CFU/ml. In pH 4.0, *L. acidophilus* LAFTI L10 showed higher tolerance with a decrease of 1.25
CFU/ml. Similarly, *B. infantis* ASCC 1912, *B. lactis* Bb12 and *L. acidophilus* ASCC 2400 also showed high tolerance to pH 4.0 with more than $10^7$ CFU/ml, whereas *L. paracasei* LAFTI L26, *L. lactis* LAFTI B94 and *L. casei* ASCC 2603 survived at $10^6$ CFU/ml. The other *Lactobacillus* and *Bifidobacterium* strains showed survival of $10^5$ CFU/ml at pH 4.0 (Table 2.1). All the *Lactobacillus* and *Bifidobacterium* strains that were screened for their tolerance to pH 2.0, pH 3.0 and pH 4.0 showed significant variations ($P < 0.01$) in their cell viability when compared with pH 6.5 (Control).

Based on the survival of *Lactobacillus* and *Bifidobacterium* strains at various pH conditions after incubating for 3 h, overall reduction of cell viability was calculated to determine the potential probiotic strains. *Lactobacillus acidophilus* LAFTI L10, *L. paracasei* LAFTI L26, *L. lactis* LAFTI B94, *B. infantis* ASCC 1912, *B. lactis* Bb12 and *L. acidophilus* ASCC 2400 exhibited greater tolerance to various pH conditions, particularly at pH 2.0 with survival more than $10^4$ CFU/ml. As shown in Table 2.1, top 6 bacterial strains were selected to further investigate their probiotic properties.

### 2.4.2. Tolerance to bile salts

The effect of bile salts on the growth of *Lactobacillus* and *Bifidobacterium* strains are shown in Table 2.2. The exposure of bile salts at 0.5% and 1.0% for 6 h was less detrimental to *L. paracasei* LAFTI L26, which demonstrated less reduction in overall cell viability. All *Lactobacillus* and *Bifidobacterium* strains in 1.0% bile salts showed less than 1.0 CFU/ml reduction in survival with exception to *L. acidophilus* ASCC 2400, which exhibited a decrease of 1.15 CFU/ml in cell viability. The resistance of
Lactobacillus and Bifidobacterium strains towards 0.5% bile salts also showed reduction in cell survival, which was not higher than 1.0 CFU/ml. The overall reduction of cell viability in different bile salts demonstrated that L. paracasei LAFTI L26 was a potential probiotic strain with high tolerance to bile salts, whereas B. lactis LAFTI B94 and L. acidophilus LAFTI L10 showed similar overall reduction of survival at different bile salts concentrations. However, L. acidophilus LAFTI L10 showed better tolerance to 1.0% bile salts compared to B. lactis LAFTI B94.
Table 2.1. Survival of *Lactobacillus* and *Bifidobacterium* strains in simulated gastric conditions for 3 h.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Cell count(^a,b)</th>
<th>pH 6.5(^b)</th>
<th>pH 4.0(^b)</th>
<th>pH 3.0(^b)</th>
<th>pH 2.0(^b)</th>
<th>Overall reduction(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. acidophilus</em> LAFTI L10</td>
<td>8.78 ± 0.24</td>
<td>8.62 ± 0.13</td>
<td>7.37 ± 0.16*</td>
<td>6.67 ± 0.18*</td>
<td>5.67 ± 0.21*</td>
<td>6.15</td>
</tr>
<tr>
<td><em>L. paracasei</em> LAFTI L26</td>
<td>8.96 ± 0.11</td>
<td>8.82 ± 0.21</td>
<td>6.78 ± 0.10*</td>
<td>6.30 ± 0.04*</td>
<td>5.11 ± 0.07*</td>
<td>8.27</td>
</tr>
<tr>
<td><em>B. lactis</em> LAFTI B94</td>
<td>8.88 ± 0.26</td>
<td>8.76 ± 0.17</td>
<td>6.72 ± 0.19*</td>
<td>6.11 ± 0.11*</td>
<td>4.87 ± 0.16*</td>
<td>8.58</td>
</tr>
<tr>
<td><em>B. infantis</em> ASCC 1912</td>
<td>8.98 ± 0.18</td>
<td>8.87 ± 0.15</td>
<td>7.22 ± 0.11*</td>
<td>6.37 ± 0.41*</td>
<td>4.36 ± 0.05*</td>
<td>8.66</td>
</tr>
<tr>
<td><em>B. lactis</em> Bb12</td>
<td>9.21 ± 0.14</td>
<td>9.14 ± 0.27</td>
<td>7.13 ± 0.16*</td>
<td>6.44 ± 0.25*</td>
<td>4.76 ± 0.17*</td>
<td>9.09</td>
</tr>
<tr>
<td><em>L. acidophilus</em> ASCC 2400</td>
<td>9.62 ± 0.19</td>
<td>9.45 ± 0.42</td>
<td>7.22 ± 0.33*</td>
<td>6.59 ± 0.17*</td>
<td>4.26 ± 0.21*</td>
<td>10.28</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> ASCC 2625</td>
<td>8.46 ± 0.22</td>
<td>8.37 ± 0.14</td>
<td>5.54 ± 0.24*</td>
<td>4.85 ± 0.04*</td>
<td>3.79 ± 0.14*</td>
<td>10.93</td>
</tr>
<tr>
<td><em>B. bifidum</em> ASCC 1903</td>
<td>8.41 ± 0.12</td>
<td>8.29 ± 0.11</td>
<td>5.24 ± 0.16*</td>
<td>4.22 ± 0.19*</td>
<td>3.65 ± 0.24*</td>
<td>11.76</td>
</tr>
<tr>
<td><em>B. longum</em> ASCC 5188</td>
<td>8.57 ± 0.29</td>
<td>8.48 ± 0.33</td>
<td>5.74 ± 0.32*</td>
<td>5.11 ± 0.24*</td>
<td>2.32 ± 0.21*</td>
<td>12.27</td>
</tr>
<tr>
<td><em>L. paracasei</em> ASCC 5437</td>
<td>8.23 ± 0.14</td>
<td>8.12 ± 0.46</td>
<td>5.58 ± 0.61*</td>
<td>4.09 ± 0.32*</td>
<td>1.32 ± 0.14*</td>
<td>13.37</td>
</tr>
<tr>
<td>Strains</td>
<td>Cell count (^{a,b})</td>
<td>pH 6.5(^{b})</td>
<td>pH 4.0(^{b})</td>
<td>pH 3.0(^{b})</td>
<td>pH 2.0(^{b})</td>
<td>Overall reduction(^{c})</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td><em>L. casei</em> ASCC 2603</td>
<td>8.30 ± 0.08</td>
<td>6.10 ± 0.28(^*)</td>
<td>4.15 ± 0.16(^*)</td>
<td>ND(^d)</td>
<td>14.23</td>
<td></td>
</tr>
<tr>
<td><em>B. breve</em> ASCC 1900</td>
<td>9.39 ± 0.12</td>
<td>5.26 ± 0.19(^*)</td>
<td>3.85 ± 0.21(^*)</td>
<td>1.58 ± 0.15(^*)</td>
<td>17.12</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Initial bacterial cell concentration.

\(^b\)Values (Log\(_{10}\) CFU ml\(^{-1}\)) are mean ± SEM (n = 6).

\(^c\)Expressed as Log\(_{10}\) values using the formula: (pH 6.5 – pH 4.0) + (pH 6.5 – pH 3.0) + (pH 6.5 – pH 2.0).

\(^d\)Not detected. Detection limit was 10\(^1\) CFU/ml.

*Mean values were significantly different (P < 0.01) from the pH 6.5 (Control).
Table 2.2. Survival of *Lactobacillus* and *Bifidobacterium* strains in milk-yeast medium with oxgall.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Cell count(^a,b)</th>
<th>0(^b)</th>
<th>0.5(^b)</th>
<th>1.0(^b)</th>
<th>Overall reduction(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. paracasei</em> LAFTI L26</td>
<td>8.93 ± 0.16</td>
<td>8.80 ± 0.10</td>
<td>8.61 ± 0.18</td>
<td>8.31 ± 0.02</td>
<td>0.68</td>
</tr>
<tr>
<td><em>B. lactis</em> LAFTI B94</td>
<td>8.91 ± 0.13</td>
<td>8.79 ± 0.08</td>
<td>8.47 ± 0.11</td>
<td>8.04 ± 0.06*</td>
<td>1.07</td>
</tr>
<tr>
<td><em>L. acidophilus</em> LAFTI L10</td>
<td>8.99 ± 0.25</td>
<td>8.93 ± 0.13</td>
<td>8.57 ± 0.02</td>
<td>8.21 ± 0.04*</td>
<td>1.08</td>
</tr>
<tr>
<td><em>B. infantis</em> ASCC 1912</td>
<td>8.68 ± 0.19</td>
<td>8.55 ± 0.05</td>
<td>8.04 ± 0.14*</td>
<td>7.88 ± 0.06*</td>
<td>1.18</td>
</tr>
<tr>
<td><em>B. lactis</em> Bb12</td>
<td>9.20 ± 0.27</td>
<td>9.06 ± 0.09</td>
<td>8.72 ± 0.09</td>
<td>8.16 ± 0.17*</td>
<td>1.24</td>
</tr>
<tr>
<td><em>L. acidophilus</em> ASCC 2400</td>
<td>9.39 ± 0.28</td>
<td>9.24 ± 0.15</td>
<td>8.51 ± 0.06*</td>
<td>8.09 ± 0.11*</td>
<td>1.88</td>
</tr>
</tbody>
</table>

\(^a\)Initial bacterial cell concentration.

\(^b\)Values (Log\(_{10}\) CFU ml\(^{-1}\)) are mean ± SEM (n = 6).

\(^c\)Expressed as Log\(_{10}\) values using the formula: (0% - 0.5%) + (0% - 1.0%).

\(^*\)Mean values were significantly different (\(P < 0.01\)) from the 0% bile salts (Control).
2.4.3. Surface hydrophobicity of *Lactobacillus* and *Bifidobacterium* strains

The cell surface hydrophobicity of *Lactobacillus* and *Bifidobacterium* strains to *n*-hexadecane was investigated as shown in Figure 2.1. The results indicate that *B. lactis* Bb12 (25%) showed higher surface hydrophobicity compared to less hydrophobic *B. infantis* ASCC 1912 (10%). Specifically, LAFTI strains, such as *L. acidophilus* L10, *L. paracasei* L26 and *B. lactis* B94 demonstrated similar cell surface hydrophobicity ranging between 14% – 16%. Furthermore, *L. acidophilus* ASCC 2400 also exhibited similar hydrophobic levels by 14%.

2.4.4. Autoaggregation and coaggregation of *Lactobacillus* and *Bifidobacterium* strains

The autoaggregation of bacterial strains was investigated on the basis of their sedimentation properties after 6 h as shown in Figure 2.2. All *Lactobacillus* and *Bifidobacterium* strains demonstrated the differences in their autoaggregation abilities. Particularly, *L. paracasei* LAFTI L26, *B. lactis* LAFTI B94 and *L. acidophilus* ASCC 2400 showed higher autoaggregation ability by 33%, whereas *L. acidophilus* LAFTI L10 and *B. infantis* ASCC 1912 indicated similar autoaggregation profiles by 30%. Particularly, *B. lactis* Bb12 exhibited autoaggregation ability by 29%, which was less in comparison to other *Lactobacillus* and *Bifidobacterium* strains that were screened in this study.
Coaggregation of \textit{L. acidophilus} LAFTI L10 and \textit{L. paracasei} LAFTI L26 and individually with other potential probiotic strains such as, \textit{B. lactis} LAFTI B94, \textit{B. lactis} Bb12, \textit{B. infantis} ASCC 1912 and \textit{L. acidophilus} ASCC 2400 was examined (Fig. 2.3). The coaggregation ability was expressed as a percentage reduction in the absorbance of mixed bacterial suspension compared to individual microbial suspension. \textit{Lactobacillus acidophilus} LAFTI L10 and \textit{L. paracasei} LAFTI L26 indicated higher coaggregation ability by 75\% compared to their individual coaggregation ability with other potential probiotic strains. The coaggregation ability of \textit{L. acidophilus} LAFTI L10 and \textit{B. lactis} LAFTI B94 was increased by 54\%, which was significantly different ($P < 0.01$) compared to \textit{L. paracasei} LAFTI L26 coaggregation with \textit{B. lactis} LAFTI B94 (24\%). Simultaneously, \textit{L. acidophilus} LAFTI L10 demonstrated increased ($P < 0.01$) coaggregation by 53\% with \textit{B. lactis} Bb12 compared to \textit{L. paracasei} LAFTI L26 with \textit{B. lactis} Bb12 (18\%). The coaggregation of \textit{L. acidophilus} LAFTI L10 or \textit{L. paracasei} LAFTI L26 with \textit{B. infantis} ASCC 1912 showed no differences ($P > 0.05$) in their aggregation capabilities. However, coaggregation of \textit{L. acidophilus} LAFTI L10 or \textit{L. paracasei} LAFTI L26 with \textit{L. acidophilus} ASCC 2400 demonstrated significant differences ($P < 0.01$).
Figure 2.1. Surface hydrophobicity of *L. acidophilus* LAFTI L10, *L. paracasei* LAFTI L26, *B. lactis* LAFTI B94, *B. lactis* Bb12, *B. infantis* ASCC 1912 and *L. acidophilus* ASCC 2400. Values are mean ± SEM (n = 6).
Figure 2.2. Autoaggregation abilities of *L. acidophilus* LAFTI L10, *L. paracasei* LAFTI L26, *B. lactis* LAFTI B94, *B. lactis* Bb12, *B. infantis* ASCC 1912 and *L. acidophilus* ASCC 2400. Values are mean ± SEM (n = 6).
Figure 2.3. Coaggregation abilities of *L. acidophilus* LAFTI L10, *L. paracasei* LAFTI L26, *B. lactis* LAFTI B94, *B. lactis* Bb12, *B. infantis* ASCC 1912 and *L. acidophilus* ASCC 2400. Values are mean ± SEM (n = 6). *Mean values were significantly different (P < 0.01) between the coaggregation of *L. acidophilus* LAFTI L10 or *L. paracasei* LAFTI L26 individually with other bacterial strains.*
2.5. DISCUSSION

In order to exert beneficial effects, probiotic bacteria must be tolerant to harsh gastric conditions and high bile salt concentrations in the GI tract. In humans, pH of normal gastric juice can be below 3.0, which can significantly prevent all bacterial growth. Although the pH of stomach could be as low as 1.0, several studies reported the preferred pH as 3.0 for *in vitro* assays (Garriga et al., 1998; Suskovic et al., 1997). Usman and Hosono, (1999) also suggested the survival of probiotic bacteria at pH 3.0 for 2 h as an optimal screening condition for characterising probiotic strains towards acid tolerance.

In the present study, overall reduction in cell viability of bacterial strains at various pH conditions was considered as selection criteria, which lead to the identification of 6 *Lactobacillus* and *Bifidobacterium* strains. Particularly, these 6 *Lactobacillus* and *Bifidobacterium* strains showed better survival at pH 2.0 with cell viability higher than $10^4$ CFU/ml (Table 2.1). *Lactobacillus acidophilus* LAFTI L10 remained highly tolerant to various pH conditions with less overall decrease in cell viability. In pH 2.0, *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 survived by more than $10^5$ CFU/ml, whereas other bacterial strains that were selected remained at $10^4$ CFU/ml. Strain differences at various pH conditions were observed, which were consistent to the earlier studies that reported similar strain-specific variations to simulated gastric conditions (Truelstrup Hansen et al., 2002; Mishra and Prasad, 2005).

Several studies reported the effects of various bile salt concentrations on the growth of probiotic bacteria (Clark and Martin, 1994; Lankaputhra and Shah, 1995; Prasad
et al., 1998; Truelstrup Hansen et al., 2002). There were no reports suggesting the precise concentration of bile salts that can be used as selection criteria towards probiotic characterisation. The bile concentration varies accordingly in the human GI tract; however it was believed to be 0.3% w/v (Sjovall, 1959, Gilliland et al., 1984). In the current study, bile salt concentrations of 0.5% and 1.0% were used for screening bacterial strains towards bile tolerance.

*Lactobacillus* and *Bifidobacterium* strains used in this study survived by more than $10^8$ CFU/ml in 1.0% bile salts with exception to *B. infantis* ASCC 1912, which survived at $10^7$ CFU/ml. *Lactobacillus paracasei* LAFTI L26 showed an overall higher tolerance to various bile salt concentrations, whereas *L. acidophilus* LAFTI L10 and *B. lactis* LAFTI B94 showed similar reductions in the overall cell survivability. In contrast, *L. acidophilus* LAFTI L10 demonstrated higher tolerance to 1.0% bile salts compared to *B. lactis* LAFTI B94. Interestingly, LAFTI strains such as *L. acidophilus* L10, *L. paracasei* L26 and *B. lactis* B94 demonstrated high tolerance to simulated gastric conditions and bile salt concentrations. These characteristics demonstrate that LAFTI strains are likely to survive in varying GI tract conditions, where they could exert positive health effects on the host.

