THE EFFECT OF PROBIOTICS ON HOST MUCOSAL IMMUNE RESPONSES

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Abstract

Lactic acid bacteria (LAB) are a group of Gram-positive anaerobic bacteria that convert carbohydrates and complex sugars into lactic acid as the end product through fermentation. Several species of LAB have been used as probiotics. Probiotics are mono- or mixed cultures of live microorganisms which, when orally administered to animals or man, benefit them by improving the balance of the indigenous microflora. Lactic acid bacteria are claimed to have several beneficial effects; one of them being stimulation of the immune system. Many studies have demonstrated the immunostimulatory effects of LAB and various mechanisms have been suggested as to how LAB stimulate the immune system. These include the ability of LAB to translocate to Peyer's Patches (PP) and other gut-associated lymphoid tissues (GALT) for immunological processing by immunocompetent cells and production of cytokines.

There were three aims in our present studies. The first was to determine the effect of dose of an immunoenhancing probiotic strain *L. rhamnosus* on the mucosal and serum immune responses of mice to oral antigens cholera toxin (CT) and ovalbumin (OV). The second aim was to examine the effect of viability of *L. rhamnosus* on these responses. Various mucosal immune parameters were measured in these studies. Results indicate that the immunostimulatory effects of *L. rhamnosus* were dose-dependent and that the 1 x 10^9 cfu dose was the most appropriate dose for *L. rhamnosus* for its immunostimulatory effects. Viability also affects the immunostimulatory effects of *L. rhamnosus* as shown by the higher efficacy of viable *L. rhamnosus* than non-viable *L. rhamnosus* in stimulation of several aspects of the mucosal immune system. In some other immune parameters, non-viable *L. rhamnosus* was found to be the same as, or more effective than the viable bacteria.

These findings were significant in that they provide additional evidence of the dose- and viability-dependency of different LAB. This information will help those involved in the development of probiotic products to consider these factors when formulating their products so that the concentration of live LAB can be adjusted to ensure the product can convey the maximum beneficial effect to the consumer.

The third aim of our studies was to examine the role played by the immune system in protecting against enteric infection. It was found that *L. rhamnosus* increased the resistance of mice to *S. typhimurium* infection as demonstrated by the lower numbers of bacteria found in the liver and spleen, and a maintenance of liveweight. This was also accompanied by increased mucosal and systemic immune responses to *S. typhimurium.* This result suggests that the immune system may play an important role in mediating the protection against enteric infection. Various other mechanisms have also been postulated by which LAB protect against enteric infection, for example, production of antibacterial substances, competition for adherence to the gut wall and for nutrients. However, the precise role and relative importance of these mechanisms in mediating protection against enteric infection is unknown.

This thesis is dedicated to God to whom I owe my life and without whom I would not be able to give my best effort in completing it. Let all glory be unto Him.

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CD	Cluster differentiation
CT	Cholera toxin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
L. rhamnosus	*Lactobacillus rhamnosus*
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MLN	Mesenteric lymph nodes
OV	Ovalbumin
PHA	Phytohemagglutinin
PP	Peyer's Patches
S. typhimurium	*Salmonella typhimurim*

Chapter 1

1.1 Overview of the Immune System

The mammalian immune system is divided into the innate / non-specific immune system and the adaptive / specific immune system. The non-specific immune system is present and functional even before the host encounters foreign antigens, while the specific immune system is activated after antigens have come into contact with the host. In the following sections, the specific and non-specific immune system are discussed.

1.1.1 Non-Specific Immune System

The host has developed a variety of non-specific defense barriers against the entry of foreign antigens. These include the intact skin which is impermeable to most infectious agents. The presence of lactic acids and fatty acids in sweat and sebaceous secretions of the skin and the resultant low pH is generally not conducive to bacterial survival and growth. Mucous present on the inner surfaces of the body can prevent the adherance of pathogens or micro-organisms to the epithelial cells on mucosal surfaces and as such act as another physical barrier. Foreign particles trapped in the mucous can be removed by the movement of cilia that line mucosal surfaces, or by coughing and sneezing. Secreted body fluids such as tears, saliva and urine also have protective effects due to the presence of antibacterial substances such as acid, spermine, lactoperoxidase and lysozyme (Abbas *et al*, 1991; Roitt, 1991).

The cells that are involved in the non-specific immune system are phagocytes and natural killer (NK) cells. There are two main types of phagocytes, mononuclear phagocytes and polymorphonuclear phagocytes. Mononuclear phagocytes include macrophages and monocytes, which originate from the bone marrow and have simple shaped nuclei. Polymorphonuclear phagocytes have segmented nuclei and they include neutrophils, basophils and eosinophils.

Phagocytes surround and engulf foreign antigens through the process of phagocytosis. The engulfed antigens are killed by either the O_2-dependent or the O_2-independent mechanisms. In the O_2-dependent mechanisms, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) are produced to destroy the antigens while in the O_2-independent mechanisms, lysozymes and proteolytic enzymes are produced (Roitt, 1991). These substances digest antigens into peptide fragments for presentation to T cells. Macrophages thus also act as antigen presenting cells (APC) (Roitt, 1991). Natural killer cells originate from the bone marrow and have a role in immunosurveillance. They can kill virus-infected and tumour cells. They employ similar mechanisms for killing as cytotoxic T cells (CTL), namely by granule exocytosis and cytotoxin secretion. Natural killer cell granules contain pore-forming proteins, cytotoxins, serine esterases and proteoglycans (Abbas *et al*, 1991) which act to destroy the target cells (virus-infected and tumour cells).

1.1.2 Specific Immune System

1.1.2.1 General Properties of Specific Immune Responses

The specific immune system has several important features. These are i) specificity, ii) memory, iii) diversity, iv) self - regulation and v) discrimination.

i) Specificity - immune responses display specificity in that specific responses are induced by different antigens. For example, a host protected against infection by a particular organism will not necessarily be protected against infection by another organism unless they are antigenically related to one another. B and T lymphocytes (B and T cells) are able to recognise subtle differences between different antigens through their cell surface receptors (Abbas *et al*, 1991; Austyn & Wood, 1993; Roitt, 1991).

ii) Memory - the immune response to an antigen is greater if the antigen has been encountered before by the host; i.e. the immune system 'remembers' the antigen that it had encountered previously and mounts a more rapid and greater response (secondary response) the second or subsequent time it meets the same antigen (Abbas *et al*, 1991; Roitt, 1991).

iii) Diversity - the immune system is able to mount an immune response against a very large number of different antigens. This is due to the large variation found in the antigen binding sites of the cell surface receptors of lymphocytes that are specific for the antigen (Abbas *et al*, 1991; Austyn & Wood, 1993 and Roitt, 1991).

iv) Self-regulation - immune responses are self-regulated, i.e. they will decrease with time following stimulation with antigen. This allows the immune system to go back to its resting state to enable it to respond effectively and efficiently to other antigen(s) that may be encountered.

v) Discrimination of "self" from "non self" - the immune system is able to differentiate between foreign antigens and self antigens. It is able to respond to foreign antigens (non self) but is normally unresponsive to antigenic substances in our body that are recognised as indigenous to the body (self) (Abbas *et al*, 1991).

1.1.2.2 Phases of the Specific Immune Response

The specific immune response has three phases, cognitive, activation and effector, as shown in Figure 1.

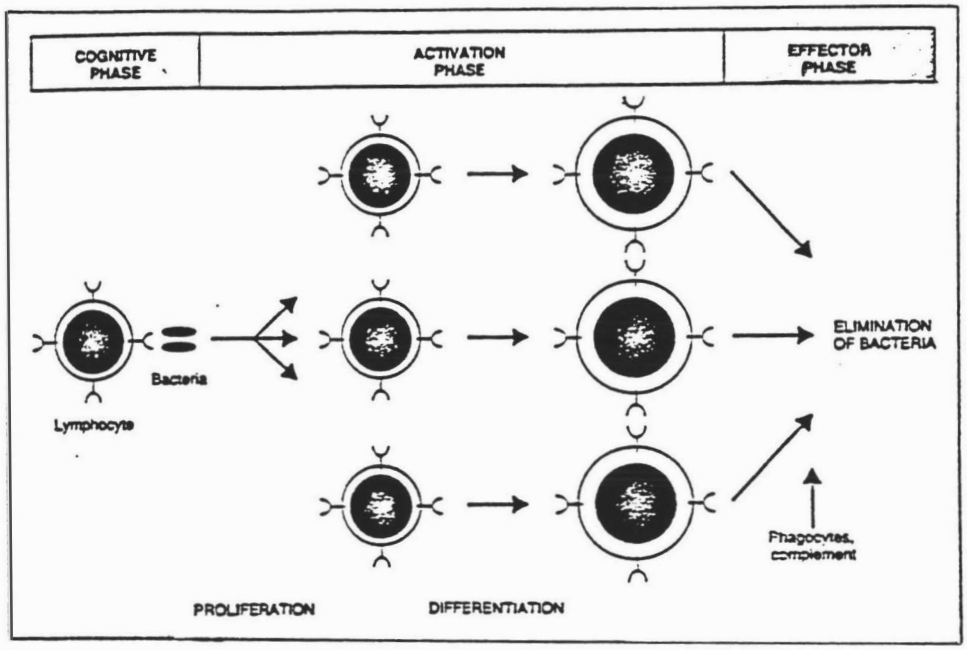

Figure 1 Phases of Specific Immune Responses. This example shows an immune response to bacteria but the same phases apply in all specific immune responses [adapted from Abbas *et al*, 1991].

The cognitive phase is where mature lymphocytes recognise and bind to foreign antigens through the specific receptors present on their cell surface. For example, B cells express immunglobulin (Ig) molecules on their cell surface that can bind to foreign antigens.

The activation phase is where lymphocytes make two changes in response to the recognition of antigens in the cognitive phase. The first is the proliferation of lymphocytes to expand the antigen-specific lymphocyte clones, and to amplify the response. The second is the differentiation of lymphocytes to effector cells that will eliminate the antigen. For example, antigen-specific B lymphocytes will differentiate to antibody-secreting cells where the secreted antibody will eliminate or neutralise the antigen.

Finally, the effector phase is where lymphocytes, activated by antigen, eliminate the antigen; these lymphocytes are termed effector cells. Some of these effector functions need the participation of other non-lymphoid cells / accessory cells, such as macrophages and natural killer cells (Abbas *et al*, 1991).

1.1.2.3 Cells of the Specific Immune System

The cells of the specific immune system are the lymphocytes which include B and T lymphocytes. Both B and T lymphocytes originate from the bone marrow; T lymphocytes migrate and mature in the thymus while B lymphocytes mature in the bone marrow (Abbas *et al*, 1991). The specific immune system is divided into the humoral and cell-mediated immune responses. Antibodies produced by B cells are responsible for humoral responses while the T cells are responsible for cell-mediated responses. T cells are divided into CTL / suppressor T cells (T_S), helper T cells (T_H), and delayed hypersensitivity T cells (T_D). Being effector cells, CTLs are responsible for killing virus-infected cells (target cells). Helper T cells have regulatory functions and they help other cells of the immune system to function efficiently as effector cells. They do this by secreting cytokines or lymphokines. Helper T cells can activate CTL precursors to CTL and also activate B cell proliferation and differentiation to plasma cells and facilitate the effector functions of macrophages (Abbas *et al*, 1991; Austyn & Wood, 1993; Roitt, 1991). There are 2 types of helper T cell clones, T_{H1} and T_{H2}. T_{H1} synthesise interleukin-2 (IL-2), interferon-γ (IFN-γ), IL-3 and lymphotoxin cytokines whereas T_{H2} synthesise IL-4, IL-5 and IL-3 (Mosmann & Coffman, 1987). Both cells facilitate antibody production by B cells, however, T_{H1} cells suppress IgE and IgG_1 production while T_{H2} clones facilitate the IgE, IgG_1 and IgA production.