Adhesion of bacterial strains to GI tract is necessary to colonise and induce beneficial effects on the host. Several direct and indirect *in vitro* methods were used to study the adherence ability of probiotic bacteria, but still there was no consensus about defining an optimal method that could predict the *in vivo* microbial adhesion. In the current study, microbial adhesion to *n*-hexadecane was used to predict the adherence ability of *Lactobacillus* and *Bifidobacterium* strains. *Bifidobacterium*
lactis Bb12 showed higher hydrophobicity when compared with other bacterial strains. The LAFTI strains, L. acidophilus L10, L. paracasei L26 and B. lactis B94 demonstrated similar hydrophobic abilities ranging between 14% – 16%. Particularly, B. infantis ASCC 1912 showed less hydrophobicity compared to other bacterial strains screened in the current study. The differences in hydrophobic ability of bacterial strains observed in this study could be due to variations in the chemical composition of cell surface components (Pelletier et al., 1997), particularly lipoteichoic acid-protein complex (Sherman and Savage, 1986) or presence of cell surface hydrophobic protein moiety (Ronner et al., 1990). Furthermore, Gomez Zavaglia et al. (2002) demonstrated the influence of bile salts, which decreased the autoaggregation ability of Bifidobacterium strains.

Earlier, several studies reported the composition, structure and forces of interaction that are related to bacterial adherence with intestinal epithelial cells (Greene and Klaenhammer, 1994; Pelletier et al., 1997; Perez et al., 1998). Moreover, the ability of aggregation is related to cell surface characteristics of bacterial strains (Vandevoorde et al., 1992; Del Re et al., 2000). Microbial aggregation is considered as a desirable characteristic of probiotic bacteria (Drago et al., 1997; Mastromarino et al., 2002; Castagliuolo et al., 2005). In the present study, autoaggregation ability of bacterial strains was examined, which could be considered as an indication of adherence characteristics. All the strains used in this study showed differences in their autoaggregation abilities. Interestingly, LAFTI strains, L. acidophilus L10, L. paracasei L26 and B. lactis B94 showed similarities in their autoaggregation and hydrophobic abilities. These results were consistent with the earlier reports, which suggested similarities in bacterial autoaggregation and hydrophobicity (Gomez
Zavaglia et al., 2002; Aslim et al., 2007). However, \textit{B. lactis} Bb12 demonstrated higher hydrophobicity and lower autoaggregation ability compared to other bacterial strains used in the current study. Similar differences in autoaggregation and hydrophobicity of bacterial strains was also reported earlier (Del Re et al., 2000).

The coaggregation ability of bacterial strains isolated from human dental plaque was the first study to suggest the process of adhesion between genetically distinct bacterial strains. However, coaggregation is now being investigated among the bacteria that were isolated from human oral cavity, mammalian GI tracts and human urogenital tract (Rickard et al., 2003). Drago et al. (1997) demonstrated the coaggregation ability of \textit{Lactobacillus} strains with enterotoxigenic \textit{E. coli}, \textit{Salmonella enteritidis} and \textit{Vibrio cholerae}. Earlier studies suggested the phenomenon of coaggregation between bacterial strains isolated from dental plaque of healthy individuals (Kolenbrander, 2000) and beneficial effects of cell aggregation in promoting the colonisation of lactobacilli in GI and vaginal tracts (Vandevoorde et al., 1992; Kmet and Lucchini, 1997; Cesena et al., 2001; Jankovic et al., 2003).

In the present study, coaggregation of \textit{L. acidophilus} LAFTI L10 with \textit{B. lactis} LAFTI B94, \textit{B. lactis} Bb12 and \textit{L. acidophilus} ASCC 2400 showed significant differences compared to \textit{L. paracasei} LAFTI L26. Vizoso Pinto et al. (2007) demonstrated higher degree of coaggregation ability of \textit{L. johnsonii} compared to \textit{L. casei} Shirota and \textit{L. rhamnosus} GG with human pathogens such as \textit{E. coli}, \textit{L. monocytogenes}, \textit{Staphylococcus aureus} and \textit{Salmonella} Typhimurium. In the current study, coaggregation of \textit{L. acidophilus} LAFTI L10 with \textit{L. paracasei} LAFTI L26 demonstrated higher coaggregating ability compared to their individual
coaggregation abilities with other bacterial strains. Especially, coaggregation abilities of *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 with pathogenic bacteria need to be investigated, which may constitute as a protective mechanism against infection (Reid et al., 1988; Schachtsiek et al., 2004).
2.6. CONCLUSIONS

*Lactobacillus acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 were selected for subsequent *in vivo* studies based on their overall tolerance to various pH conditions, especially due to their higher survival at pH 2.0. These strains also showed better tolerance to bile conditions particularly to 1.0% bile salts. All bacterial strains demonstrated the correlation of adherence abilities such as hydrophobicity and autoaggregation with exception to *B. lactis* Bb12. *Lactobacillus acidophilus* LAFTI L10 showed higher coaggregation abilities with *L. paracasei* LAFTI L26 and also with other probiotic bacterial strains.
CHAPTER 3

Effect of *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 on gut and systemic immunity and bacterial translocation in mice
3.1. ABSTRACT

The immune enhancing properties of *Lactobacillus acidophilus* LAFTI L10 and *Lactobacillus paracasei* LAFTI L26 in mice were investigated. Each mouse (BALB/c) was orally fed with cultures of either *L. acidophilus* or *L. paracasei* at $10^8$ CFU/50µl per day for 14-days. Immunofluorescence assay of the small intestine in mice demonstrated the capability of both *Lactobacillus* strains in enhancing immunoglobulin (Ig)-A, interleukin (IL)-10 and interferon (IFN)-γ producing cells. In systemic immune response, proliferative responses of splenocytes to concanavalin A (ConA; T-cell mitogen) and lipopolysaccharide (LPS; B-cell mitogen) were significantly higher in mice fed with *L. acidophilus*. ConA-induced splenocyte proliferative responses were increased significantly in mice fed with *L. paracasei*, however there was no significant difference of splenocyte proliferative responses to LPS compared to control mice. In mice fed with either *L. acidophilus* or *L. paracasei*, IL-10 and IFN-γ levels in blood serum and splenocytes stimulated with ConA were increased compared to the control group. Both *Lactobacillus* strains showed similar patterns in activating the phagocytic activity of peritoneal macrophages. *Lactobacillus acidophilus* or *L. paracasei*-fed mice modulated the translocation of *Lactobacillus* spp. and total anaerobes to Peyer’s patches and mesenteric lymph nodes when compared to control mice. Furthermore, there was no indication of disruption of intestinal mucosal integrity and thus no bacterial translocation to spleen, liver or blood in mice fed with *L. acidophilus* or *L. paracasei*. The results of this study indicate that *L. acidophilus* and *L. paracasei* are potential enhancers of gut and systemic immunity and are non-pathogenic, as suggested by their bacterial translocation profiles in healthy mice.
3.2. INTRODUCTION

The mucous covering of gastrointestinal (GI) tract has various important functions such as providing a well balanced habitat for beneficial intestinal microflora and protecting the host from pathogenic bacterial infection (Corfield et al., 2001). Lactic acid bacteria (LAB) are known to stimulate immune responses of gut associated lymphoid tissues (GALT) through lymphoid cells of GI tract (Herias et al., 1999; Perdigon et al., 1994; Perdigon et al., 1999). Evidence is accumulating to demonstrate that certain LAB promote health benefits by stimulating immune responses through phagocytosis (Perdigon et al., 1986), enhanced release of cytokines (Marin et al., 1998; Miettinen et al., 1998; Kato et al., 1999; Tejada-Simon et al., 1999) and increased mucosal and secretory antibody levels in response to Escherichia coli 0157:H7 infection (Shu and Gill, 2002) and Shigella sonnei (Nader de Macias et al., 1992). Several reports have shown that LAB influences the balance of Th1/Th2 cytokines (Borreul et al., 2003; Morita et al., 2002; Wallace et al., 2003). Stimulation of cells to produce cytokines and elimination of toxic compounds are related to the surface adherence properties of LAB (Gomez Zavaglia et al., 2002).

The effect of probiotic bacteria on immune responses is often evaluated through in vitro and ex vivo measurements of cytokine or immunoglobulin production, T- or B-cell proliferation. Gill et al. (2000) demonstrated that orally administered LAB enhances proliferative responses of splenocytes to concanavalin A (ConA; T-cell mitogen) and lipopolysaccharide (LPS; B-cell mitogen). However, Kirjavainen et al. (1999) reported that oral administration of LAB inhibits lymphocyte proliferation. Several scientific studies reported the influence of LAB in inducing cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL-12
and IL-18 in human blood mononuclear cells (Miettinen et al., 1996, Solis-Pereyra et al., 1997; Miettinen et al., 1998). Furthermore, LAB influenced the enhancement of IFN-γ levels in ConA stimulated human peripheral blood lymphocytes (De Simone et al., 1986).

Macrophages are regulatory cells that play an important role in inflammation and host defence-related functions such as phagocytosis, production of cytokines and mediators, antigen presentation, antimicrobial and tumoricidal activity. Phagocytosis is a critical function of mononuclear phagocytes and neutrophils. The ability of lactobacilli to induce cytokine production by macrophages (Marin et al., 1998; Hessle et al., 1999) and stimulation of macrophages through NF-kappa B and STAT transcription factor has been demonstrated (Mestecky et al., 1999). Human volunteers fed with either L. acidophilus La1 or B. bifidum Bb12 enhanced the phagocytosis of peripheral blood leukocytes, which persisted for 6 weeks after oral ingestion of these strains (Schiffrin et al., 1997).

Intestinal bacterial translocation is the passage of viable indigenous bacteria from gastrointestinal lumen to lymphatic organs (Peyer’s patches and mesenteric lymph nodes), spleen, liver, peritoneal cavity and blood stream (Berg, 1992). Translocation of microorganisms includes E. coli and certain members of Enterobacteriaceae and rarely anaerobes (Wells et al., 1988). Bacterial translocation in healthy mice occurs continuously at a low rate, which is a highly regulated biological process (Ma et al., 1990). The disruption of intestinal barrier or incompetent host immune system that is unable to confine an infection could lead the pathogenic bacteria to bloodstream and cause septicemia (Berg, 1999). However, translocation of LAB from gut to GALT is
considered as a normal and beneficial physiological process associated with the stimulation of immune responses (Bengmark and Jeppsson, 1995). Furthermore, scientific studies also reported the potential role of LAB in protecting gut barrier functions in animal models (Luyer et al., 2005; Daniel et al., 2006; Zareie et al., 2006).

Earlier reports showed the influence of probiotic bacteria on host immune functions in a strain dependent manner and therefore it is inappropriate to generalise the probiotic beneficial effects that are specific to genus or species (Perdigon et al., 2001; Schiffrin and Blum, 2002). As shown in Chapter 2, Lactobacillus and Bifidobacterium strains were screened to select bacterial strains that are tolerant to simulated gastric juices and bile salts. After subsequent screening of the bacterial strains, L. acidophilus LAFTI L10 and L. paracasei LAFTI L26 were identified as potential probiotic strains based on their tolerance to low pH and high bile salts. Lactobacillus acidophilus L10 and L. paracasei L26 also demonstrated better adhesive abilities such as autoaggregation and coaggregation, which are known to be important in bacterial adhesion to intestinal mucosa in order to influence host immune functions. The commercial probiotic strains may lose their potential probiotic properties and also change their physiological processes due to rigorous industrial processing conditions and repeated subculturing. Earlier in vitro studies reported that L. acidophilus L10 and L. paracasei L26 inhibited the growth of Listeria monocytogenes and E. coli 0111 (Pidcock et al., 2002). Further studies conducted using L. acidophilus L10 in mice showed the ability to inhibit the growth of L. monocytogenes in the GI tract (Mahoney and Henriksson, 2003). Elahi et al. (2005) demonstrated that mice fed with L. acidophilus L10 enhanced the clearance of
Candida albicans from oral cavities, which are correlated with increased levels of IFN-γ and nitric oxide in saliva.

There were no scientific reports that suggested the ability of L. acidophilus L10 or L. paracasei L26 in stimulating in vivo gut immune responses. However, little was known about the capability of L. acidophilus L10 or L. paracasei L26 in stimulating distant immune responses at systemic levels (Cayzer et al., 2001; Clancy et al., 2006) and furthermore no scientific studies reported the influence of these strains on bacterial translocation. Therefore, this study investigated the effects of L. acidophilus L10 and L. paracasei L26 on various indices of immune functions in gut and systemic immunity and their influence on bacterial translocation in healthy mice.
3.3. MATERIALS AND METHODS

3.3.1. Animals

Male 6 - 8 wks old BALB/c mice were obtained from Biological Resources Centre, The University of New South Wales, Sydney, Australia. Control and experimental groups consisted of six mice each, which were caged separately at 22 ± 1°C and housed under 12 h light-dark photoperiod. All mice were fed with standard mouse chow (Gordon's Specialty Stock Feeds, Sydney, Australia) and provided ad libitum access to water. The University of Western Sydney (Sydney, Australia) Animal Care and Ethics Committee approved all protocols for the animal experiment.

3.3.2. Microorganisms

*Lactobacillus acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 strains were obtained from DSM culture collection (DSM Food Specialties Ltd., Sydney, Australia). *Lactobacillus acidophilus* and *L. paracasei* were grown anaerobically in gas jars using GasPak System (Oxoid, Adelaide, Australia) for 24 h at 37°C in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Adelaide, Australia). The bacterial cells were harvested at 4000 x g for 15 min at 4°C and washed twice with sterile 0.01 M phosphate buffered saline (PBS) solution.

3.3.3. Feeding procedure

After acclimatisation for 1 week, mice were fed daily by oral gavage with either *L. acidophilus* or *L. paracasei* of 10⁸ colony-forming units (CFU) in 50µl of 10% (w/v) skimmed milk powder (SMP) for 14-days. Control group mice received 50µl of SMP without lactic acid bacteria. The viability of bacteria was determined by spread plate
count of bacterial serial dilutions on MRS agar (Oxoid, Adelaide, Australia), which were incubated anaerobically in gas jars using GasPak System at 37°C for 48 h.

3.3.4. Histological samples

At the end of 14-day feeding trial, mice were euthanized by carbon dioxide inhalation and small intestines were removed, dissected into segments and stored in 95% ethanol (v/v). The tissues were then embedded in paraffin blocks and the sections were processed for immunofluorescence assays.

3.3.5. Immunofluorescent detection of IgA cells

The number of immunoglobulin (Ig)-A secreting cells was determined in histological sections of the mice small intestine by direct immunofluorescence assay. Histological sections (4-µm) were deparaffinised by immersion in xylene and rehydrated in ethanol, which were incubated with 1% blocking solution of bovine serum albumin (Sigma-Aldrich, Sydney, Australia) for 30 min at room temperature. The sections were then incubated with 1/100 dilution of anti-IgA mono-specific antibody (α-chain specific) conjugated with fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Sydney, Australia) in 0.01 M PBS solution for 30 min at 37°C. The sections were washed three times with 0.01 M PBS solution and the immune cells were observed by using a fluorescence light microscope. Results were expressed as number of positive fluorescent cells in 10 fields of vision at 1000x magnification.
3.3.6. Detection of cytokine producing cells in small intestine

Interleukin–10 and IFN-γ producing cells were detected in histological sections of the mice small intestine by indirect immunofluorescence assay. After deparaffinization and rehydration in ethanol, histological sections (4-µm) were incubated with 1/100 dilution of rabbit anti-mouse IL-10 and IFN-γ polyclonal antibodies (Peprotech Inc., Rocky Hill, USA) for 60 min at room temperature. The incubation was followed by washing twice with 0.01 M PBS solution. The sections were then incubated with 2/100 dilution of goat anti-rabbit antibody conjugated with FITC (Jackson ImmunoResearch Laboratories Inc., West Grove, USA) for 45 min at room temperature. The number of fluorescent cells was counted using a fluorescence light microscope and expressed as positive fluorescent cells in 10 fields of vision at 1000x magnification.

3.3.7. Splenocytes

Spleens from mice were removed aseptically and placed individually in complete RPMI-1640 medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Splenocytes were isolated by chopping the spleen tissue into small pieces with sterile scissors and forcing the spleen cell suspension through a 5 ml syringe. The resulting spleen cell suspension was then transferred into a tube containing 5 ml of complete RPMI-1640 medium and centrifuged at 300 x g for 10 min. To lyse erythrocytes, cells were resuspended in ACK lysis buffer (Tris-NH₄Cl) and incubated at room temperature for 5 min followed by washing the cells twice in complete RPMI-1640 medium. The splenic lymphocyte suspensions were adjusted to a final concentration of 2 x 10⁶ cells/ml in complete RPMI-1640 medium. Trypan blue exclusion method was used to determine the cell viability.
3.3.8. Lymphocyte proliferation assay

A 50 µl of complete RPMI-1640 medium with $2 \times 10^6$ cells ml$^{-1}$ were added in triplicates to the wells of a 96-well tissue culture plate and cultured in the presence or absence of T- and B-cell mitogens. A 50 µl/well of pre-optimised concentrations of mitogens, concanavalin A (ConA, 2.5 µg/ml; Sigma, Sydney, Australia) and lipopolysaccharide (LPS, 5 µg/ml, derived from *E. coli*; Sigma, Sydney, Australia) were added to the wells. Control wells received 50 µl of complete RPMI-1640 medium. The cells were cultured for 72 h at 37°C in a humidified incubator with CO$_2$ and air (5:95, v/v). The cell proliferation over the final 4 h of culture was determined by using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, USA). The absorbance of each well was read at 492nm using a microtitre plate reader (Multiskan Titertek, Huntsville, USA).

3.3.9. Estimation of cytokines in blood and splenocytes

Blood was collected upon euthanizing the mice after 14-day feeding trial and left to coagulate overnight at 4°C. Serum was then processed from blood by centrifugation at 2000 x g for 10 min and stored at -20°C until cytokines were assayed. Serum was used to estimate the presence of IL-10 and IFN-γ using mouse IL-10 and mouse IFN-γ sandwich ELISA kits (Chemicon International Inc., Temecula, USA).