Suppressor T cells act to control immune responses by preventing excessive antibody production. Delayed hypersensitivity T cells are involved in destroying target cells indirectly with the help of accessory cells, for example, macrophages or by releasing mediators that will destroy the target cells (Sell, 1987).

1.1.3 Tissues of the Immune System

In mammals, the tissues of the immune system comprise of the primary and secondary lymphoid tissues. The primary lymphoid tissue consists of the bone marrow and thymus where mature lymphocytes are produced. The secondary lymphoid tissue consists of the spleen, lymph nodes, the gut-associated lymphoid tissue (GALT) which consists of Peyer's Patches (PP) in the intestine (Austyn & Wood, 1993); bronchus-associated lymphoid tissue (BALT), which consists of the palatine and pharyngeal tonsils (Walker, 1994), and Waldeyer's ring which comprises the tonsils and adenoids (Austyn & Wood, 1993).

1.1.4 The Mechanisms by which the Specific and Non-Specific Immune System Eliminate Antigens

The specific and non-specific immune systems are separate systems; functioning on their own but also working together very closely to eliminate antigens or pathogens. A simplified schematic diagram (Figure 2) explains the process of antigen recognition and elimination by the cells and molecules of the specific and non-specific immune system.

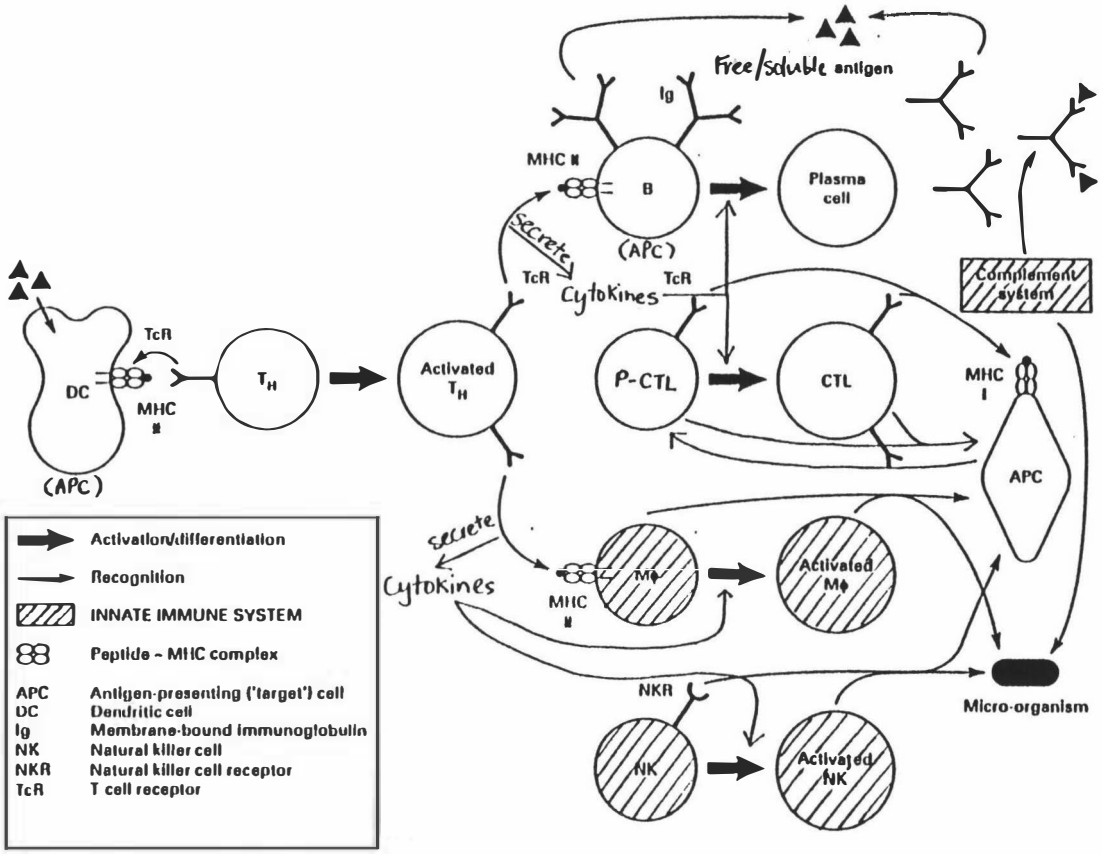

Figure 2 Process of Antigen Recognition and Elimination by the Specific and Non-Specific Immune System (adapted from Austyn & Wood, 1993)

1.1.4.1 Mechanisms of Elimination of Antigens by the Non-Specific Immune System

The entry of foreign antigens results in inflammation which triggers the entry of soluble molecules such as complement and lysozyme, and phagocytes from the blood to the site of injury. Macrophages phagocytose antigens and destroy them using the O_2-dependent or independent mechanisms. Antigens are also internalised and processed into peptides to form the peptide-MHC II complex by macrophages. The complex is recognised by activated T_H cells. This recognition activates the macrophages so that phagocytic activity is enhanced (Figure 2). Natural killer cells are able to recognise viral infected cells through altered cell surface molecules on the infected cells. Natural killer cells are activated by interferons (cytokines) produced by viral infected cells and the ability to destroy the cells is enhanced (Figure 2). Interferons can also help protect uninfected cells in that they make these cells resistant to viral infection (Roitt *et al*, 1985).

Activated complement can form pores in the cell wall of foreign antigens / pathogens via the so called 'alternative' pathway. The antigens / pathogens are destroyed by the process of osmotic lysis (Austyn & Wood, 1993). Activated complement can also facilitate phagocytosis through opsonisation, by coating the surface of the foreign organism. Phagocytes, (eg. macrophages) possess specific membrane receptors for complement and so can recognise and phagocytose the antigens coated with complement (Austyn & Wood, 1993; Roitt, 1991).