Spleen cell suspensions ($2 \times 10^6$ cells ml$^{-1}$) were added in triplicates to the wells of a 96-well tissue culture plate and cultured in the presence of ConA (2.5 µg/ml) for 48 h at 37°C in a humidified incubator with CO$_2$ and air (5:95, v/v). The supernatant fractions were harvested from cell suspensions and stored at -80°C until cytokine
analysis. Estimation of IL-10 and IFN-γ in the supernatants was determined using mouse IL-10 (Chemicon International Inc., Temecula, USA) and mouse IFN-γ (Assay Designs Inc., Ann Arbor, USA) sandwich ELISA kits.

3.3.10. Phagocytosis

Peritoneal macrophages were isolated from the peritoneal cavity of each mouse by flushing with 5 ml of RPMI-1640 medium. Peritoneal macrophages were washed twice and adjusted to a final concentration of $10^6$ cells/ml in RPMI-1640 medium. Heat killed (100°C, 15 min) *Candida albicans* ATCC 10231 suspension ($10^6$ cells ml$^{-1}$) of 0.2 ml opsonised with 10% mouse autologous serum for 15 min at 37°C was added to 0.2 ml of each macrophage suspension at a concentration of $10^6$ cells/ml and incubated for 30 min at 37°C. The results were expressed as percentage of phagocytosis that was determined by counting over 100 macrophages using a Nikon optical microscope.

3.3.11. Bacterial translocation assay

Bacterial translocation to Peyer’s patches, mesenteric lymph nodes (MLN), spleen, liver and blood was determined in control and experimental group mice. The Peyer’s patches, MLN, spleen and a sample of liver tissue were aseptically removed from each animal and washed twice with 0.01 M PBS solution, which were transferred to pre-weighed tubes containing 0.01 M PBS. The organs were weighed, homogenised and serially diluted in 0.01 M PBS. Blood collected through cardiac puncture was also serially diluted in 0.01 M PBS. Tissue homogenates and blood suspensions from each mouse were plated separately in triplicates on MRS agar for detection of
*Lactobacillus* spp., Wilkins-Chalgren (WC) Anaerobe agar (Oxoid, Adelaide, Australia) containing 5% horse blood (Oxoid, Adelaide, Australia) for detection of total anaerobes and MacConkey agar (Oxoid, Adelaide, Australia) for detection of enteric bacteria. Plates were incubated under anaerobic (MRS and WC agar plates) and aerobic (MacConkey agar plates) conditions at 37°C for 48 h. Colonies were enumerated on appropriate agar plates and expressed as either colony forming units per g (CFU g⁻¹) or per ml (CFU ml⁻¹).

### 3.3.12. Statistical analysis

The results were expressed as mean ± standard error of mean (SEM). The data were analysed using the one-way analysis of variance procedure of SPSS (Version 12.0.1). Significant differences between groups were identified by Duncan’s multiple range test (SPSS, Version 12.0.1).
3.4. RESULTS

3.4.1. Determination of IgA producing cells

The effect of orally administered LAB on IgA producing cells in lamina propria of the small intestine was determined by direct immunofluorescence assay (Fig. 3.1A, B). In comparison to control mice, mice orally fed with *L. acidophilus* or *L. paracasei* significantly enhanced (*P* < 0.001) the IgA producing cells in the small intestine. *Lactobacillus acidophilus*-fed mice showed an increase of 68% in IgA cells compared to control mice, whereas *L. paracasei*-fed mice demonstrated 20% increase in IgA cells. The differences between *L. acidophilus* and *L. paracasei* experimental groups were significant (*P* < 0.001) in enhancing the number of IgA cells.

3.4.2. Effect of LAB on cytokines in gut

The effect of oral administration of LAB in enhancing cytokine producing cells in lamina propria of the small intestine was determined by indirect immunofluorescence assay (Fig. 3.2A, B). Significant increase (*P* < 0.001) of IL-10 and IFN-γ producing cells were detected in the small intestine of mice fed with *L. acidophilus* or *L. paracasei* when compared to the control mice. Specifically, *L. acidophilus*-fed mice showed an increase of 63% of IL-10 producing cells and more than 200% of IFN-γ producing cells compared to control group. Simultaneously, *L. paracasei* demonstrated an increase of 18% of IL-10 and 95% of IFN-γ producing cells, however the increase was lesser compared to mice fed with *L. acidophilus*. 
3.4.3. Lymphocyte proliferative responses

The proliferative responses of spleen cells stimulated with ConA and LPS were estimated to determine the effect of orally administered LAB on T- and B-cell functions in each experimental group (Table 3.1). The proliferative responses of splenocytes stimulated with ConA were significantly higher in mice fed with *L. acidophilus* (*P* < 0.01) or *L. paracasei* (*P* < 0.05) compared to control mice. In comparison to control mice, LPS induced proliferative responses were increased (*P* < 0.05) in mice fed with *L. acidophilus*. The splenocyte proliferative responses induced by LPS were higher in mice fed with *L. paracasei* but were not significantly different (*P* > 0.05) compared to control mice. In contrast, *L. acidophilus*-fed mice demonstrated higher (*P* < 0.01) proliferative responses when stimulated with ConA and LPS compared to mice fed with *L. paracasei*.

3.4.4. Effect of LAB on cytokines in blood serum and splenocytes

The effects of *L. acidophilus* and *L. paracasei* in systemic immunity were determined by estimating the serum cytokines. *Lactobacillus acidophilus* or *L. paracasei* demonstrated a significant increase (*P* < 0.001) of IL-10 and IFN-γ levels in serum compared to control mice (Fig. 3.3A, B). The increase of IL-10 and IFN-γ levels detected in serum of mice fed with *L. acidophilus* were higher (*P* < 0.001) compared to mice fed with *L. paracasei*.

The effects of ConA induced cytokine production in spleen cells from mice fed with *L. acidophilus* or *L. paracasei* were estimated (Fig. 3.4A, B). In mice fed with *L. acidophilus*, production of IL-10 (*P* < 0.01) and IFN-γ (*P* < 0.05) by splenocytes were enhanced when compared to control mice. Splenocytes from mice fed with *L.
*paracasei* also demonstrated significantly higher levels of IL-10 and IFN-γ (*P* < 0.05) compared to control mice. The differences between mice fed with *L. acidophilus* and *L. paracasei* in enhancing IL-10 production was significant (*P* < 0.01). However, there were no differences (*P* > 0.05) between *L. acidophilus* and *L. paracasei* in inducing IFN-γ levels.
Figure 3.1. Effect of A) *L. acidophilus* L10 and B) *L. paracasei* L26 on immunoglobulin (Ig)-A producing cells in the small intestine of mice. Values are mean ± SEM (n = 4). *Mean values were significantly different (P < 0.001) from the control group. #Significant differences (P < 0.001) between *L. acidophilus* L10 and *L. paracasei* L26 experimental groups.
**Figure 3.2.** Effect of orally administered *L. acidophilus* L10 or *L. paracasei* L26 on A) interleukin (IL)-10 and B) interferon (IFN)-γ cytokine producing cells in the small intestine of mice. Values are mean ± SEM (n = 6). *Mean values were significantly different (*P < 0.001*) from the control group. #Significant differences (*P < 0.001*) between *L. acidophilus* L10 and *L. paracasei* L26 experimental groups.
Table 3.1. Proliferative responses of spleen cells stimulated with ConA and LPS in mice fed with either *L. acidophilus* L10 or *L. paracasei* L26.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mitogen stimulated splenocytes (Absorbance – OD 492 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ConA</td>
</tr>
<tr>
<td>Control</td>
<td>3.28 ± 0.19</td>
</tr>
<tr>
<td><em>L. acidophilus</em> L10</td>
<td>5.04 ± 0.29 **.*, #</td>
</tr>
<tr>
<td><em>L. paracasei</em> L26</td>
<td>4.25 ± 0.32 *., #</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6).
Significantly different from the control mice *P < 0.05; **P < 0.01.
Significant differences between *L. acidophilus* L10 and *L. paracasei* L26 experimental groups *P < 0.01.
Figure 3.3. Estimation of cytokines A) interleukin (IL)-10 and B) interferon (IFN)-γ in serum of mice fed with *L. acidophilus* L10 or *L. paracasei* L26. Values are mean ± SEM (n = 6). *Mean values were significantly different (P < 0.001) from the control group. #Significant differences (P < 0.001) between *L. acidophilus* L10 and *L. paracasei* L26 experimental groups.
Figure 3.4. Production of A) interleukin (IL)-10 and B) interferon (IFN)-γ by spleen cells from mice orally fed with either *L. acidophilus* L10 or *L. paracasei* L26. Values are mean ± SEM (n = 6). Significantly different from the control mice *P < 0.05; **P < 0.01. Significant differences between *L. acidophilus* L10 and *L. paracasei* L26 experimental groups #P < 0.01.
3.4.5. Phagocytic activity

The phagocytic activity of peritoneal macrophages in mice fed with either *L. acidophilus* or *L. paracasei* was determined (Fig. 3.5A, B). Macrophages isolated from the peritoneal cavity of mice fed with *L. acidophilus* or *L. paracasei* showed significantly increased (*P* < 0.01) phagocytic activity against *Candida albicans* when compared with the control mice. In contrast, *L. acidophilus* and *L. paracasei* strains showed no differences (*P* > 0.05) in inducing phagocytic activity of the peritoneal macrophages.

3.4.6. Bacterial translocation

Bacterial translocation to Peyer’s patches, MLN, spleen, liver and blood was determined in mice fed with *L. acidophilus* or *L. paracasei* to evaluate possible adverse effects that could lead to systemic dysfunction. Translocation of *Lactobacillus* spp. to Peyer’s patches was significantly different in mice fed with *L. acidophilus* (*P* < 0.01) or *L. paracasei* (*P* < 0.05) compared to control mice (Table 3.2). In mice fed with *L. acidophilus* or *L. paracasei*, *Lactobacillus* spp. translocated to MLN was also increased (*P* < 0.01) compared to control mice. There were no differences (*P* > 0.05) between mice fed with *L. acidophilus* and *L. paracasei* in relation to the increased translocation of *Lactobacillus* spp. to Peyer’s patches and MLN. Furthermore, there was no incidence of translocation of *Lactobacillus* spp. to spleen, liver and blood in mice fed with either *L. acidophilus* or *L. paracasei* and control group (Table 3.2).

Total anaerobes translocated to Peyer’s patches in *L. acidophilus*-fed mice was higher (*P* < 0.01) compared to control mice (Table 3.3). Translocation of total
anaerobes to Peyer’s patches was also increased in mice fed with *L. paracasei* but not significantly different (*P* > 0.05) when compared with control mice. Mice fed with either *L. acidophilus* or *L. paracasei* showed increased (*P* < 0.01) translocation of total anaerobes to MLN compared to control mice (Table 3.3). There were no significant differences (*P* > 0.05) between mice fed with *L. acidophilus* and *L. paracasei* in relation to increased translocation of total anaerobes to Peyer’s patches. However, *L. acidophilus* and *L. paracasei* strains demonstrated the differences in translocation of total anaerobes to MLN. There was no evidence of translocation of total anaerobes to spleen, liver and blood in mice fed with either *L. acidophilus* or *L. paracasei* and control group (Table 3.3).

In mice fed with *L. acidophilus* or *L. paracasei* and control group, translocation of enteric bacteria to Peyer’s patches, MLN, spleen, liver and blood was not detected (Table 3.4).
Figure 3.5. Effect of A) *L. acidophilus* L10 and B) *L. paracasei* L26 on phagocytic activity of the peritoneal macrophages. Results are the mean ± SEM (n = 6). Significantly different from the control mice *P* < 0.01.
Table 3.2. Translocation of *Lactobacillus* spp. to Peyer’s patches, mesenteric lymph nodes (MLN), spleen, liver and blood in mice orally fed with either *L. acidophilus* L10 or *L. paracasei* L26 for 14-days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peyer’s patches&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MLN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Spleen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Liver&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Blood&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.61 ± 0.08</td>
<td>4.08 ± 0.06</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>L. acidophilus</em> L10</td>
<td>4.93 ± 0.16&lt;sup&gt;**&lt;/sup&gt;</td>
<td>4.42 ± 0.14&lt;sup&gt;**&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>L. paracasei</em> L26</td>
<td>4.77 ± 0.06&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.49 ± 0.10&lt;sup&gt;**&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6).
Significantly different from the control mice *P* < 0.05; **P** < 0.01.

<sup>a</sup>Detection limit was 10<sup>2</sup> CFU g<sup>-1</sup>
<sup>b</sup>Detection limit was 10<sup>2</sup> CFU ml<sup>-1</sup>
<sup>c</sup>Not detected.
Table 3.3. Translocation of total anaerobes to Peyer’s patches, mesenteric lymph nodes (MLN), spleen, liver and blood in mice orally fed with either *L. acidophilus* L10 or *L. paracasei* L26 for 14-days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Organ</th>
<th>Peyer’s patches&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MLN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Spleen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Liver&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Blood&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>5.01 ± 0.12</td>
<td>4.24 ± 0.04</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>L. acidophilus</em> L10</td>
<td></td>
<td>5.36 ± 0.19&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.51 ± 0.02&lt;sup&gt;*,#&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>L. paracasei</em> L26</td>
<td></td>
<td>5.15 ± 0.12</td>
<td>4.69 ± 0.03&lt;sup&gt;*,#&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6).

Significantly different from the control mice *P* < 0.01.

Significant differences between *L. acidophilus* and *L. paracasei* experimental groups #P < 0.01.

<sup>a</sup>Detection limit was 10<sup>2</sup> CFU g<sup>-1</sup>

<sup>b</sup>Detection limit was 10<sup>2</sup> CFU ml<sup>-1</sup>

<sup>c</sup>Not detected.
Table 3.4. Translocation of enteric bacteria to Peyer’s patches, mesenteric lymph nodes (MLN), spleen, liver and blood in mice orally fed with either *L. acidophilus* L10 or *L. paracasei* L26 for 14-days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peyer’s patches(^a)</th>
<th>MLN(^a)</th>
<th>Spleen(^a)</th>
<th>Liver(^a)</th>
<th>Blood(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND(^c)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>L. acidophilus</em> L10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>L. paracasei</em> L26</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6).
\(^a\)Detection limit was $10^2$ CFU $g^{-1}$
\(^b\)Detection limit was $10^2$ CFU $ml^{-1}$
\(^c\)Not detected.
3.5. DISCUSSION

The external secretions of IgA in GI tract play an active role in protecting the surfaces of mucus membrane from pathogenic infections and carcinogens (Mestecky and McGhee, 1987). The formation of immune complexes in intestinal lumen to bind antigens was the protective effect of antigen-specific secretory IgA, which interferes with the adherence of pathogenic microorganisms to intestinal epithelial cells and also helps in neutralising toxins and viruses (Mestecky et al., 1999). In the current study, *L. acidophilus* and *L. paracasei* strains enhanced the number of IgA producing cells in the small intestine of mice (Fig. 3.1A, B), which suggest that these strains could protect the GI tract from pathogenic microorganisms through increased IgA response. In comparison to *L. paracasei*, *L. acidophilus* showed an increased number of IgA producing cells in the lamina propria that could be beneficial to the host in conferring greater protection against pathogenic microorganisms. Earlier, it was reported that peptides derived from *L. helveticus* fermented milk enhanced the IgA secretion in gut and systemic immunity in mice challenged with *E. coli* O157:H7 (Leblanc et al., 2004).

The Th1 cells produce important factors such as IL-2, IFN-γ and tumor necrosis factor for cell-mediated immunity. Cytokines like IL-4, IL-5 and IL-10 associated with humoral immunity and allergic responses are produced by Th2 cells (Kidd, 2003). The Th1 cytokine, IFN-γ is a multifunctional pro-inflammatory cytokine that triggers innate immune responses such as phagocytosis and antimicrobial activity, whereas Th2 cytokine, IL-10 regulates a number of events including IFN-γ and antibody production (Kidd, 2003). Both IL-10 and IFN-γ were considered as pivotal cytokines in inducing Th1 and Th2 responses (Perdigon et al., 2002). In this study, *L.*
acidophilus and L. paracasei strains were effective in activating mucosal and systemic immune responses through the release of IL-10 and IFN-γ cytokines (Fig. 3.2; 3.3; 3.4). Perdigon et al. (1990) reported that mice fed with fermented milk consisting of LAB were protected against Salmonella Typhimurium infection. Furthermore, Gould and Sonnenfeld, (1987) demonstrated that mice pre-treated with IFN-γ were also protected against Salmonella Typhimurium strain LT-2. In the present study, increase of IFN-γ producing cells in the intestinal mucosa of mice fed with L. acidophilus or L. paracasei could therefore contribute to the protection of mucosal surfaces against harmful pathogenic microorganisms.

Lymphocytes play an important role in the regulation of immune responses. T-lymphocytes are the mediators of cellular immunity, whereas B-lymphocytes develop into antibody producing cells in response to foreign antigens. In the current study, mitogen-stimulated lymphocyte proliferative responses were used to assess the T- and B-cell functions. Mice fed with L. acidophilus or L. paracasei exhibited the enhancement of splenocyte proliferative responses to T-cell mitogen (ConA) and B-cell mitogen (LPS). Although, LPS stimulated B-cell proliferative responses in L. paracasei-fed mice was higher, but not significantly different from the control mice (Table 3.1). The present study demonstrated the differences in mitogen-stimulated proliferative responses of splenocytes, which could be due to strain-specific variations. Kirjavainen et al. (1999) demonstrated similar strain-specific effects of mitogen-stimulated splenocyte proliferative responses of T- and B-lymphocytes.