The mechanisms of eliminating foreign antigen during non-specific immune responses are shown in Figure 3.

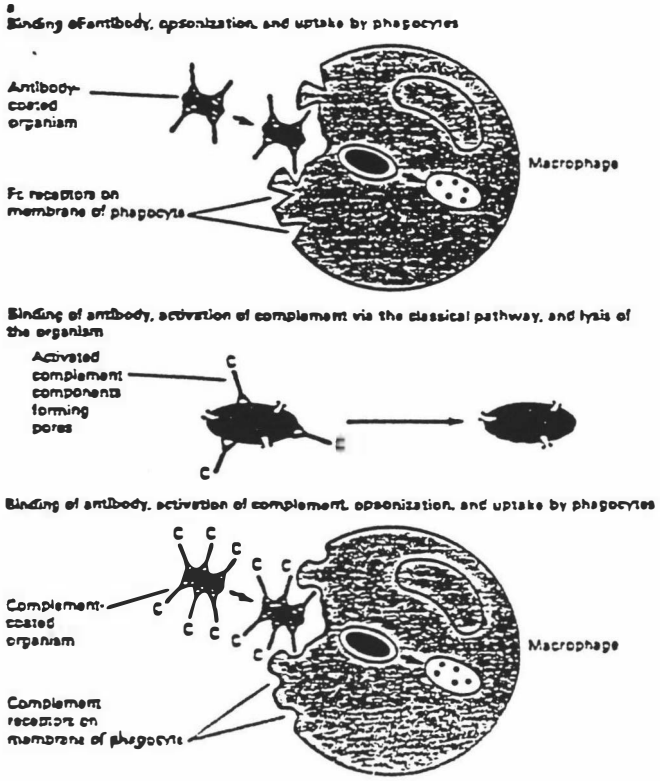

Figure 3 Some Mechanisms of Elimination of Antigens During Non-Specific Immune Responses [Austyn & Wood, 1993].

1.1.4.2 Mechanisms of Elimination of Antigens by the Specific Immune System

B and T lymphocytes play important roles in the specific immune responses, working together very closely to effect the elimination of foreign antigens as shall be seen from the discussion that follows.

Refer to Figure 2 for the description of the following processes. Soluble / free antigens are recognised by APCs which include macrophages (M), dendritic cells (DC) and B cells (B). The antigens recognised by B cells are internalised and processed into peptides which will then associate with the major histocompatibility complex (MHC) class II molecules (top part of figure). The antigen peptides bound to MHC class II molecules are presented to T_H cells. Helper T cells are activated when antigens recognised by DC are processed and the peptide-MHC II complex are recognised by T_H (middle left of figure). Activated T_H cells secrete cytokines that stimulate the proliferation and differentiation of B cells into antibody producing cells (plasma cells) (Austyn & Wood, 1993). Macrophages and other accessory cells are also able to secrete cytokines. The antibodies produced eliminate the antigens by the process of neutralisation, agglutination or opsonisation. There are several different types of

antibodies and they perform specific functions. For example, IgA protects against infectious agents encountered at mucosal surfaces and IgG protects the newborn who is immunologically immature against foreign antigens, and also fixes complement and facilitates phagocytosis by macrophages (Stites *et al*, 1994). Figure 4 shows the process of eliminating antigens through the humoral immune response. Figure 5 shows some of the mechanisms by which antibodies can act to eliminate / neutralise antigens.

Cytokines secreted by T cells can also act on effector cells such as precursor CTL, NK cells and macrophages (Abbas *et al*, 1991).

Those antigens (produced within cells) associated with MHC Class I molecules are recognised by pre-CTLs (P-CTL). Pre-CTLs differentiate when they recognise the antigen associated with MHC Class I molecules and when they encounter the cytokines produced by helper T cells (middle right of figure). These differentiated CTLs develop membrane bound cytoplasmic granules (Abbas *et al*, 1991) and deliver the contents of the granules to the target cell. After delivery of the granule contents, differentiated CTLs detach themselves from the target cell. The cytolysins and cytotoxins released from the granules lead to the destruction of the target cell (Abbas *et al*, 1991; Austyn & Wood, 1993).

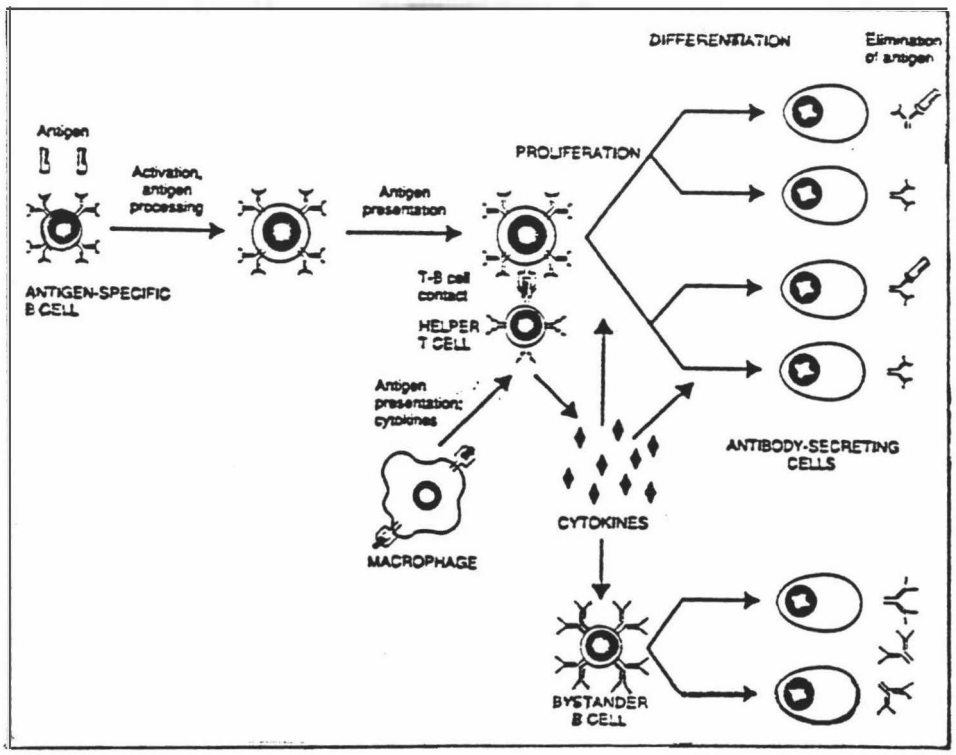

Figure 4 Process of Humoral Immune Responses to Protein Antigens (Abbas *et al*, 1991).