It is widely known that the balance between Th1 and Th2 responses was important to elevate immunological responses against various diseases. Lactic acid bacteria are
known to modulate the balance of Th1 and Th2 immune responses (Maassen et al., 2000; Perdigon et al., 2002). Human volunteers fed with lyophilised lactobacilli showed an increased production of IFN-γ in serum (De Simone et al., 1993). Schultz et al. (2003) reported that oral administration of *L. rhamnosus* GG to healthy human volunteers stimulated an increased secretion of anti-inflammatory cytokines (IL-10 and IL-4) and decreased secretion of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-6). In the present study, IL-10 was significantly increased in serum as well as in splenocytes stimulated with ConA of mice fed with *L. acidophilus* or *L. paracasei*. The magnitude in the increment of IL-10 was higher than Th1 cytokine IFN-γ, which may be necessary to control the increase of the pro-inflammatory cytokine IFN-γ (Fig. 3.3; 3.4).

In the current study, splenocytes from mice fed with *L. acidophilus* or *L. paracasei* significantly increased the production of IL-10 and IFN-γ cytokines (Fig. 3.4A, B). Roller et al. (2004) reported similar enhancement of IL-10 and IFN-γ production in Peyer’s patches of rats fed with synbiotics (Probiotics and prebiotics). In another study, CD4⁺ T-cells from Peyer’s patches of mice fed with dietary fructooligosaccharides showed dose-dependent increased production of IL-10 and IFN-γ levels (Hosono et al., 2003). The current study demonstrates that *L. acidophilus* and *L. paracasei* were able to influence the regulation of Th1 and Th2 cytokines in gut and systemic immunity through increased production of IL-10 and IFN-γ (Fig. 3.2; 3.3; 3.4). In contrast, *L. acidophilus*-fed mice stimulated higher levels of IL-10 and IFN-γ compared to the increase detected in mice fed with *L. paracasei*. 
The influence of LAB on phagocytosis could be strain-specific, dose dependent or survivability in the GI tract (Gill et al., 2000; Gill and Rutherfurd, 2001; Medici et al., 2005). The differences in cell surface structures of *Lactobacillus* strains lead to strain-specific variations in modulating immune responses (Sato et al., 1988). The ability of LAB in stimulating phagocytic cells also depends on its survival and persistence in GI tract and their adherence to intestinal epithelial cells (Perdigon and Alvarez, 1992; Schiffrin et al., 1997). In the present study, orally administered *L. acidophilus* or *L. paracasei* strains enhanced the phagocytic activity of peritoneal macrophages (Fig. 3.5A, B), which suggests that these strains are tolerant to gastric conditions of GI tract and can persist through the GI passage to induce host immunological responses. Mice fed with *L. acidophilus* showed an increase of 21% activation of peritoneal macrophages compared to control mice, whereas *L. paracasei*-fed mice showed only 6% activation. However, there were no significant differences observed between *L. acidophilus* and *L. paracasei* in inducing macrophage activity.

Gut mucosal integrity is crucial in host immune defence mechanisms, such as protection against pathogenic bacteria through interfering their adherence to intestinal epithelial cells (Mestecky et al., 1999). Translocation of indigenous microflora to distant intestinal sites such as spleen, liver and blood could occur due to intestinal inflammation that causes the disruption of intestinal mucosal surfaces. Vinderola et al. (2004) reported the translocation of various doses of different LAB to liver, which activated gut and systemic immune responses. In the present study, there was no evidence of translocation of intestinal microflora to spleen, liver or blood in both the experimental group mice (Table 3.2; 3.3), which suggests that oral
administration of *L. acidophilus* or *L. paracasei* at 10^8 CFU/day did not induce adverse effects such as disruption of immune defence mechanisms that leads to inflammation of intestinal mucosal layers and bacterial translocation to sterile organs.

The absence of bacterial translocation to spleen, liver or blood also demonstrates the active role of immune defence mechanisms that could have inhibited the translocation beyond MLN. Additionally, enteric bacteria were not detected in Peyer’s patches, MLN, spleen, liver and blood either in control or experimental group mice (Table 3.4).

Systemic dissemination of bacteria in mice has been correlated with the adhesion of probiotic bacteria to intestinal epithelial cells (Wagner et al., 1997). Earlier reports suggested that adhesion of bacterial strains to gut epithelial cells is necessary to modulate immune responses (Perdigon and Alvarez, 1992; Schiffrin et al., 1997). In the present study, *L. acidophilus*-fed mice showed increased translocation of *Lactobacillus* spp. to Peyer’s patches (7%) and MLN (8%) compared to control mice, whereas *L. paracasei*-fed mice demonstrated the translocation of *Lactobacillus* spp. with an increase of 3% to Peyer’s patches and 10% to MLN (Table 3.2). However, increased translocation of *Lactobacillus* spp. in mice fed with *L. acidophilus* or *L. paracasei* were statistically significant compared to control mice but showed very small differences. Similar small differences were also observed in the translocation of total anaerobes to Peyer’s patches and MLN in both the experimental groups with respect to control mice (Table 3.3). The results of this study suggest that *L. acidophilus* and *L. paracasei* have potential adhesive properties
that can lead to the translocation of *Lactobacillus* spp. to Peyer’s patches and MLN, which may contribute to the enhancement of various systemic immune functions.
3.6. CONCLUSIONS

Orally administered *L. acidophilus* and *L. paracasei* demonstrated the enhancement of gut mucosal immunity in relation to the increase of IgA and cytokines (IL-10 and IFN-γ). In systemic immunity, *L. acidophilus* and *L. paracasei* enhanced the phagocytic activity of peritoneal macrophages and release of IL-10 and IFN-γ cytokines from serum and splenocytes. In contrast, *L. acidophilus* showed higher stimulation of gut and systemic immune responses compared to *L. paracasei*. Additionally, there was no evidence of bacterial translocation to spleen, liver or blood in mice fed with either *L. acidophilus* or *L. paracasei*. Therefore, *L. acidophilus* and *L. paracasei* strains are considered non-pathogenic, as indicated by their effects on bacterial translocation in healthy mice.
CHAPTER 4

Immunostimulatory responses of *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 in cholera toxin mice
4.1. ABSTRACT

Mice were orally fed with either *L. acidophilus* or *L. paracasei* for 14-days, during which they were immunized twice with 10 µg of cholera toxin (CT). *Lactobacillus acidophilus*-fed mice showed an increase (*P < 0.01*) in serum nitric oxide (NO) levels. The increment of NO levels in intestinal fluid and serum of *L. paracasei*-fed mice was significantly different compared to control mice. Immunoglobulin (Ig)-A response to CT in intestinal fluid and serum were significantly enhanced by *L. acidophilus* or *L. paracasei*. Cytokine producing cells in small intestine of mice fed with either *L. acidophilus* or *L. paracasei* demonstrated the regulation of Th1 and Th2 cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-6 and IL-10. The results of this study demonstrate that *L. acidophilus* and *L. paracasei* are capable of enhancing gut immune responses as well as serum NO and IgA in systemic levels of CT immunized mice.
4.2. INTRODUCTION

Gastrointestinal (GI) tract is considered to be a complex ecosystem consisting of various resident microorganisms such as *Escherichia coli*, *Clostridium* spp., *Streptococcus* spp., *Lactobacillus* spp., *Bacteroides* spp., and *Bifidobacterium* spp., which are involved in the development of gut microflora and maintains host health by exerting specific gut functions (McCracken and Lorenz, 2001). The microbial population in GI tract, specifically, *Lactobacillus* and *Bifidobacterium* spp. were considered as probiotic due to their beneficial health effects such as inhibiting the growth of *Escherichia coli* (Forestier et al., 2001), *Helicobacter pylori* (Wang et al., 2004) and resistance to diseases (Shanahan, 2002; de Waard et al., 2003). Microorganisms of GI tract have the potential to influence immunoglobulin (Ig)-A production for the development and activation of gut immune system (Dogi and Perdigon, 2006). Furthermore, commensal bacteria also help in promoting the development of B-cell population in Peyer’s patches that were not developed in germ-free animals. In comparison to germ-free animals, IgA producing cells were higher in intestinal lamina propria of animals possessing normal intestinal microflora (Crabbe et al., 1968; Jiang et al., 2004). Due to the significance of IgA in host immune functions, it is important to screen the capability of probiotic bacteria in enhancing antibody response in gut and systemic immunity.

Oral administration of appropriate microbial strains could potentially restore and maintain oral tolerance and host immune functions (Sudo et al., 1997; Kalliomaki et al., 2001). Korhonen et al. (2001) reported that *L. rhamnosus* GG induced the production of nitric oxide (NO), which played a functional role in protective mechanisms of intestinal mucosa and regulating immune functions. In rats, dietary
supplementation of food with lactobacilli and nitrate increased the NO levels in small intestine and caecum (Sobko et al., 2006). The endogenous NO was known to have potential role in regulating various host functions including water and electrolyte transport (Mourad et al., 1999) and motility (Mashimo et al., 2000). Scientific reports speculated that some of the beneficial effects exerted by probiotic bacteria were mediated through NO release in intestinal lumen (Xu and Verstraete, 2001; Lamine et al., 2004; Sobko et al., 2005). Sobko et al. (2005) suggested the relationship between increased number of intestinal microflora with higher NO levels in the intestine of healthy new born infants. The source of NO detected in the lumen of new born infants however remains unclear.

Gastrointestinal epithelial cells act as physical and chemical barriers by protecting the host from harmful microorganisms that could hijack the regulation of cellular molecules and signalling pathways (Cossart, 1997; Finlay and Falkow, 1997). Epithelial cells of GI tract were considered important in mucosal immune system due to their interdependency on adjacent lymphoid cells (Shanahan, 1999). Cytokines such as interleukin (IL)-6, IL-10 and tumor necrosis factor (TNF)-α were few of the several cytokines secreted by intestinal epithelial cells (McCracken and Gaskins, 1999). Enhanced secretory antibodies and variations in mucous layers along the intestine could be crucial in strengthening the intestinal barrier of GI tract (Yuan and Walker, 2004). Earlier studies reported the capability of probiotic bacteria in normalising and reinforcing gut barrier functions and protecting the host from pathogenic bacteria (Terpend et al., 1999; Madsen et al., 2001).
Cholera toxin (CT), the enterotoxin of *Vibrio cholerae*, which affects epithelial cells by inducing massive salt and water secretion results in severe diarrhoea. The toxin is considered as a potent adjuvant and immunogen that can influence mucosal and systemic immune functions (Elson and Ealding, 1984). Several scientific reports demonstrated CT as a mucosal adjuvant by enhancing IgA and IgG immune responses, increasing T-cell antigen priming and inhibiting oral tolerance to unrelated antigens (Elson and Ealding, 1984; Lycke et al., 1985; Hornqvist et al., 1991). Mucosal adjuvants are also known to activate immune responses by stimulating cytokines that are involved in innate and acquired immunity (Elson and Dertzbaugh, 1994). The degradation of orally administered antigens could occur in GI tract due to constant changes such as low gastric pH and high bile salts, which further may lead to either ineffective or minimal immune responses. Considerable attention was given to lactic acid bacteria as an adjuvant due to their general use as safe food grade microorganisms in the manufacturing of fermented dairy products. Several studies have shown the differences between LAB strains in inducing adjuvant effects when delivered orally with antigens (McCracken and Gaskins, 1999; Tejada-Simon et al., 1999a; Plant and Conway, 2002).

As shown in chapter 3, *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 demonstrated the stimulation of various immune functions in gut and systemic immunity. Particularly, *L. acidophilus* L10 demonstrated higher stimulation of gut and systemic immune responses with respect to *L. paracasei* L26. However, it is unclear whether *L. acidophilus* L10 and *L. paracasei* L26 have the potential to alter the regulation of B- and T-cell functions after oral immunisation with an antigen in BALB/c mice. Therefore, present study aimed to use CT as an oral antigen to
determine the immune responses enhanced by oral administration of *L. acidophilus* L10 and *L. paracasei* L26 in mice. The effects of *L. acidophilus* L10 and *L. paracasei* L26 on various indices of immune functions such as NO, CT-specific IgA response and Th1/Th2 cytokine producing cells in the small intestine and serum NO and CT-specific IgA levels were evaluated.
4.3. MATERIALS AND METHODS

4.3.1. Mice

Male 6-week old BALB/c mice were purchased from Biological Resources Centre, The University of New South Wales, Sydney, Australia. Mice were housed at 22 ± 1°C under 12 h light-dark photoperiod and fed with standard mouse chow (Gordon’s Speciality Stock Feeds, Sydney, Australia) and provided ad libitum access to water. Control and experimental groups consisted of six mice each, which were randomly allocated. All experiments were performed with the approval from Animal Care and Ethics Committee of University of Western Sydney, Sydney, Australia.

4.3.2. *Lactobacillus* strains and feeding procedure

*Lactobacillus acidophilus* LAFTI L10 and *Lactobacillus paracasei* LAFTI L26 used in this study were obtained from DSM culture collection (DSM Food Specialties Ltd., Sydney, Australia). The bacterial strains were grown under anaerobic conditions in gas jars using GasPak System (Oxoid, Adelaide, Australia) at 37°C for 24 h in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Adelaide, Australia) and washed twice with sterile 0.01 M phosphate buffered saline (PBS; Sigma-Aldrich, Sydney, Australia) solution following centrifugation at 4000 x g for 15 min at 4°C. The viability of bacteria was determined by spread plate count of bacterial serial dilutions on MRS agar (Oxoid, Adelaide, Australia), which were incubated anaerobically for 48 h at 37°C. After acclimatisation for 1 week, mice (six per group) were fed daily by oral gavage with $10^8$ colony-forming units (CFU) of either *L. acidophilus* or *L. paracasei* in 50 μl of 10% (w/v) skimmed milk powder (SMP) for 14-days. Control group mice received 50 μl of SMP without lactic acid bacteria.
4.3.3. Cholera toxin immunisation

Control and experimental group mice were orally immunized with 10 µg of cholera toxin (Sigma-Aldrich, Sydney, Australia) in 25 µl of 0.1 M sodium bicarbonate (Sigma-Aldrich, Sydney, Australia) on day 0 and 7. Mice were sacrificed by carbon dioxide inhalation after 14-day feeding trial and intestinal fluid, blood and small intestines were collected.

4.3.4. Intestinal fluid and serum preparation

Intestinal fluid of small intestine in each mouse was collected by flushing the intestinal contents with 2 ml of 0.01 M PBS and centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was collected and stored at -20°C until use.

Blood was collected upon euthanizing the mice and left to coagulate overnight at 4°C and centrifuged at 2000 x g for 10 min (4°C). The serum was collected and stored at -20°C until use.

4.3.5. Determination of nitric oxide

Nitric oxide was determined by measuring nitrite accumulation in intestinal fluid and serum using Griess reagent system (Promega, Sydney, Australia). Briefly, 50 µl of intestinal fluid supernatant or serum were added in triplicates to the wells, which were incubated with 50 µl of sulfanilamide solution for 10 min at room temperature while protected from light. After adding 50 µl of N-1-napthylethylenediamine dihydrochloride solution to all the wells, absorbance was read at 540 nm using a microtitre plate reader (Multiskan Titertek, Huntsville, USA).
4.3.6. Estimation of Immunoglobulin A

The CT-specific IgA antibodies in intestinal fluid and serum were detected by ELISA. Briefly, each well of the 96-well microtitre plate was coated with 100 µl of CT (10 µg/ml) in 0.05 M carbonate-bicarbonate buffer, pH 9.6. For standard, 100 µl of goat anti-mouse IgA (Bethyl Laboratories Inc., Montgomery, USA) was added to the wells in triplicates at 10 µg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6. The plates were incubated overnight at 4°C and washed three times with 0.01 M PBS containing 0.05% Tween 20 (PBS-T; Sigma-Aldrich, Sydney, Australia). Then the plates were incubated for 2 h at 37°C with either 100 µl of mouse IgA-kappa (Sigma-Aldrich, Sydney, Australia) as a standard or 100 µl of intestinal fluid or serum, which were added in triplicates. The plates were washed three times with PBS-T and incubated with 100µl of goat anti-mouse IgA horseradish peroxidase conjugate (Sigma-Aldrich, Sydney, Australia) for 90 min at 37°C. Incubation was followed by washing the plates three times with PBS-T and 200µl of o-phenylenediamine dihydrochloride peroxide substrate (Sigma-Aldrich, Sydney, Australia) was added to the wells and incubated for 30 min at room temperature. The absorbance was read at 450 nm using a microtitre plate reader (Multiskan Titertek, Huntsville, USA).

4.3.7. Histological samples

Mice small intestines were removed, dissected into segments and stored in 95% ethanol (v/v). The tissues were then embedded in paraffin blocks and the sections were processed for immunofluorescence assays.
4.3.8. Immunofluorescent detection of cytokine producing cells

The number of cytokine secreting cells in small intestine of mice was determined by indirect immunofluorescence assay. The paraffin sections (4-μm) were deparaffinised in xylene and rehydrated in ethanol, which were then washed twice with 0.01 M PBS solution and incubated with a blocking solution (0.01 M PBS containing 1% bovine serum albumin) for 30 min at room temperature. The sections were incubated with 0.2 μg/ml (diluted in 0.01 M PBS) of rabbit anti-mouse IL-4, IL-6, IL-10, interferon (IFN)-γ and TNF-α (Peprotech Inc., Rocky Hill, USA) polyclonal antibodies for 75 min at 37°C. The incubation was followed by washing the sections twice with 0.01 M PBS. The sections were then incubated with goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC; Jackson Immuno Research Labs Inc., West Grove, USA) for 45 min at room temperature and washed twice with 0.01 M PBS. The number of fluorescent cells was counted by using fluorescence light microscope (Olympus Optical Co., Tokyo, Japan) and the results were expressed as number of positive fluorescent cells in 10 fields of vision.