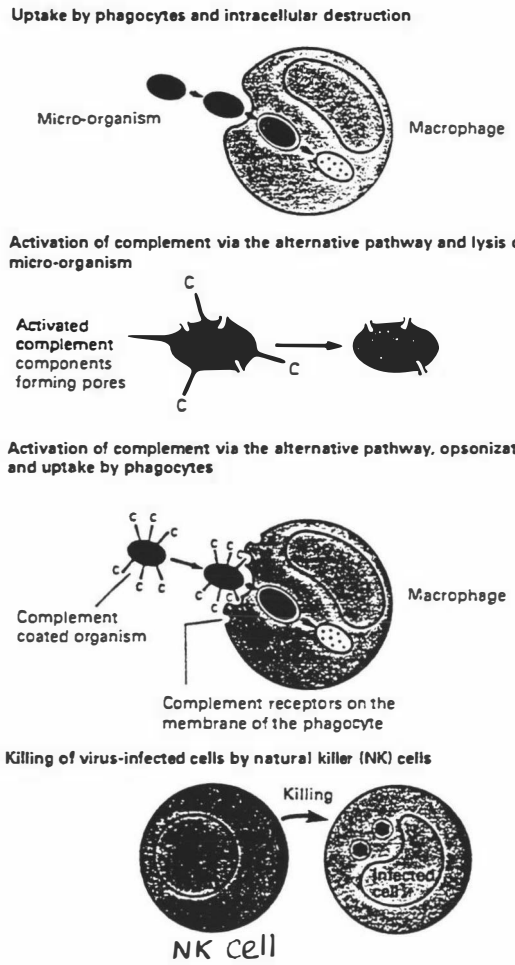

Figure 5 Some Mechanisms of Elimination of Antigens During Specific Immune Responses (Austyn & Wood, 1993).

The following sections will first differentiate between the systemic and secretory immune responses followed by a discussion on the mucosal immune system.

1.1.5 Systemic and Mucosal Immune Responses

1.1.5.1 Differences Between Systemic and Mucosal Immune Responses

The systemic immune response is induced when antigen(s) enter the host parenterally (i.e. not orally), although the response can also be induced when an orally administered antigen enters the circulation. The secretory / mucosal immune response is activated when antigens are encountered at mucosal sites such as the gastrointestinal (GI), respiratory and urogenital tracts. Systemic immune responses occur at sites that are distinct from the mucosal immune response. The lymphoid tissues that are involved in the systemic response include the spleen, lymph nodes, blood and lymphatics while those involved in the mucosal response include the GALT and BALT (Stites *et al*, 1994). In the systemic response, antigens are received from the blood / lymphatics (Stites *et al*, 1994) while in the mucosal response, antigens are received from the mucosal epithelium. In the systemic immune system, non-specific responses are mediated by phagocytic cells such as macrophages, neutrophils and NK cells through processes that have been mentioned previously (Section 1.1.4.1). Specific immune responses are mediated by B and T lymphocytes through mechanisms that have been mentioned earlier in Section 1.1.4.2. Antibodies that are involved in the systemic immune response are primarily of the IgG and IgM isotypes (Mestecky, 1987). The mucosal immune response is mainly a humoral response mediated by antibodies, especially secretory IgA (sIgA). The events of the humoral response have already been discussed in Section 1.1.4.2.

1.1.5.2 The Mucosal Immune System

The mucosal immune system is divided into two parts, the organised lymphoid tissue and the diffuse lymphoid tissue. The organised lymphoid tissues consist of the GALT and BALT while the diffuse lymphoid tissues consist of cells in both the intraepithelial lymphocyte (IEL) and the lamina propria lymphocyte (LPL) compartments (Abbas *et al*, 1991; Stites *et al*, 1994). The organised lymphoid tissues are sites where antigen(s) are encountered by immunocompetent cells, while the diffuse lymphoid tissues are sites where sIgA are produced and where antigen(s) are eliminated. The GALT and BALT have many similarities as well as many differences (McGhee *et al*, 1992; Walker, 1994; Bienenstock *et al*, 1973). The remaining discussion will be focused on the GALT.

Peyer's Patches are the most important structural units of the GALT as they are the sites where the mucosal immune responses are induced (Stites *et al*, 1994). A flattened specialised epithelium enriched with microfold or M cells is found to overlay the PP (McGhee *et al*, 1992). The dome region which is located below the epithelium is rich in lymphocytes, macrophages and other accessory cells. The follicular region located below the dome region contains germinal centres where there are many IgA^+ highly differentiated B cells.

The intraepithelial lymphocytes consist mainly of $CD3^+$ and $CD2^+$ T cells while there are equal numbers of T and B cells in the LP. In the LPL compartment, there are mainly IgA^+ B cells and plasma cells. There are also IgM^+, IgG^+ and IgE^+ B and plasma cells, and $CD4^+$ and $CD8^+$ T cells, and macrophages in the LP (Stites *et al*, 1994). There is however, a lack of NK cells in human LP, although there are low levels of activity in the LP of rodents and primates. There are also lymphokine-activated killer cells (LAK) and mast cells in the LP (Stites *et al*, 1994).