4.3.9. Statistical analysis

The results were expressed as mean ± standard error of mean (SEM). The data were analysed using the one-way analysis of variance procedure of SPSS (Version 12.0.1). Significant differences between groups were identified by Duncan’s multiple range test (SPSS, Version 12.0.1).
4.4. RESULTS

4.4.1. Nitric oxide production in intestinal fluid and serum

The effect of orally administered Lactobacillus strains on NO levels was determined by Griess reagent system. As shown in Fig. 4.1A, L. paracasei showed significant increase ($P < 0.01$) of NO levels in intestinal fluid compared to control mice. Nitric oxide levels in intestinal fluid of mice fed with L. acidophilus were also increased but not significantly different when compared to control mice. The serum NO levels were increased significantly ($P < 0.01$) in mice fed with either L. acidophilus or L. paracasei (Fig. 4.1B). The differences between L. acidophilus and L. paracasei were significant ($P < 0.01$) in enhancing NO levels in intestinal fluid and serum.

4.4.2. Immunoglobulin A antibodies in intestinal fluid and serum

The IgA antibodies specific to CT in mice fed with L. acidophilus or L. paracasei were estimated by ELISA (Fig. 4.2). In intestinal fluid, CT-specific IgA antibodies were increased significantly ($P < 0.01$) in mice fed with L. acidophilus or L. paracasei compared to control mice (Fig. 4.2A). In systemic response, strong serum IgA response to CT was detected in mice fed with L. acidophilus or L. paracasei, which differed significantly ($P < 0.001$) from control mice (Fig. 4.2B). There were no significant differences ($P > 0.05$) between L. acidophilus and L. paracasei in inducing CT-specific IgA response in intestinal fluid. In contrast, L. acidophilus and L. paracasei demonstrated the differences ($P < 0.01$) in enhancing CT-specific IgA antibodies in serum.
Figure 4.1. Nitric oxide production in A) intestinal fluid and B) serum of mice fed with *L. acidophilus* L10 or *L. paracasei* L26. The results shown are mean ± standard error of mean (n = 6). Mean values were significantly different in comparison with the control mice *P* < 0.01. Significant differences between *L. acidophilus* L10 and *L. paracasei* L26 experimental groups #*P* < 0.01.
Figure 4.2. Immunoglobulin (Ig)-A antibody response to cholera toxin in A) intestinal fluid and B) serum of mice fed with *L. acidophilus* L10 or *L. paracasei* L26. The results shown are mean ± standard error of mean (n = 6). Mean values were significantly different in comparison with the control mice *P* < 0.01; **P** < 0.001. Significant differences between *L. acidophilus* L10 and *L. paracasei* L26 experimental groups #P < 0.01.
4.4.3. Detection of cytokine producing cells in small intestine

Cytokine producing cells in small intestine of mice fed with *L. acidophilus* or *L. paracasei* were shown in Figures 4.3; 4.4. Th1 cytokine, IFN-γ producing cells were significantly increased in mice fed with *L. acidophilus* (*P* < 0.001) or *L. paracasei* (*P* < 0.01) compared to control mice (Fig. 4.3A). The TNF-α was increased in mice fed with *L. acidophilus*, but not significantly different from control mice. However, TNF-α producing cells in *L. paracasei*-fed mice were significantly increased (*P* < 0.01) with respect to control mice (Fig. 4.3A).

In *L. paracasei*-fed mice, Th2 cytokine, IL-4 producing cells were enhanced (*P* < 0.01) when compared with control mice. Significant differences (*P* > 0.05) were not detected in IL-4 producing cells when compared between mice fed with *L. acidophilus* and control group (Fig. 4.3B). In comparison to control mice, IL-6 producing cells were increased in mice fed with either *L. acidophilus* (*P* < 0.05) or *L. paracasei* (*P* < 0.001) (Fig. 4.3B). The IL-10 producing cells were also enhanced (*P* < 0.01) in mice fed with *L. acidophilus* compared to control mice. The increase of IL-10 producing cells of mice fed with *L. paracasei* did not show significant differences (*P* > 0.05) when compared to control mice (Fig. 4.3B). There were no significant differences (*P* > 0.05) between *L. acidophilus* and *L. paracasei* in enhancing cytokines such as IFN-γ, TNF-α, IL-4, IL-10 with exception to the significant difference (*P* < 0.05) between *L. acidophilus* and *L. paracasei* in increasing IL-6 cytokine producing cells in small intestine of mice.
A

Figure 4.3. (Continued)
Figure 4.3. Effect of *L. acidophilus* L10 and *L. paracasei* L26 on (A) interferon (IFN)-γ and tumor necrosis factor (TNF)-α; (B) interleukin (IL)-4, IL-6 and IL-10 in the small intestine of mice. The results shown are mean ± standard error of mean (n = 6). Mean values were significantly different in comparison with the control mice *P* < 0.05; **P** < 0.01; ***P** < 0.001. Significant differences between *L. acidophilus* and *L. paracasei* experimental groups #*P* < 0.05.
Figure 4.4. Histological sections of the small intestine in mice fed with *L. acidophilus* L10 showing A) interleukin (IL)-10 and B) interferon (IFN)-γ producing cells labelled with rabbit anti-mouse IL-10 and IFN-γ respectively and secondary antibody as goat anti-rabbit conjugated with FITC, which fluoresces. Magnification 400X.
4.5. DISCUSSION

The NO in human body acts as a mediator in regulating various physiological and pathophysiological processes (Moncada and Higgs, 1995). In physiological levels, NO was essential in protecting intestinal mucosa through regulating mucus, bicarbonate secretion and maintaining mucosal blood flow (Hutcheson et al., 1990; Alican and Kubes, 1996; Lefer and Lefer, 1999). McCafferty et al. (1997) suggested that NO was important in resolving gut inflammation and may also protect the GI tract in inflammatory mechanisms. Several scientific reports indicated the involvement of NO in protecting against intestinal mucosal permeability associated with reperfusion of postischemic intestine (Payne and Kubes, 1993), preventing mucosal damage (Conforti et al., 1993), and regulating GI tract protective mechanisms (Korhonen et al., 2001; Lamine et al., 2004).

Elahi et al. (2005) correlated the increase of NO and IFN-γ in saliva with enhanced clearance of Candida albicans from oral cavities of L. acidophilus L10-fed mice. Interestingly, results of the current study showed the capability of L. acidophilus and L. paracasei in inducing NO levels in intestinal fluid and serum with increased IFN-γ producing cells in small intestine (Fig. 4.1; Fig. 4.3A). However, L. acidophilus-fed mice showed no significant differences in increasing NO levels in intestinal fluid compared to control mice. In relation to inducing NO levels in GI tract, L. paracasei could be considered as a potential enhancer compared to L. acidophilus. Moreover, both Lactobacillus strains showed significant enhancement of serum NO levels in systemic immune response.
Gastrointestinal immune system consists of Peyer’s patches, mesenteric lymph nodes, immunocompetent cells of lamina propria and mucosal epithelium. Lamina propria and intraepithelial spaces are the main effector sites in the intestine. In lamina propria, Peyer’s patch derived B-cells differentiate into dimeric IgA secreting plasma cells, which were transported across epithelial cells and released as secretory IgA. The secretory IgA is necessary in host defence mechanisms such as antigen binding and preventing the adherence of pathogenic bacteria to intestinal epithelial cells (Hudault et al., 1997; Gopal et al., 2001; Shu and Gill, 2002). In this study, *L. acidophilus* and *L. paracasei* significantly enhanced CT-specific IgA response in intestinal fluid and serum (Fig. 4.2). Intestinal lymphoid organ, Peyer’s patches were located in small intestine that was considered as principal inductive sites of immune responses upon oral administration of an antigen. In contrast to large intestine, small intestine is anatomically connected to systemic immune system through lymphatic and blood circulation by which the immune responses induced in small intestine could further lead to distant mucosal sites in systemic immunity.

In the current study, increase of CT-specific IgA response was observed in intestinal fluid of small intestine and blood serum of mice fed with *L. acidophilus* or *L. paracasei*, which suggests that the release of CT-specific IgA antibodies in small intestine could possibly lead into blood circulation. Therefore, blood serum could be regarded as a potential marker to study the immunomodulation of orally administered antigens. Furthermore, enhancement of CT-specific IgA response in intestinal fluid also suggests the potential capability of *L. acidophilus* and *L. paracasei* strains, which could protect the GI tract towards pathogenic microbial infections (Macpherson et al., 2001).
Goodrich and McGee, (1999) reported the ability of IL-6 in inducing terminal differentiation of B-lymphocytes to IgA secreting cells. However, B-cells switching and differentiation leading to plasmocyte secreting IgA also occurs in high IL-4, IL-5 and transforming growth factor (TGF)-β environment (Blum et al., 1999). Despite the capability of macrophages and T-cells to produce IL-6, intestinal epithelial cells are also known to produce IL-6 (Vitini et al., 2000). In the present study, *L. acidophilus* and *L. paracasei* increased IL-6 producing cells in small intestine (Fig. 4.3B). The enhancement of IL-6 producing cells is considered to have a potential role in augmenting the CT-specific IgA response in intestinal fluid and serum (Fig. 4.2). The increase of IL-6 and IgA levels observed in this study is consistent with the earlier studies that reported similar effects of lactic acid bacteria in enhancing IL-6 as well as IgA responses (Miettinen et al., 1996; Galdeano and Perdigon, 2006).

Regulatory cytokines such as IL-4 and IL-10 are associated with humoral and allergic immune responses. The IL-4 cytokine plays an important role in cell growth and regulation of immune response particularly in inhibiting cytokines such as IL-1, IL-6, IL-8 and TNF-α (Feghali and Wright, 1997). In the current study, differences in stimulating IL-6 cytokine producing cells in mice fed with *L. acidophilus* or *L. paracasei* were observed (Fig. 4.3B). The analysis of cytokine producing cells in small intestine of mice showed the capability of *L. acidophilus* and *L. paracasei* strains in modulating Th2 regulatory cytokines, such as IL-10 that plays an important role in chronic inflammatory bowel disease (Braat et al., 2003) and amelioration of colitis (Di Giacinto et al., 2005).
In the present study, pro-inflammatory cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\) were increased in mice fed with *L. acidophilus* or *L. paracasei* (Fig. 4.3A). In comparison to control mice, *L. acidophilus* induced TNF-\(\alpha\) cytokine producing cells were not significantly different. Despite the role of IFN-\(\gamma\) in inflammatory response, it can also act as an effector molecule in immune responses against solid cancers (Numata et al., 1991; Belardelli and Ferrantini, 2002). Macrophages and dendritic cells of lamina propria are known to be crucial in producing various cytokines such as IL-6, IL-10, IL-12, IFN-\(\gamma\) and TNF-\(\alpha\) (Husband et al., 1999). However, mast cells are also considered necessary in releasing cytokines such as IL-4 and TNF-\(\alpha\) that are important in initiating immune and inflammatory responses (Feger et al., 2002). These various cell populations from innate immune system might have been the source of cytokine producing cells in lamina propria of small intestine that were determined in this study. Additionally, current study also showed simultaneous stimulation of pro-inflammatory and regulatory cytokines in mice fed with *L. acidophilus* or *L. paracasei*, which could be considered necessary for maintaining a chronic and immunological balanced intestinal inflammatory response termed as physiological inflammation (Cebra et al., 2005).
4.6. CONCLUSIONS

The current study demonstrates that *L. acidophilus* and *L. paracasei* were capable of inducing NO levels in intestinal fluid and serum. Particularly, *L. paracasei*-fed mice showed higher NO levels in intestinal fluid compared to mice fed with *L. acidophilus*. *Lactobacillus acidophilus* and *L. paracasei* strains were demonstrated as potential inducers of CT-specific IgA antibodies in gut and systemic immunity. Especially, *L. acidophilus* induced higher CT-specific IgA levels in serum compared to *L. paracasei*-fed mice. The analysis of cytokines in small intestine of mice reported the capability of *L. acidophilus* and *L. paracasei* in regulating Th1 and Th2 cytokines. Particularly, differences between *L. acidophilus* and *L. paracasei* were observed in inducing IL-6 cytokine producing cells in small intestine of mice.
CHAPTER 5

Microarray analysis of *L. acidophilus* LAFTI L10 induced gene expression in the small intestine of mice
5.1. ABSTRACT

The microflora of gastrointestinal (GI) tract is known to be involved in promoting health and physiological functions of the host. The influence of *L. acidophilus* in regulating gene expression in the small intestine of normal and cholera toxin (CT) mice was examined in this study. In the first experiment, mice were fed daily with *L. acidophilus* at $10^8$ CFU for 14-days, whereas in the second experiment, mice were fed with *L. acidophilus* at $10^8$ CFU/day for 14-days, during which they were immunized on day 0 and 7 with 10 μg of cholera toxin. In both the experiments, mice were euthanized after 14-day feeding trail and small intestines were excised to determine the gene expression patterns using mouse oligonucleotide microarray. Microarray data was generated by comparing the influence of *L. acidophilus* in regulating gene expression with the respective control group mice. In the first experiment, normal mice fed with *L. acidophilus* demonstrated the regulation of genes with an up-regulation of 27 and down-regulation of 35 genes involved in various biological and molecular functions such as cell-cell signalling, cell growth and proliferation, cell death and various metabolic functions. In CT mice model, *L. acidophilus* influenced the up- and down-regulation of 28 and 32 genes that are involved in various cell functions, drug metabolism, immune responses and metabolic functions. The present study demonstrated the influence of *L. acidophilus* in regulating gene expression in the small bowel of normal and CT mice. Furthermore, this study also provides a basis to identify the gene targets that may represent a reference for further studies to define the pathways through which these bacteria influence the host.
5.2. INTRODUCTION

Probiotic bacteria are considered as beneficial microbes due to their potential with respect to different ways in which they can contribute to the positive health of GI tract. Probiotic strains such as *Lactobacillus* and *Bifidobacterium* spp. are predominantly incorporated into fermented dairy products. The interactions of probiotic bacteria in GI tract are believed to be necessary in promoting host biological functions. Several reports also suggested the probiotic effects on respiratory, subcutaneous and urogenital tract functions (Tagg and Dierksen, 2003; Sheil et al., 2004; Reid, 2005). The microflora of GI tract interacts with mucosal cell population, which includes epithelial cells (Hooper et al., 2001). Commensal microbial population that colonises the GI tract acts as a barrier in protecting the intestinal mucosa against pathogens through various mechanisms, such as occupation of niches, antimicrobial production and competition for essential nutrients.

The probiotic responses of microorganisms induced on host could be due to either microbe-microbe or microbial-host interactions. Probiotic bacteria play an important role in host defence mechanisms through activation of mucosal immune responses (Shanahan, 2002a). Previous studies reported the capability of probiotic bacteria in promoting gut barrier functions, balance of Th1/Th2 cytokines, enhancement of host immune responses and interactions with gut associated lymphoid tissues (Erickson and Hubbard, 2000; Isolauri et al., 2001). Furthermore, several studies also reported the beneficial effects of probiotic bacteria in breast cancer model (de Moreno de LeBlanc et al., 2005), respiratory infections (Racedo et al., 2006; Villena et al., 2006) and inflammatory bowel diseases (Madsen et al., 2001; Schultz et al., 2002).
Probiotic bacteria especially *Lactobacillus* strains were characterised due to their production of lactic acid, where most of them are indigenous inhabitants of human GI tract and colonise in higher populations in the small intestine. Orally administered probiotic bacteria are inducers of immune responses through potential inductive sites such as M cells or follicle associated epithelial cells of Peyer’s patches on small intestine. The immune responses induced in small intestine can effectively reach systemic immune response due to its anatomical connection through lymphatic and blood circulation, whereas immune responses induced in large intestine were mostly limited to this environment. Vinderola et al. (2006) suggested the importance of small intestine in understanding the immunomodulatory properties of orally administered kefir microflora in mice.

The molecular interactions of gut microflora with host specifically in relation to probiotic bacteria were not well understood. Microarray technology is a powerful tool, which offers a comprehensive view of biological systems through monitoring the expression of thousands of genes simultaneously and rapidly (Wu et al., 2001; Sepulveda et al., 2002). Microarray analysis of the gene expression in host is necessary to define these probiotic microorganisms as beneficial bacteria, which also provides further insights into the interactions of these microbes with epithelial cells of GI tract.

In the current study, *L. acidophilus* L10 demonstrated its potential probiotic properties such as tolerance to simulated gastric juices particularly at pH 2.0, better survival at various bile salt concentrations and coaggregation abilities with other bacterial strains (Chapter 2). *Lactobacillus acidophilus* L10 also showed its potential role in stimulating
various gut and systemic immune functions, especially non-specific and specific immune responses in normal and cholera toxin (CT) mice (Chapters 3 & 4). Therefore, *L. acidophilus* L10 was chosen to elucidate its influence on gene expression in the small intestine of mice and also to provide further insights on genes that were involved in regulating various biological and molecular functions of the host. This study was aimed to investigate the effects of *L. acidophilus* L10 on gene expression patterns in the small intestine of normal and CT mice.
5.3. MATERIALS AND METHODS

5.3.1. Animals

Male 6 - 8 wks old mice (BALB/c) were obtained from Biological Resources Centre, The University of New South Wales, Sydney, Australia. Control and experimental groups consisted of six mice each, which were caged separately at 22 ± 1°C and housed under 12 h light-dark photoperiod. All mice were fed with standard mouse chow (Gordon's Specialty Stock Feeds, Sydney, Australia) and provided ad libitum access to water. All the protocols for the animal experiment were approved by the University of Western Sydney Animal Care and Ethics Committee.