Antigens present in the intestinal lumen are delivered by M cells to the underlying lymphoid tissue where they are processed immunologically by B and T cells. The antigen activated T and B cells then leave the PP through the mesenteric lymph nodes (MLN) and thoraic duct and enter into the circulation. These cells will enter the mucosal effector sites such as the lamina propria (LP) of the respiratory, GI and reproductive tracts. At these sites, the B cell clones expand into IgA plasma cells and secrete IgA into the external secretions. This whole process from inducing a sIgA

response and its transport to effector sites is termed the Common Mucosal Immune System (Roitt, 1991; McGhee *et al*, 1992; Walker, 1994; Mestecky *et al*, 1994 and Mestecky, 1987). Figure 6 shows the pathway of the mucosal immune system (Service, 1994).

Protection of mucosal sites such as the gastrointestinal, respiratory and urogenital tracts (sites where the antigen(s) first encounter the host) is mainly mediated by sIgA (Mestecky, 1987). Polymeric IgA (pIgA) induced at mucosal surfaces interacts with the secretory component (SC) after which sIgA is produced when pIgA, connected to SC, is transported through the epithelial cells (Mestecky, 1987 and Kagnoff, 1993). Other antibodies such as IgG also play a part in protecting the mucosal surfaces (Service, 1994).

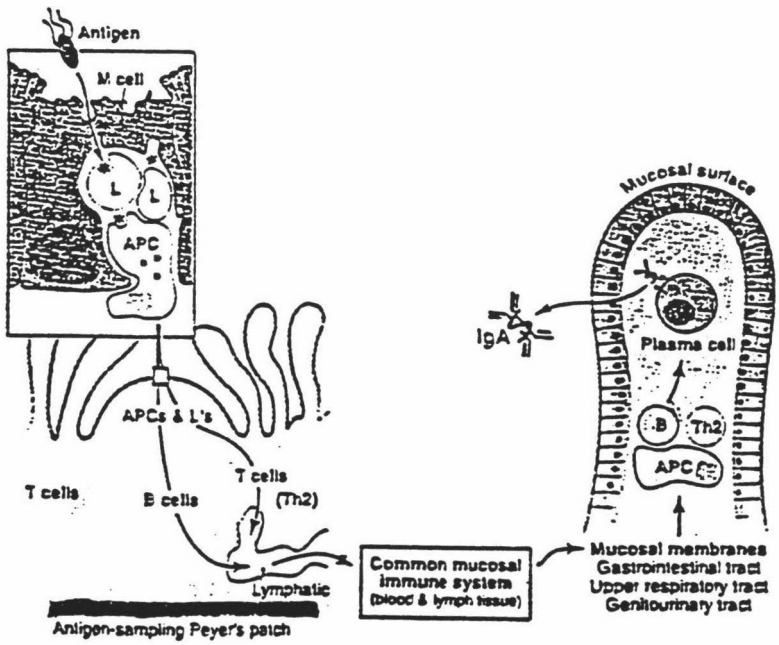

Figure 6 Pathway of the Stimulation of the Common Mucosal Immune System (Service, 1994)

1.2 Probiotics

1.2.1 Definition

"Probiotics are mono or mixed cultures of live microorganisms, which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora" (Havenaar & Huis In'T Veld, 1992).

1.2.2 Probiotic Bacteria

Table 1 shows some examples of probiotic bacteria that are commonly used in probiotic products (Tannock, 1997). Most of these belong to the group Lactic Acid Bacteria (LAB) and they are part of the indigenous gut microflora of humans and animals. Lactic acid bacteria are Gram positive anaerobic bacteria with rod and coccus morphologies. They convert carbohydrates and complex sugars into lactic acid as the end product through a process of fermentation (Salminen *et al*, 1993 and Hull, 1995). Members of the *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Pediococcus* genera are included in LAB. Lactic acid bacteria are used in the production of a variety of fermented food products. They are also present naturally on some plant surfaces and mucous membrane of humans and animals (Salminen *et al*, 1993 and Richardson, 1996).

Table 1 Examples of Microorganisms Used in Probiotic Products

Products for humans	Products for farm animals
Lactobacillus acidophilus	*Lactobacillus acidophilus*
Lactobacillus casei Shirota strain	*Lactobacillus casei*
Lactobacillus reuteri	*Lactobacillus plantarum*
Lactobacillus rhamnosus	*Lactobacillus subtilitis*
Bifidobacterium bifidum	*Streptococcus thermophilus*
Bifidobacterium infantis	*Enterococcus faecium*
Streptococcus thermophilus	*Aspergillus oryzae*

[Tannock, 1997]

Some specific examples of LAB with documented health benefits include *L. acidophilus* (NCFB 1748), *L. casei* Shirota strain, *Lactobacillus* GG (*L. GG*) (ATTCC 53103) and *L. acidophilus* LA1 (IDF, 1996 and Salminen & Saxelin, 1996).

Other new and novel strains of LAB that are being tested for health effects / benefits include strains of *L. reuteri*, *Bifidobacterium animalis*, *L. casei*, *B. bifidum* and strains of *E. faecium* (Salminen & Saxelin, 1996).

1.2.3 Characteristics of an Effective Probiotic

In order for any bacteria to be an effective probiotic, they must possess the following characteristics:

- be stable under the gastric conditions in the stomach, and resistant to bile and gastric acid (Salminen *et al*, 1993).
- have the capacity to adhere and colonise the intestinal mucosa
- be of human origin (especially for human products)
- be safe for human use
- have a generally regarded as safe (GRAS) status

1.2.4 Beneficial Effects of Probiotic Bacteria

Some of the claimed beneficial effects of LAB include prevention against bacterial infection (Salminen *et al*, 1993; O'Sullivan *et al*, 1992; Hitchins & McDonough, 1989); alleviation of lactose intolerance (O'Sullivan *et al*, 1992; Salminen *et al*, 1993); reduction of serum cholesterol levels (O'Sullivan et al, 1992; Lichtenstein & Goldin, 1993); anticarcinogenic activity (O'Sullivan *et al*, 1992); stimulation of the immune system (Richardson, 1996) and improvement in the nutritional value and quality of food (O'Sullivan *et al*, 1992). Each of these benefits are discussed in detail in the following sections.

1.2.4.1 Prevention of Bacterial Infection

Many studies have shown that LAB can protect against enteric infections in humans (Saavedra *et al*, 1994; Siitonen *et al*, 1990; Majamaa *et al*, 1995; Kaila *et al*, 1992; Oksanen *et al*, 1990) and in animals (Cole & Fuller, 1984; Hitchins *et al*, 1985; Perdigon *et al*, 1990a; Nader de Macias *et al*, 1992; Nader de Macias *et al*, 1993; Link-Amster *et al*, 1994; Clements *et al*, 1981; Paubert-Braquet *et al*, 1995). *In vitro* studies have also shown the antagonistic effects of LAB on pathogenic bacteria (Hudault *et al*, 1997; Gilliland & Speck, 1977a; Bernet *et al*, 1994; Apella *et al*, 1992).

A number of studies have examined the effect of LAB on the prevention of antibiotic-asssociated diarrhoea and different effects (beneficial / no effect) have been reported. This may be due to different LAB strains and antibiotics being used; the use of LAB that may not be resistant to antibiotics (Sanders, 1993); the small number of subjects, and in some studies, results were only semiquantitative.

Results of studies on the prevention of traveller's diarrhoea have also been varied. Different locations where studies were conducted and the different pathogens that may be involved in infection (Sanders, 1993) makes it difficult to compare the results. Different doses and delivery system of LAB (Katelaris, 1996) also contribute to the difficulty in comparing the results.

Secretion of antibacterial or inhibitory substances is one of the suggested mechanisms whereby LAB may prevent bacterial infection. Some of the inhibitory substances produced by LAB include hydrogen peroxide (H_2O_2) (Gilliland & Speck, 1977a), carbon dioxide (CO_2) (Mishra & Lambert, 1996), diacetyl; antimicrobial substances such as bacteriocins (IDF, 1991; Vincent *et al*, 1959 and Silva *et al*, 1987) and organic acids such as lactic and acetic acids (Shahani & Chandan, 1979; Helander *et al*, 1997; Mishra & Lambert, 1996 and Gilliland & Speck, 1977a). These substances employ various mechanisms to destroy pathogens and their toxins. For example, the inhibitory effect of H_2O_2 may be due to the toxic reactive O_2 species such as O_2^- and (hydroxyl radical) OH· (Hollang *et al*, 1987). Diacetyl's inhibitory effect is due to its ability to react with the arginine-binding proteins of Gram negative bacteria thus disturbing the usage of arginine by these bacteria (Jay, 1986 cited by Mishra & Lambert, 1996). Lactic and

acetic acids' inhibitory effects are due to the low pH generated by these acids which prevents the growth of some pathogenic bacteria (Mishra & Lambert, 1996).

A number of studies have shown that LAB stimulate the immune response to pathogenic bacteria (Perdigon *et al*, 1990a; Nader de Macias *et al*, 1992; Saucier *et al*, 1992; De Petrino *et al*, 1995) and this constitutes another mechanism for the antibacterial effects of LAB. The host employs the non-specific and specific immune defenses to eliminate or neutralise pathogenic bacteria.

1.2.4.2 Alleviation of Lactose Intolerance

Lactose intolerance is a condition that is the result of the absence or lack of sufficient amounts of the β-galactosidase enzyme in the intestine of some individuals (Gilliland, 1989; Shahani & Chandan, 1979 and Hamilton-Miller, 1996), who are then unable to digest lactose. Lactic acid bacteria's beneficial effect on lactose intolerance in man is one of the best established health benefits of LAB (Marteau & Rambaud, 1993). Many studies have shown LAB's ability to increase lactose digestion in lactose intolerant human subjects and normal animals (IDF, 1991; Garvie *et al*, 1984; Goodenough & Kleyn, 1976; Marteau *et al*, 1990; Savaiano *et al*, 1984; Lin *et al*, 1991; Kolars *et al*, 1984).

Lactic acid bacteria's ability to increase digestion of lactose is due to the production of lactase (a β-galactosidase) by LAB which hydrolyses lactose (IDF, 1991).

1.2.4.3 Reduction of Serum Cholesterol Levels

Studies on LAB's effect on serum cholesterol levels have shown positive as well as negative (no effect) results. Rao *et al* (1981) found a significant decrease in the plasma cholesterol levels in rats fed milk fermented by *S. thermophilus* while Gorbach *et al.* (1988) cited from IDF (1991), found decreased cholesterol levels in healthy human volunteers fed two weeks with a fermented product containing *L. GG.* Hepner *et al* (1979) showed that pasteurised and non-pasteurised yoghurt were able to significantly decrease plasma cholesterol levels in humans but there was no effect on cholesterol levels in subjects fed buttermilk. In another study, Grunewald (1982) found that feeding

skim milk fermented with *L. acidophilus* to rats for four weeks decreased the plasma cholesterol levels. No such effect was found for unfermented skim milk. However, many other studies found no effect of yoghurt or LAB preparations on the plasma lipid and lipoprotein levels, and total plasma cholesterol levels (Halpern *et al*, 1991, cited from Marteau & Rambaud, 1993; Thompson *et al*, 1982).

Negative results shown in some studies could be due to various reasons. For example, differences in the efficacy of the different LAB strains used, studies were not double blind and placebo controlled, limited subjects were used, studies done in animals were extrapolated to humans, baseline serum cholesterol levels of subjects were not determined before starting the trial etc.