5.3.2. Lactic acid bacteria

Lactobacillus acidophilus LAFTI L10 used in this study was obtained from DSM culture collection (DSM Food Specialties Ltd., Sydney, Australia). Lactobacillus acidophilus was grown anaerobically in gas jars using GasPak System (Oxoid, Adelaide, Australia) for 24 h at 37°C in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Adelaide, Australia). The bacterial cells were harvested at 4000 x g for 15 min at 4°C and washed twice with sterile 0.01 M phosphate buffered saline (PBS) solution.

5.3.3. Experimental design

Two experiments were conducted. In the first experiment, mice (n = 6) were fed daily by oral gavage with 10⁸ CFU of L. acidophilus in 50 μl of 10% (w/v) skimmed milk
powder (SMP) for 14-days. Control group mice received 50 µl of SMP without lactic acid bacteria.

In the second experiment, mice (n = 6) were fed daily by oral gavage with $10^8$ CFU of *L. acidophilus* in 50 µl of 10% (w/v) SMP for 14-days. Control group mice received 50 µl of SMP without lactic acid bacteria. Experimental and control group mice were orally immunized with 10 µg of cholera toxin (Sigma-Aldrich, Sydney, Australia) in 25 µl of 0.1 M sodium bicarbonate (Sigma-Aldrich, Sydney, Australia) on day 0 and 7.

The viability of bacteria was determined by spread plate count of bacterial serial dilutions on MRS agar (Oxoid, Adelaide, Australia), which were incubated anaerobically in gas jars using GasPak System at 37°C for 48 h. In both the experiments, mice were euthanized by carbon dioxide inhalation after 14-day feeding trial and small intestines were removed for gene expression analysis.

### 5.3.4. RNA isolation

Total RNA was isolated from the small intestine of each mouse using TRIzol reagent (Invitrogen, Melbourne, Australia) according to the manufacturer’s instructions. The total RNA was further purified by RNeasy mini kit (Qiagen, Melbourne, Australia). The purity and concentration of RNA was determined by spectrophotometer readings at 260 and 280 nm. The RNA integrity was confirmed by agarose gel electrophoresis. For microarray analysis, equal amounts of RNA was pooled from all the mice (n = 6) of experimental and control groups.
5.3.5. Microarray analysis

Microarray analysis was performed with RNA extracted from the experimental and control groups using a compugen mouse oligonucleotide microarray consisting of 22,000 probes. A list of oligonucleotides immobilized on the array can be obtained from the web-site: http://www.microarray.adelaide.edu.au/libraries.

5.3.6. Synthesis of cRNA probes

The total RNA pooled from individual experimental and control group mice was amplified using a SuperScript Indirect RNA Amplification System (Invitrogen, Melbourne, Australia) according to the manufacturer’s instructions. The purified cRNA was dried under reduced pressure, dissolved in 9 µl of 0.1 M NaHCO₃ (pH 9.0), mixed with Cy3 or Cy5 and left in the dark to couple at room temperature for 60 min. The labelled cRNA was mixed with 41 µl of Milli Q water and purified using a QIAquick PCR purification kit (Qiagen, Melbourne, Australia). The purified fluorescent cRNA samples were eluted into a clean tube with 90 µl of Milli Q water and dried under reduced pressure.

5.3.7. Hybridisation of probes to oligonucleotide microarray

Prior to hybridization, each microarray slide was immersed in 50 ml of hot (60-95°C) Milli-Q water for 1 min, then each array was dried by centrifugation at 650 x g for 5 min. The labelled cRNA probes were then mixed with 0.64 µl of 25 mg/ml yeast tRNA, 4 µl of 2 mg/ml poly A and 20 µl of 1 mg/ml mouse Cot-1 DNA, dried under reduced
pressure, resuspended in 16 µl of formamide and 16 µl of 6.25 X SSC, denatured by heating to 100°C for 3 min and transferred directly to ice, followed by addition of 0.5 µl of 10% SDS. The probes were then applied to the array and incubated at 42°C overnight in a humidified chamber. The arrays were washed in 0.5 X SSC containing 0.01% SDS for 5 min, 0.5 X SSC for 3 min and rinsed in 0.2 X SSC for 3 min. The slides were dried by centrifugation at 650 x g for 5 min and were then scanned using an Axon 4000B microarray scanner. Data analysis was performed by using the single image tif files.

5.3.8. Data Analysis

The Cy5 and Cy3 fluorescent signal intensity of each gene on the array was extracted using SPOT software (CSIRO, Australia). After the subtraction of morphological background fluorescence, ratio of signal intensities (Cy5/Cy3) was calculated. Each probe ratio was log 2 transformed and normalised using a LOWESS algorithm (Locally weighted scatterplot smoothing) within each slide using LIMMA (Linear models for microarray analysis). Between the arrays, normalisation was performed to have the log-ratios with same median-absolute-deviation (MAD) across all arrays. Linear modelling combined with Bayesian statistical analysis of the normalised data produced a ranked list of genes that were likely to be consistently differentially expressed on all 4 arrays.
5.4. RESULTS

In both the experiments, microarray analysis of gene expression in the small bowel of mice was analysed using oligonucleotide microarray consisting of 22,000 genes. Analysis of data from arrays ranked the genes in order of likelihood of being differentially expressed. In both the experiments, top 100 genes that were likely to be differentially expressed were selected and their functional and pathway analysis were determined through Ingenuity Pathways Analysis (Ingenuity systems, www.ingenuity.com).

In the first experiment, 62 of the top 100 genes were mapped to Ingenuity Pathways Knowledge Base associated with known biological functions. In 62 genes, 27 and 35 genes were up- and down-regulated (Table 5.1). Orally administered *L. acidophilus* in normal mice altered the expression of genes that are involved in various biological and molecular functions such as cell-cell signalling, cell growth, molecular transport, tissue development, immune response and various metabolic functions (Table 5.2; 5.3). Genes such as MAP4K1, H3 histone family 3A and HSPA1A, which are involved in p38 MAPK signalling, TGF-β signalling and ERF/MAPK signalling pathways were up-regulated in *L. acidophilus*-fed mice (Table 5.4).

Genes related to cytochrome P450 family (CYP2B6, CYP2C8, CYP2C19) that are involved in linoleic acid, fatty acid and arachidonic acid metabolic pathways were down-regulated by *L. acidophilus*. Several other genes such as fibroblast growth factor 19 (FGF signalling), carboxyl ester lipase (Bile acid metabolism), AMY2A and ENPP3
(Starch and sucrose metabolism) and glutathione S-transferase A5 (Glutathione metabolism) were also down-regulated in *L. acidophilus*-fed mice (Table 5.4).
Table 5.1. Effect of *L. acidophilus* L10 on gene expression (Up- and down-regulated) in the small intestine of normal mice.

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<th>Gene name</th>
<th>Fold change</th>
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<td>Attractin (<em>ATRN</em>)</td>
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<td>S100 calcium binding protein G (S100G)</td>
<td>1.7 ↓</td>
</tr>
<tr>
<td>D83146</td>
<td>Sine oculis-related homeobox 5 homolog (Drosophila) (SIX5)</td>
<td>1.4 ↑</td>
</tr>
<tr>
<td>NM_019481</td>
<td>Solute carrier family 13 (sodium/sulfate symporters), member 1 (SLC13A1)</td>
<td>1.4 ↓</td>
</tr>
<tr>
<td>AF026489</td>
<td>Spectrin, beta, non-erythrocytic 2 (SPTBN2)</td>
<td>1.4 ↑</td>
</tr>
<tr>
<td>AK003083</td>
<td>Syncollin (SYCN)</td>
<td>2.1 ↓</td>
</tr>
<tr>
<td>S76673</td>
<td>Transcription factor binding to IGHM enhancer 3 (TFE3)</td>
<td>1.4 ↑</td>
</tr>
<tr>
<td>NM_009363</td>
<td>Trefoil factor 2 (spasmyolytic protein 1) (TFF2)</td>
<td>1.6 ↓</td>
</tr>
<tr>
<td>AK016603</td>
<td>Transketolase-like 2 (TKTL2)</td>
<td>1.6 ↓</td>
</tr>
<tr>
<td>BC006649</td>
<td>Ubiquitination factor E4A (UFD2 homolog, yeast) (UBE4A)</td>
<td>1.5 ↓</td>
</tr>
</tbody>
</table>
Table 5.2. Functional analysis of differentially expressed genes (Up-regulated) after oral administration of *L. acidophilus* L10 in normal mice.

<table>
<thead>
<tr>
<th>Functions &amp; Diseases</th>
<th>Genes, n</th>
<th>Functions &amp; Diseases</th>
<th>Genes, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small molecule biochemistry</td>
<td>5</td>
<td>Tissue development</td>
<td>2</td>
</tr>
<tr>
<td>Genetic disorder</td>
<td>4</td>
<td>Amino acid metabolism</td>
<td>1</td>
</tr>
<tr>
<td>Cell-cell signalling and interaction</td>
<td>3</td>
<td>Carbohydrate metabolism</td>
<td>1</td>
</tr>
<tr>
<td>Hematological system development and function</td>
<td>3</td>
<td>Cardiovascular disease</td>
<td>1</td>
</tr>
<tr>
<td>Cell signalling</td>
<td>2</td>
<td>Cell death</td>
<td>1</td>
</tr>
<tr>
<td>Cellular assembly and organisation</td>
<td>2</td>
<td>Cell morphology</td>
<td>1</td>
</tr>
<tr>
<td>Cellular development</td>
<td>2</td>
<td>Connective tissue disorders</td>
<td>1</td>
</tr>
<tr>
<td>Cellular growth and proliferation</td>
<td>2</td>
<td>Endocrine system disorder</td>
<td>1</td>
</tr>
<tr>
<td>Developmental disorder</td>
<td>2</td>
<td>Gene expression</td>
<td>1</td>
</tr>
<tr>
<td>DNA replication, recombination and repair</td>
<td>2</td>
<td>Immune response</td>
<td>1</td>
</tr>
<tr>
<td>Embryonic development</td>
<td>2</td>
<td>Infectious disease</td>
<td>1</td>
</tr>
<tr>
<td>Immune and lymphatic development and function</td>
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<td>Inflammatory disease</td>
<td>1</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>2</td>
<td>Metabolic disease</td>
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<tr>
<td>Molecular transport</td>
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<td>Skeletal and muscular development and function</td>
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<tr>
<td>Post-translational modification</td>
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<td>Viral function</td>
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</table>
Table 5.3. Functional analysis of differentially expressed genes (Down-regulated) after oral administration of *L. acidophilus* L10 in normal mice.

<table>
<thead>
<tr>
<th>Functions &amp; Diseases</th>
<th>Genes, n</th>
<th>Functions &amp; Diseases</th>
<th>Genes, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small molecule biochemistry</td>
<td>14</td>
<td>Skeletal and muscular disorders</td>
<td>2</td>
</tr>
<tr>
<td>Lipid metabolism</td>
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<td>Tissue morphology</td>
<td>2</td>
</tr>
<tr>
<td>Molecular transport</td>
<td>6</td>
<td>Cardiovascular disease</td>
<td>1</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>5</td>
<td>Cell cycle</td>
<td>1</td>
</tr>
<tr>
<td>Drug metabolism</td>
<td>4</td>
<td>Cell morphology</td>
<td>1</td>
</tr>
<tr>
<td>Cancer</td>
<td>3</td>
<td>Cell-cell signalling and interaction</td>
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<tr>
<td>Cellular movement</td>
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<td>Cellular assembly and organisation</td>
<td>1</td>
</tr>
<tr>
<td>Organismal development</td>
<td>3</td>
<td>Cellular growth and proliferation</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin and mineral metabolism</td>
<td>3</td>
<td>Connective tissue development and function</td>
<td>1</td>
</tr>
<tr>
<td>Amino acid metabolism</td>
<td>2</td>
<td>Connective tissue disorders</td>
<td>1</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>2</td>
<td>Digestive system development and function</td>
<td>1</td>
</tr>
<tr>
<td>Cellular development</td>
<td>2</td>
<td>Endocrine system disorders</td>
<td>1</td>
</tr>
<tr>
<td>Developmental disorder</td>
<td>2</td>
<td>Gastrointestinal disease</td>
<td>1</td>
</tr>
<tr>
<td>Embryonic development</td>
<td>2</td>
<td>Hematological system development and function</td>
<td>1</td>
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<tr>
<td>Endocrine system development and function</td>
<td>2</td>
<td>Hepatic system disease</td>
<td>1</td>
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<tr>
<td>Genetic disorder</td>
<td>2</td>
<td>Organ development</td>
<td>1</td>
</tr>
<tr>
<td>Inflammatory disease</td>
<td>2</td>
<td>Organismal injury and abnormalities</td>
<td>1</td>
</tr>
<tr>
<td>Organ morphology</td>
<td>2</td>
<td>Skeletal and muscular development and function</td>
<td>1</td>
</tr>
<tr>
<td>Post-translation modification</td>
<td>2</td>
<td>Tissue development</td>
<td>1</td>
</tr>
<tr>
<td>Respiratory disease</td>
<td>2</td>
<td>Tumor morphology</td>
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</table>
Table 5.4. Pathway analysis of differentially expressed genes (Up- and down-regulated) after oral administration of *L. acidophilus* L10 in normal mice.

<table>
<thead>
<tr>
<th>Up-regulation</th>
<th>Genes, $n$</th>
<th>Down-regulation</th>
<th>Genes, $n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38 MAPK signalling</td>
<td>2</td>
<td>Linoleic acid metabolism</td>
<td>4</td>
</tr>
<tr>
<td>TGF-β signalling</td>
<td>1</td>
<td>Xenobiotic metabolism signalling</td>
<td>4</td>
</tr>
<tr>
<td>SAPK/JNK signalling</td>
<td>1</td>
<td>Arachidonic acid metabolism</td>
<td>3</td>
</tr>
<tr>
<td>ERF/MAPK signalling</td>
<td>1</td>
<td>Fatty acid metabolism</td>
<td>3</td>
</tr>
<tr>
<td>Wnt/β-catenin signalling</td>
<td>1</td>
<td>Glycerolipid metabolism</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tryptophan metabolism</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Starch and sucrose metabolism</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bile acid metabolism</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGF signalling</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutathione metabolism</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycerophospholipid metabolism</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pentose phosphate metabolism</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein ubiquitination pathway</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Riboflavin metabolism</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sulphur metabolism</td>
<td>1</td>
</tr>
</tbody>
</table>
In the second experiment, regulation of gene expression by *L. acidophilus* in the small intestine of CT mice model was examined. Sixty of the top 100 genes were mapped to the Ingenuity Pathways Knowledge Base associated with known biological functions. In 60 genes, 28 and 32 genes were up- and down-regulated (Table 5.5). *Lactobacillus acidophilus* influenced the expression of genes that are involved in various cell functions such as cell death, cellular movement, cell cycle, cellular growth and proliferation. Several other genes regulated are also involved in functions related to molecular transport, immune responses, immunological and inflammatory diseases, gastrointestinal diseases and various metabolic functions (Table 5.6; 5.7).

Genes related to cytochrome P450 family (CYP2C9, CYP2C19, CYP2C38, CYP3A5) that are involved in various metabolic pathways such as arachidonic acid metabolism, fatty acid metabolism, linoleic acid metabolism and tryptophan metabolism were up-regulated in the small bowel of *L. acidophilus*-fed mice (Table 5.8). Additionally, genes involved in oxidative phosphorylation such as COX7C, NDUFC1, NDUFC2, PPA1 and UCRC were up-regulated in mice fed with *L. acidophilus*. Fibronectin 1, which is involved in integrin and actin cytoskeleton signalling pathways was also up-regulated in mice fed with *L. acidophilus*.

Orally administered *L. acidophilus* down-regulated genes such as BIRC3, TNFRSF1A, CD74 that are involved in apoptosis, death receptor signalling, antigen presenting pathway, IL-6 and p38 MAPK signalling pathways. Furthermore, *L. acidophilus* also down-regulated several genes that are involved in various functions related to eicosanoid signalling (DPEP1), glutathione metabolism (ANPEP), N-Glycan biosynthesis
(MAN1B1), starch and sucrose metabolism (SI), cAMP-mediated signalling and G-protein coupled receptor signalling pathways (ADORA1) (Table 5.8).
Table 5.5. Effect of *L. acidophilus* L10 on gene expression (Up- and down-regulated) in the small intestine of cholera toxin mice model.

<table>
<thead>
<tr>
<th>GenBank</th>
<th>Gene name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>U05671</td>
<td>Adenosine A1 receptor (<em>ADORA1</em>)</td>
<td>1.4 ↓</td>
</tr>
<tr>
<td>NM_013778</td>
<td>Aldo-keto reductase family 1, member C13 (<em>AKR1C13</em>)</td>
<td>1.6 ↑</td>
</tr>
<tr>
<td>NM_007442</td>
<td>Aristaless-like homeobox 4 (<em>ALX4</em>)</td>
<td>1.4 ↑</td>
</tr>
<tr>
<td>NM_007446</td>
<td>Amylase, alpha 1A (salivary) (<em>AMY1A</em>)</td>
<td>1.6 ↑</td>
</tr>
<tr>
<td>NM_009669</td>
<td>Amylase, alpha 2A (pancreatic) (<em>AMY2A</em>)</td>
<td>1.7 ↑</td>
</tr>
<tr>
<td>AK010761</td>
<td>Ankyrin repeat domain 11 (<em>ANKRD11</em>)</td>
<td>1.7 ↓</td>
</tr>
<tr>
<td>NM_008486</td>
<td>Alanyl (membrane) aminopeptidase (<em>ANPEP</em>)</td>
<td>1.4 ↓</td>
</tr>
<tr>
<td>X15191</td>
<td>Apolipoprotein B (including Ag(x) antigen) (<em>APOB</em>)</td>
<td>1.6 ↓</td>
</tr>
<tr>
<td>AY033514</td>
<td>Butyrobetaeine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaeine hydroxylase) 1 (<em>BBOX1</em>)</td>
<td>1.9 ↑</td>
</tr>
<tr>
<td>NM_007464</td>
<td>Baculoviral IAP repeat-containing 3 (<em>BIRC3</em>)</td>
<td>1.4 ↓</td>
</tr>
<tr>
<td>NM_007643</td>
<td>CD36 molecule (thrombospondin receptor) (<em>CD36</em>)</td>
<td>1.4 ↑</td>
</tr>
<tr>
<td>NM_009690</td>
<td>CD5 molecule-like (<em>CD5L</em>)</td>
<td>1.3 ↓</td>
</tr>
<tr>
<td>X00496</td>
<td>CD74 molecule, major histocompatibility complex, class II invariant chain (<em>CD74</em>)</td>
<td>1.3 ↓</td>
</tr>
<tr>
<td>NM_011926</td>
<td>Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) (<em>CEACAM1</em>)</td>
<td>1.4 ↓</td>
</tr>
<tr>
<td>NM_013492</td>
<td>Clusterin (<em>CLU</em>)</td>
<td>1.3 ↓</td>
</tr>
<tr>
<td>NM_007749</td>
<td>Cytochrome c oxidase subunit VIIc (<em>COX7C</em>)</td>
<td>1.3 ↑</td>
</tr>
<tr>
<td>AK003088</td>
<td>Carboxypeptidase A1 (pancreatic) (<em>CPA1</em>)</td>
<td>1.8 ↑</td>
</tr>
<tr>
<td>NM_025583</td>
<td>Chymotrypsinogen B1 (<em>CTRBI</em>)</td>
<td>2.4 ↑</td>
</tr>
<tr>
<td>NM_023182</td>
<td>Chymotrypsin-like (<em>CTRL</em>)</td>
<td>1.6 ↑</td>
</tr>
<tr>
<td>AF197159</td>
<td>Cubilin (intrinsic factor-cobalamin receptor) (<em>CUBN</em>)</td>
<td>1.4 ↓</td>
</tr>
<tr>
<td>NM_010003</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 19 (<em>CYP2C19</em>)</td>
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</tr>
<tr>
<td>AF047725</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 38 (<em>CYP2C38</em>)</td>
<td>1.9 ↑</td>
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</tbody>
</table>

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<table>
<thead>
<tr>
<th>GenBank</th>
<th>Gene name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_007815</td>
<td>Cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9)</td>
<td>1.6 ↑</td>
</tr>
<tr>
<td>NM_017396</td>
<td>Cytochrome P450, family 3, subfamily A, polypeptide 5 (CYP3A5)</td>
<td>1.3 ↑</td>
</tr>
<tr>
<td>AY039762</td>
<td>24-dehydrocholesterol reductase (DHCR24)</td>
<td>1.4 ↓</td>
</tr>
<tr>
<td>NM_007876</td>
<td>Dipeptidase 1 (renal) (DPEP1)</td>
<td>1.5 ↓</td>
</tr>
<tr>
<td>NM_030238</td>
<td>Dynein, cytoplasmic 1, heavy chain 1 (DYNC1H1)</td>
<td>1.3 ↓</td>
</tr>
<tr>
<td>M27347</td>
<td>Elastase 1, pancreatic (ELA1)</td>
<td>1.7 ↑</td>
</tr>
<tr>
<td>BC004798</td>
<td>ELK4, ETS-domain protein (SRF accessory protein 1) (ELK4)</td>
<td>1.6 ↓</td>
</tr>
<tr>
<td>NM_008375</td>
<td>Fatty acid binding protein 6, ileal (gastrotropin) (FABP6)</td>
<td>2.0 ↓</td>
</tr>
<tr>
<td>X93167</td>
<td>Fibronectin 1 (FN1)</td>
<td>1.4 ↑</td>
</tr>
<tr>
<td>NM_008181</td>
<td>Glutathione S-transferase A5 (GSTA5)</td>
<td>1.9 ↑</td>
</tr>
<tr>
<td>NM_013541</td>
<td>Glutathione S-transferase pi (GSTP1)</td>
<td>1.3 ↑</td>
</tr>
<tr>
<td>NM_008218</td>
<td>Hemoglobin, alpha 2 (HBA2)</td>
<td>1.4 ↑</td>
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<tr>
<td>NM_016868</td>
<td>Hypoxia inducible factor 3, alpha subunit (HIF3A)</td>
<td>1.3 ↓</td>
</tr>
<tr>
<td>NM_015783</td>
<td>ISG15 ubiquitin-like modifier (ISG15)</td>
<td>1.4 ↑</td>
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<tr>
<td>BC006645</td>
<td>Mannosidase, alpha, class 1B, member 1 (MAN1B1)</td>
<td>1.7 ↓</td>
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<tr>
<td>NM_008585</td>
<td>Meprin A, alpha (PABA peptide hydrolase) (MEP1A)</td>
<td>1.4 ↓</td>
</tr>
<tr>
<td>AK016915</td>
<td>Mesoderm induction early response 1 homolog (Xenopus laevis) (MI-ER1)</td>
<td>1.3 ↓</td>
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<tr>
<td>X82786</td>
<td>Antigen identified by monoclonal antibody Ki-67 (MKI67)</td>
<td>1.4 ↓</td>
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<tr>
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<td>NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1, 6kDa (NDUFC1)</td>
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</tr>
<tr>
<td>BC002097</td>
<td>NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5kDa (NDUFC2)</td>
<td>1.4 ↑</td>
</tr>
<tr>
<td>NM_021543</td>
<td>Protocadherin 8 (PCDH8)</td>
<td>1.3 ↓</td>
</tr>
<tr>
<td>NM_018874</td>
<td>Pancreatic lipase-related protein 1 (PNLIPRP1)</td>
<td>1.4 ↑</td>
</tr>
<tr>
<td>NM_026438</td>
<td>Pyrophosphatase (inorganic) 1 (PPA1)</td>
<td>1.3 ↑</td>
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<tr>
<td>AB009661</td>
<td>Protease, serine, 2 (trypsin 2) (PRSS2)</td>
<td>1.8 ↑</td>
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<tr>
<td>NM_023333</td>
<td>Protease, serine, 3 (mesotrypsin) (PRSS3)</td>
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<td>GenBank</td>
<td>Gene name</td>
<td>Fold change</td>
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<td>-----------</td>
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<td>-------------</td>
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<td>Protease, serine, 7 (enterokinase) (PRSS7)</td>
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<tr>
<td>M31441</td>
<td>RAD52 homolog (S. cerevisiae) (RAD52)</td>
<td>1.5↓</td>
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<tr>
<td>NM_009042</td>
<td>Regenerating islet-derived 1 alpha (pancreatic stone protein, pancreatic thread protein) (REG1A)</td>
<td>2.1↑</td>
</tr>
<tr>
<td>NM_009789</td>
<td>S100 calcium binding protein G (S100G)</td>
<td>1.5↑</td>
</tr>
<tr>
<td>AB030906</td>
<td>Sex comb on midleg homolog 1 (Drosophila) (SCMH1)</td>
<td>1.3↓</td>
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<tr>
<td>AK008441</td>
<td>Sucrase-isomaltase (alpha-glucosidase) (SI)</td>
<td>1.5↓</td>
</tr>
<tr>
<td>NM_011388</td>
<td>Solute carrier family 10 (sodium/bile acid cotransporter family), member 2 (SLC10A2)</td>
<td>1.6↓</td>
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<td>NM_011402</td>
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<td>1.6↓</td>
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<td>NM_019754</td>
<td>Transgelin 3 (TAGLN3)</td>
<td>1.4↓</td>
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<td>NM_011609</td>
<td>Tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A)</td>
<td>1.5↓</td>
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<td>AK003881</td>
<td>Ubiquinol-cytochrome c reductase complex (7.2 kD) (UCRC)</td>
<td>1.3↑</td>
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<tr>
<td>AK013986</td>
<td>Ubiquitin specific peptidase 45 (USP45)</td>
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<tr>
<td>NM_009509</td>
<td>Villin 1 (VIL1)</td>
<td>1.3↓</td>
</tr>
</tbody>
</table>
Table 5.6. Functional analysis of differentially expressed genes (Up-regulated) after oral administration of *L. acidophilus* L10 in cholera toxin mice model.

<table>
<thead>
<tr>
<th>Functions &amp; Diseases</th>
<th>Genes, n</th>
<th>Functions &amp; Diseases</th>
<th>Genes, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small molecule biochemistry</td>
<td>11</td>
<td>Genetic disorder</td>
<td>2</td>
</tr>
<tr>
<td>Drug metabolism</td>
<td>6</td>
<td>Hepatic system disease</td>
<td>2</td>
</tr>
<tr>
<td>Cancer</td>
<td>6</td>
<td>Immune and lymphatic development and function</td>
<td>2</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>5</td>
<td>Immunological disease</td>
<td>2</td>
</tr>
<tr>
<td>Cell death</td>
<td>4</td>
<td>Skeletal and muscular disorder</td>
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</tr>
<tr>
<td>Cell-cell signalling and interaction</td>
<td>4</td>
<td>Tissue development</td>
<td>2</td>
</tr>
<tr>
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<td>Cell morphology</td>
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<td>Cellular assembly and organisation</td>
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<td>Cellular compromise</td>
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<tr>
<td>Molecular transport</td>
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<tr>
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<td>Connective tissue disorders</td>
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<td>Cell cycle</td>
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<td>Organ development</td>
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<tr>
<td>Cellular function and maintenance</td>
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<td>Organ morphology</td>
<td>1</td>
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<tr>
<td>Cellular growth and proliferation</td>
<td>2</td>
<td>Organismal injury and abnormalities</td>
<td>1</td>
</tr>
<tr>
<td>Connective tissue development and functions</td>
<td>2</td>
<td>Protein trafficking</td>
<td>1</td>
</tr>
<tr>
<td>DNA replication, recombination and repair</td>
<td>2</td>
<td>Tissue morphology</td>
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<tr>
<td>Gene expression</td>
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<td>Tumor morphology</td>
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Table 5.7. Functional analysis of differentially expressed genes (Down-regulated) after oral administration of *L. acidophilus* L10 in cholera toxin mice model.

<table>
<thead>
<tr>
<th>Functions &amp; Diseases</th>
<th>Genes, n</th>
<th>Functions &amp; Diseases</th>
<th>Genes, n</th>
</tr>
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<tr>
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<td>Digestive system development and function</td>
<td>2</td>
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<tr>
<td>Cancer</td>
<td>7</td>
<td>Drug metabolism</td>
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</tr>
<tr>
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<td>2</td>
</tr>
<tr>
<td>Cellular movement</td>
<td>6</td>
<td>Endocrine system disorders</td>
<td>2</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>6</td>
<td>Immune and lymphatic development and function</td>
<td>2</td>
</tr>
<tr>
<td>Molecular transport</td>
<td>6</td>
<td>Organ development</td>
<td>2</td>
</tr>
<tr>
<td>Cell-cell signalling and interaction</td>
<td>5</td>
<td>Organ morphology</td>
<td>2</td>
</tr>
<tr>
<td>Cellular assembly and organisation</td>
<td>5</td>
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<td>2</td>
</tr>
<tr>
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<td>Respiratory disease</td>
<td>2</td>
</tr>
<tr>
<td>Cellular compromise</td>
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<td>Skeletal and muscular disorders</td>
<td>2</td>
</tr>
<tr>
<td>Hematological disease</td>
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<td>1</td>
</tr>
<tr>
<td>Immune response</td>
<td>4</td>
<td>Cell cycle</td>
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<td>Tumor morphology</td>
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<td>Cell signalling</td>
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<tr>
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<td>Connective tissue disorders</td>
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<td>Cellular growth and proliferation</td>
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<td>DNA replication, recombination and repair</td>
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<td>Inflammatory disease</td>
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<td>Metabolic disease</td>
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<td>Organismal injury and abnormalities</td>
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<td>Tissue development</td>
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<td>Cell morphology</td>
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<td>Cellular function and maintenance</td>
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<td>Vitamin and mineral metabolism</td>
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</tr>
</tbody>
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Table 5.8. Pathway analysis of differentially expressed genes (Up- and down-regulated) after oral administration of *L. acidophilus* L10 in cholera toxin mice model.

<table>
<thead>
<tr>
<th>Canonical Pathway Analysis</th>
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</thead>
<tbody>
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<td><strong>Up-regulation</strong></td>
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<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>Xenobiotic metabolism signalling</td>
</tr>
<tr>
<td>Arachidonic acid metabolism</td>
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<tr>
<td>Fatty acid metabolism</td>
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<tr>
<td>Linoleic acid metabolism</td>
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<tr>
<td>Tryptophan metabolism</td>
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<tr>
<td>Glutathione metabolism</td>
</tr>
<tr>
<td>Starch and sucrose metabolism</td>
</tr>
<tr>
<td>Ubiquinone biosynthesis</td>
</tr>
<tr>
<td>Actin cytoskeleton signalling</td>
</tr>
<tr>
<td>Glycerolipid metabolism</td>
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<tr>
<td>Integrin signalling</td>
</tr>
<tr>
<td>Lysine degradation</td>
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</table>
5.5. DISCUSSION

The GI tract harbours a well balanced microflora, which is in close and continuous contact with epithelial and immune cells that are essential for a functionally balanced immune system. Earlier studies suggested that complex modifications of epithelial cells could occur due to the interactions between probiotic bacteria and enterocytes (Lu and Walker, 2001). The cross-talk between microorganisms and enterocytes is required to influence the mucosal barrier functions, which could favour positive health or cause disease (Kohler et al., 2003). Lactobacilli remain as a major component of human intestinal microflora that are frequently considered as good candidates in the preparation of functional foods. The ability of several LAB strains in modulating host innate and acquired immune responses has been demonstrated in many in vivo animal models. However, influence of LAB on gene expression of the intestinal epithelial cells remains unclear.

The present study investigated the effect of orally administered L. acidophilus on gene expression in the small intestine of mice. Compugen mouse oligonucleotide microarray was used to identify genes that were influenced by L. acidophilus through comparing the gene expression profiles in the small intestine of L. acidophilus-fed mice with the control group. Orally administered L. acidophilus induced the expression of genes that are involved in immune and lymphatic development such as attractin and chemokine (C-X-C motif) ligand 15. Attractin is a normal serum glycoprotein of 175 kDa, which is expressed on activated T-cells. Attractin gene encoded protein is involved in initial immune cell clustering during inflammatory responses, which may also regulate chemotactic activity of chemokines. Attractin protein is also crucial in mediating the
clustering of non-proliferative T-lymphocytes and spreading of monocytes (Duke-Cohan et al., 1998). Furthermore, Nagle et al. (1999) reported the potential role of attractin in decreasing the obesity that was increased by diet in mice. Chemokine receptors are suggested to be involved in allergic diseases (Dulkys et al., 2004). Chemokine CXCL12 and its receptor are known to be important in the migration of intestinal epithelial cells, development of enterocytes and promoting mucosal barrier integrity (Smith et al., 2005). The expression of chemokine (C-X-C motif) ligand 15 (CXCL15) was enhanced by \textit{L. acidophilus}, which is also known to be regulated by the influence of CD14 gene in immune cells (Benhnia et al., 2005).

Several genes involved in various cellular functions such as cell-cell signalling, cell development, cell death and cell morphology were up-regulated by \textit{L. acidophilus}. As an example, Protocadherin 8 (PCDH8) gene involved in cell signalling was enhanced after oral administration of \textit{L. acidophilus}. Protocadherin 8 belongs to protocadherin gene family, a subfamily of cadherin superfamily that are crucial in cell interactions and morphogenesis of epithelial tissue (Yagi and Takeichi, 2000; Rhee et al., 2003). Heat shock protein 1A (HSPA1A) gene involved in cell signalling mechanism was up-regulated by \textit{L. acidophilus}. The heat shock proteins are known to be capable of increasing tumor necrosis factor (TNF)-\(\alpha\) protein in macrophages (Zheng et al., 2004).

Additionally, genes involved in cell signalling mechanism such as protein tyrosine phosphatase, receptor type D (PTPRD) and mitogen-activated protein kinase kinase kinase kinase 1 (MAP4K1) were up-regulated by \textit{L. acidophilus}. The PTPRD gene is a member of protein-tyrosine phosphatase (PTP) family, which are signalling molecules
involved in variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. The protein-tyrosine phosphatases are also involved in dephosphorylation of amino acids (Pulido et al., 1995). The MAPK family is a major signalling system, which is considered as an intracellular mediator of inflammation and also known to play a functional role in c-Jun amino-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs) pathway (Hu et al., 1996; van den Blink et al., 2002).

Other genes up-regulated by *L. acidophilus* are involved in lipid metabolism such as angiopoietin-like 4 (ANGPTL4) and ATP-binding cassette, sub-family A, member 12 (ABCA12). The ANGPTL4 is a member of angiopoietin/angiopoietin-like gene family, which is a blood-borne hormone directly involved in regulating glucose homeostasis, lipid metabolism, and insulin sensitivity. Human clinical trials reported the decrease of ANGPTL4 serum levels compared to healthy subjects, which suggested that lower ANGPTL4 levels could be a causative factor for type 2 diabetes (Xu et al., 2005). Furthermore, Kaneda et al. (2002) reported that aberrant methylation of this gene is associated with human gastric cancers. The ABCA12 gene encoded protein is a member of ATP-binding cassette transporters superfamily, which is involved in transporting various molecules across extra- and intracellular membranes.

Another gene enhanced by oral administration of *L. acidophilus* is lysozyme, which is generally regarded as an anti-microbial agent found in human milk, spleen, white blood cells and plasma. Several studies reported the importance of lysozyme expression in
human B-lymphocytes and gastric adenocarcinoma tissues (Hogerkorp et al., 2003; Oien et al., 2003).

*Lactobacillus acidophilus* down-regulated genes involved in lipid metabolism such as pancreatic lipase (PNLIP), carboxyl ester lipase (CEL) and phospholipase A2, group IB (PLA2G1B). Pancreatic lipase (PNLIP) is a member of lipase gene family, which encodes a carboxyl esterase that is essential for efficient digestion of dietary fats. Pancreatic triglyceride lipase deficiency was shown to have minimal affect on dietary fat absorption, however dietary cholesterol absorption was decreased in mice (Huggins et al., 2003). The CEL is a glycoprotein secreted into digestive tract from pancreas, which is necessary in the catabolism of cholesterol (Ikeda et al., 2002) and also influence the secretion and assembly of lipoprotein through ceramide hydrolysis in mice intestine (Kirby et al., 2002). Moreover, PLA2G1B gene involved in the catalyses of releasing fatty acids from glycero-3-phosphocholines was down-regulated by *L. acidophilus*. Several forms of PLA2 are known to be present in spleen, macrophages, leukocytes and erythrocytes. *In vitro* studies using HL-60 cells reported the influence of interferon (IFN)-γ protein in increasing PLA2G1B protein activity (Visnjic et al., 1997).

*Lactobacillus acidophilus* down-regulated trefoil factor 2 (TFF2) gene, which is a member of trefoil family that are commonly known as stable secretory proteins expressed in gastrointestinal mucosa. Trefoil family secreted proteins play an active role in protecting the mucosa by stabilising mucus layer and healing epithelial cells. *In vitro* studies using human bronchial epithelial cell line, BEAS-2B demonstrated the capability of TFF-peptides in modulating the inflammatory responses by regulating TNF-α induced
secretion of interleukin (IL)-6 and IL-8 (Graness et al., 2002). Furthermore, matrix metallopeptidase 2 (MMP2) gene was also down-regulated by *L. acidophilus*. The proteins that belong to MMP family are involved in tissue remodelling, arthritis and metastasis disease processes. Several studies reported the role of MMP activity in apoptosis of epithelial cells (Wiseman et al., 2003) and cancer (Egeblad and Werb, 2002).

In the second experiment, *L. acidophilus* induced gene expression in the small intestine was determined in cholera toxin mice using compugen mouse oligonucleotide microarray. *Lactobacillus acidophilus* induced gene expression profiles in the small intestine of mice were identified by comparing with the control group. Mice fed with *L. acidophilus* demonstrated the up-regulation of CD36 gene, which is involved in cell-cell signalling mechanism. The CD36 encoded protein acts as a receptor for thrombospondin in various cell lines. The CD36 protein also involves in various adhesive processes due to its functional role as cell adhesion molecule. Recent study reported the role of CD36 in proinflammatory responses, particularly as a recognition receptor that mediates microglial and macrophage response to beta-amyloid (El Khoury et al., 2003).

Several genes involved in cell death such as glutathione S-transferase pi (GSTP1), fibronectin 1 (FN1) and ISG15 ubiquitin-like modifier (ISG15) were up-regulated by *L. acidophilus*. The GSTP1 is a polymorphic gene, which belongs to GST family of enzymes that are crucial in catalysing the conjugation of many hydrophobic and electrophilic compounds through reduced glutathione. The GSTP1 gene is known to be functional in xenobiotic metabolism and is also involved in the regulation of cell
signalling pathways (Gate et al., 2004). The FN1 gene is a glycoprotein, which involves in cell adhesion and migration processes including host defence functions. Earlier it was reported that fibronectin type III A1A2 domain was capable of regulating T-lymphocyte proliferation and cytokine production (Puente Navazo et al., 2001). Furthermore, fibronectin protein also showed the influence in wound healing process (Rybarczyk et al., 2003). Moreover, ISG15 gene was up-regulated, which is involved in cell death mechanism. The ISG15 gene, which is a member of cytokine cascade mechanism, plays an important role in several host immune functions such as innate immune response and regulating interferon signalling. D'Cunha et al. (1996) also suggested the role of ISG15 gene in immunomodulatory effects of IFN-α or IFN-β.

*Lactobacillus acidophilus* down-regulated genes involved in cell growth and proliferation such as carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), clusterin (CLU) and tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A). The CEACAM1 gene belongs to carcinoembryonic antigen (CEA) gene family, which is a cell surface molecule known to have regulatory effects on T-cell functions. Earlier studies reported the involvement of CEACAM1 protein in decreasing tumor development and oxazolone colitis (Fournes et al., 2001; Iijima et al., 2004). Furthermore, CEACAM1 protein also acts as a signal-transducing receptor, which could regulate early maturation and activation of dendritic cells (Kammerer et al., 2001). The CLU is a glycoprotein of 75-80 kDa in size secreted in physiological fluids, which is known to be involved in apoptosis and colorectal cancer (Scaltriti et al., 2004; Andersen et al., 2007). Moreover, *L. acidophilus* also down-regulated TNFRSF1A gene, which is a member of TNF-receptor superfamily that are potential regulators of apoptosis, a
process considered to be important in maintaining immune homeostasis. Particularly, TNFRSF1A protein is known to have a functional role as a regulator in inflammatory responses. Several studies also reported the involvement of TNF-receptor proteins in decreasing obesity, liver inflammation and tumor formation (Chan et al., 2003; Francis et al., 2003; Arnott et al., 2004).

Oral administration of *L. acidophilus* down-regulated villin 1 (VIL1), which is an actin-binding protein localises to the brush borders of intestine. Villin 1 gene is a member of gelsolin family of proteins, which acts as a regulator in hepatocyte growth factor-induced epithelial cell motility (Athman et al., 2003). The CD74 gene was also down-regulated by *L. acidophilus*, which is considered to be important in the activation of ERK-1/2 MAP kinase cascade and cell proliferation (Leng et al., 2003). Furthermore, *L. acidophilus* down-regulated apolipoprotein B (APOB), which occurs in two forms in plasma known as APOB-48 and APOB-100. Interestingly, Farese et al. (1995) reported the potential role of APOB gene in diet-induced hypercholesterolemia mice model.

The present study generated a list of genes that were influenced by *L. acidophilus* in normal and cholera toxin mice. To gain further insights into the potential value of this data, it is important to analyse the gene expression in various segments of GI tract as well as in distant mucosal site such as spleen in animal models. This study evaluated the effects of a specific probiotic strain in the small intestine of mice for a specific period. Therefore, it is also reasonable to predict that various factors such as strain variations, dosage or duration of the probiotic feeding may affect in modulating the gene
expression. Furthermore, human trials with different clinical settings are necessary to understand the clinical value of the microarray data reported in this study.

Nevertheless, this study represents a basic methodology to understand the influence of *L. acidophilus* in regulating gene expression that was involved in various biological functions.
5.6. CONCLUSIONS

Oral administration of *L. acidophilus* influenced the regulation of genes involved in various biological functions of the small bowel in normal and cholera toxin mice. The current study generated a comprehensive list of genes that were affected by *L. acidophilus* in small intestine of mice, which lay a basis for further understanding the interactions between probiotic bacteria and the host.
CHAPTER 6

Concluding summary
The current study examined the probiotic properties of *L. acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 using various *in vitro* methods. The influence of orally administered *L. acidophilus* and *L. paracasei* on gut and systemic immune responses in normal and cholera toxin (CT) mice were investigated. Furthermore, *L. acidophilus* induced gene expression was examined in the small intestine of normal and CT mice.

The intestinal microflora play a significant role in influencing the structural and functional development of gut immune system. Lactic acid bacteria are non-pathogenic food grade microorganisms that are natural components of intestinal microflora. Lactic acid bacteria are important in a well balanced intestinal microbial ecosystem, which are also known for their health-promoting attributes. Earlier studies suggested that probiotic bacteria must be tolerant to adverse conditions of GI tract, especially in stomach that has lower pH and high bile salt concentrations (Prasad et al., 1998; Mishra and Prasad, 2005). Furthermore, adhesive ability of probiotic bacteria to intestinal mucosa is also considered as a prerequisite for colonisation and modulation of host immune functions (Castagliuolo et al., 2005; Aslim et al., 2007).

The initial work in this study was focused in characterising the probiotic properties of *Lactobacillus* and *Bifidobacterium* species to identify potential probiotic strains for further *in vivo* mice studies. *Lactobacillus acidophilus* LAFTI L10 and *L. paracasei* LAFTI L26 showed higher tolerance to simulated gastric conditions and bile salts (Chapter 2). *Lactobacillus acidophilus* and *L. paracasei* demonstrated the correlation between *in vitro* adhesive properties such as hydrophobicity and autoaggregation. Coaggregation abilities of *L. acidophilus* and *L. paracasei* with other bacterial strains
showed significant variations, which could be due to the differences in their cell surface proteins. Particularly, *L. acidophilus* demonstrated higher level of coaggregation with *L. paracasei* and other probiotic strains. The tolerance of *L. acidophilus* and *L. paracasei* to gastric pH 2.0 and 1.0% bile salts demonstrated the potential of these strains to survive and colonise especially in the small intestine to induce beneficial effects on the host.

Orally administered *L. acidophilus* and *L. paracasei* at $10^8$ CFU/day for 14-days in BALB/c mice stimulated the host immune functions (Chapter 3). Immunofluorescent examination of small intestine in mice demonstrated the capability of *L. acidophilus* and *L. paracasei* in enhancing immunoglobulin (Ig)-A producing B-cells, which are necessary in mucosal defence mechanisms. The T-cells in small intestine of mice fed with either *L. acidophilus* or *L. paracasei* showed an increase of anti-inflammatory cytokine, interleukin (IL)-10 and pro-inflammatory cytokine, interferon (IFN)-γ. *Lactobacillus acidophilus* and *L. paracasei* were capable of enhancing IL-10 cytokine, which is known to have a beneficial role in preventing immune hypersensitivity/atopy (Majamaa and Isolauri, 1997; Kalliomaki et al., 2001) and alleviating inflammatory bowel disease (Madsen et al., 1999; Matsumoto et al., 2001). In systemic immunity, proliferative responses of splenocytes stimulated with concanavalin A (ConA) and lipopolysaccharide (LPS) were increased in mice fed with *L. acidophilus* or *L. paracasei*. Particularly, LPS induced proliferative responses of splenocytes in mice fed with *L. paracasei* were not significantly higher compared to control mice.
Mice fed with either *L. acidophilus* or *L. paracasei* regulated the systemic immune response by increasing Th1 cytokine, IFN-\( \gamma \) and Th2 cytokine, IL-10 in serum and spleen cells (Chapter 3). In the current study, stimulation of systemic immune responses by probiotic bacteria might have occurred through the bacterial antigen uptake by M-cells of Peyer’s patches in the small intestine. In Peyer’s patches, probiotic bacterial antigens interact with lymphocytes that lead to the activation of antigen-specific lymphocytes, which could have migrated through mesenteric lymph nodes and then entered into blood stream via thoracic duct to stimulate systemic immune functions.

*Lactobacillus acidophilus* and *L. paracasei* also showed the capability in stimulating non-specific immune functions such as macrophage activity (Chapter 3). The peritoneal macrophages of mice fed with *L. acidophilus* or *L. paracasei* demonstrated the enhancement of phagocytic activity against *C. albicans* compared to control mice. Vinderola et al. (2004) suggested that lack of influence of probiotic bacteria in activating systemic immune functions such as macrophage activity could be due to the inhibitory effect of IL-10. Interestingly, inhibitory effect of IL-10 on macrophage activation was not observed in the current study, where *L. acidophilus* and *L. paracasei* enhanced the Th2 cytokine, IL-10 in serum and spleen cells (Chapter 3). As shown in chapter 2, tolerance of *L. acidophilus* and *L. paracasei* to simulated acid and bile conditions and their better adherence capabilities may have favoured them to persist, colonise and adhere in GI tract of mice to stimulate the macrophage activity (Perdigon and Alvarez, 1992; Schiffrin et al., 1997).
The differences in translocation of *Lactobacillus* spp. and total anaerobes to Peyer’s patches and MLN were observed in mice fed with *L. acidophilus* or *L. paracasei* (Chapter 3). Particularly, *L. acidophilus*-fed mice showed higher translocation of *Lactobacillus* spp. and total anaerobes to Peyer’s patches compared to *L. paracasei*-fed mice and control group. Mice fed with *L. acidophilus* or *L. paracasei* showed increased bacterial translocation to Peyer’s patches and MLN, which could be due to their adhesive properties (Wagner et al., 1997) that may have contributed to the enhancement of various gut and systemic immune functions. Furthermore, oral administration of *L. acidophilus* or *L. paracasei* at $10^8$ CFU/day for 14-days in BALB/c mice did not cause intestinal inflammation, as there was no indication of translocation of *Lactobacillus* spp. and total anaerobes to sterile organs such as spleen, liver and blood.

The influence of orally administered *L. acidophilus* and *L. paracasei* on host immune responses was investigated in CT mice (Chapter 4). *Lactobacillus acidophilus* and *L. paracasei* showed the differences in inducing nitric oxide (NO) production in intestinal fluid and serum. Particularly, *L. paracasei* demonstrated as a potential inducer of NO levels in intestinal fluid and serum compared to *L. acidophilus*. The potential of *L. paracasei* in inducing the release of NO in intestinal fluid may be necessary to play a protective role in colitis (Wallace et al., 1999; Lamine et al., 2004). Both, *L. acidophilus* and *L. paracasei* increased the IgA response to CT in intestinal fluid and serum, which could be considered as a potential attribute necessary in host immune defence mechanisms.
As shown in chapter 4, immunofluorescent studies demonstrated the potential of *L. acidophilus* and *L. paracasei* in regulating Th1- and Th2-cytokines in the small intestine of CT mice. Both *L. acidophilus* and *L. paracasei* enhanced the IL-6 producing cells in small intestine, which may have contributed to the stimulation of IgA response to CT in intestinal fluid and serum. *Lactobacillus acidophilus* and *L. paracasei* showed the regulation of anti-inflammatory cytokine, IL-10 and pro-inflammatory cytokine, IFN-γ in the small intestine of CT mice. Similar effects of *L. acidophilus* and *L. paracasei* in regulating IL-10 and IFN-γ was also observed in the small intestine of normal mice (Chapter 3). In the current study, stimulation of IFN-γ producing cells in small intestine of normal and CT mice fed with *L. acidophilus* or *L. paracasei* may have contributed to the increase in uptake of bacterial antigens into Peyer’s patches (Sutas et al., 1997), which lead to the stimulation of gut and systemic immune functions (Chapters 3 & 4). Moreover, mice fed with *L. acidophilus* or *L. paracasei* also enhanced the stimulation of tumor necrosis factor (TNF-α) and IL-4 cytokines in small intestine of CT mice. Further studies on dose-dependent oral administration of *L. acidophilus* and *L. paracasei* are needed to understand the cytokine profiles at different time intervals.

Considering the *in vitro* probiotic characteristics of *L. acidophilus* and its immune enhancing properties in normal and CT mice, *L. acidophilus* induced gene expression in the small intestine of normal and CT mice was investigated using mouse oligonucleotide microarray (Chapter 5). In normal mice, *L. acidophilus* altered the gene expression in the small intestine through up- and down-regulation of 27 and 35 genes. Orally administered *L. acidophilus* affected the genes that are important in cell signalling such as HSPA1A and MAP4K1. The up-regulation of HSPA1A by *L. acidophilus* showed its
potential in interacting with intestinal epithelial cells to maintain cytoskeletal integrity. Particularly, *L. acidophilus*-fed mice up-regulated MAP4K1 gene, which is an intracellular signalling molecule that may have influenced the expression of HSPA1A in intestinal epithelial cells (Tao et al., 2006). Moreover, *L. acidophilus*-fed mice up-regulated the genes involved in immune responses (attractin and CXCL15) and cell signalling (PCDH8), whereas the down-regulated genes are functional in lipid metabolism (CEL, PLA2G1B and PNLIP) and apoptosis (MMP2).

In CT mice model, *L. acidophilus* modulated the gene expression in small intestine through an up- and down-regulation of 28 and 32 genes. Orally administered *L. acidophilus* up-regulated CD36 gene, which is involved in cell-cell signalling and known to have a significant role in pro-inflammatory responses (El Khoury et al., 2003). Other genes up-regulated by *L. acidophilus* are involved in cell death such as FN1, GSTP1 and ISG15. Particularly, genes that belong to cytochrome P450 family (CYP2C9, CYP2C19, CYP2C38 and CYP3A5) were also up-regulated by *L. acidophilus*. Interestingly, *L. acidophilus* down-regulated TNFRSF1A gene, which is considered to have a regulatory role in inflammatory mechanisms (Chan et al., 2003). Additionally, *L. acidophilus* also down-regulated genes such as VIL1, CD74 and APOB.

The present study investigated the influence of *L. acidophilus* on gene expression in the small intestine of mice, since it is considered as an important site for probiotic bacterial interactions with intestinal epithelial cells. The analysis of *L. acidophilus* induced gene expression in distant mucosal site such as spleen could be useful in further elucidating the mechanisms through which these probiotic bacteria are beneficial to the host.
Moreover, the current study generated the pool of genes in small intestine of normal and CT mice that were modified by oral administration of *L. acidophilus*. *Lactobacillus acidophilus* induced gene expression was analysed in normal and CT mice. Therefore, it is reasonable to suggest that the analysis of gene expression modulated by *L. acidophilus* was limited and could possibly differ in various animal models. Furthermore, validating the influence of *L. acidophilus* on gene expression in human clinical trials is necessary to unravel the beneficial role of probiotic bacteria.
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