The mechanisms by which LAB may reduce cholesterol levels are not fully understood. However, several mechanisms have been suggested. One of the mechanisms is the ability of LAB to directly assimilate cholesterol and thus remove it from the intestine (O'Sullivan *et al*, 1992; Gorbach, 1990; Gilliland *et al*, 1985).

Another mechanism is the ability of LAB to deconjugate bile acids which participate in the formation of intestinal cholesterol (Gilliland & Speck, 1977b). Deconjugated bile acids do not absorb lipids as well as the conjugated bile acids from the intestinal tract and so the absorption of cholesterol from the intestine is reduced (Eyssen, 1973).

Milk factors present in fermented milk such as dihydroxy-methyl-glutaric acid (HMG) and orotic acid could be another mechanism whereby LAB exert their hypocholesterolemic effects (Mann, 1977; Richardson, 1978). Dihydroxy-methyl-glutaric acid acts by preventing cholesterol biosynthesis as it inhibits the rate limiting enzyme, HMG-CoA-reductase that is involved in cholestrol synthesis. Orotic acid also acts by inhibiting cholesterol synthesis; in addition, it interferes with the lipoprotein and apolipoprotein synthesis from lipids or lipoprotein from the liver (Richardson, 1978).

1.2.4.4 Anticarcinogenic Effects

Several studies have shown the anti-carcinogenic activities of LAB in humans and animals (Reddy *et al*, 1983; Friend & Shahani, 1984; Farmer *et al*, 1975). However, the results of some other studies not mentioned here are varied and inconclusive. This may be due to different strains of LAB used in studies, the use of different animal species (IDF, 1991), the lack of human studies and the difficulty in extrapolating *in vitro* results to the *in vivo* conditions in animals or humans, especially in studies on the antimutagenic effects of LAB (Hosono *et al*, 1987).

Several mechanisms have been suggested whereby LAB may exert anticarcinogenic activity. Inactivating carcinogenic or procarcinogenic compounds such as azo dyes and nitrosamines that may have originated from the diet or be produced in the intestine is one of the mechanisms. Lactic acid bacteria have been shown by Dodds & Collins-Thompson (1984) to decrease nitrite *in vitro*. Nitrite is a pro-carcinogen and can be converted into nitrosamines (a carcinogen) in the intestine. By directly reducing pro-carcinogens in the intestine, LAB exert their anti-carcinogenic activity.

Anticarcinogenic components can also inactivate carcinogenic or pro-carcinogenic compounds. Sekine *et al* (1985) and Hosono e*t al* (1987) suggested that the anti-tumour activity of LAB could be induced by the cell wall components of LAB such as peptidoglycans and polysaccharides. These components act by stimulating the immune system (refer to Section 3.5.2).

Ayebo *et al* (1981) have reported the antitumour activity of a component of LAB in yoghurt that had a molecular weight of < 14,000. Short chain fatty acids such as butyrate have also been suggested to play a role in protecting against colon cancer as shown by various studies (McIntyre *et al*, 1993 and Young, 1996).

Decreasing faecal bacterial enzymes such as nitroreductase and azoreductase that can convert procarcinogens to carcinogens (O'Sullivan *et al*, 1992; Gorbach, 1990) may contribute to the anticarcinogenic effect of LAB. Many studies have shown the efficacy of LAB in decreasing faecal bacterial enzymes (Goldin & Gorbach, 1984; Goldin *et al*, 1980 and as reviewed by IDF, 1991). A concensus panel of experts on the health attributes of LAB fermented milk have agreed that some LAB fermented products can

protect against colon cancer by decreasing certain faecal bacterial enzymes (Sanders, 1993), but further studies are needed to support the anti-cancer claim of LAB. A study by Goldin & Gorbach (1977), cited from Adachi (1992) showed that feeding rats a diet rich in beef with supplements of viable *L. acidophilus* significantly decreased the activities of β-glucuronidase, nitroreductase and azoreductase enzymes in the faecal microflora. Another study by Goldin *et al* (1980), cited from Salminen *et al* (1993) showed similar results.

Stimulation / enhancement of the host immune system (IDF, 1991; Adachi, 1992) can also contribute to the anticarcinogenic effect of LAB. In a study by Perdigon *et al* (1995), it was found that feeding yoghurt inhibited tumour development in the intestine and this was accompanied by increased IgA producing cells and T lymphocytes in the large intestine for all feeding periods. Macrophages were also greatly activated in the treated group. In another study, Fernandes & Shahani (1990) found that mice treated with hydrocortisone (an immunosuppressant) and given *L. acidophilus* had lower tumour suppression compared to mice given just *L. acidophilus*. Other studies have also shown that macrophages and NK cells are involved in the antitumor effect of LAB (Kato *et al*, 1981; Kuwabara *et al*, 1988, cited by Adachi, 1992; Kato *et al*, 1984).

Carcinogenesis can also be initited by carcinogen induced mutations. By suppressing mutagenesis and inactivating mutagens, carcinogenesis may be prevented. Very little information is available on the antimutagenic activity of LAB and the activity is also not well understood (Hosono *et al*, 1987). Several studies have shown that LAB have anti-mutagenic activities (Hosono & Kashina, 1986; Hosono *et al,* 1988; Hayatsu & Hayatsu, 1993). Some mechanisms have been suggested as to how LAB suppresses mutagens or mutagenesis (Hayatsu & Hayatsu, 1993). Lactic acid bacteria can bind to heterocyclic amines (mutagens) in the intestine thus preventing the absorption of mutagens. Zhang & Ohta (1990) have shown that some mutagens can bind to LAB. Metabolic function of the intestinal bacteria or other activities of LAB could also destroy mutagens thereby reducing the exposure to mutagens in the intestine (Hayatsu & Hayatsu, 1993).

Other suggested mechanisms of LAB's anticarcinogenic effects include the lowering of the intestinal pH by LAB, and changing the motility of the colon and the transit time of carcinogenic or procarcinogenic substances in the colon (McIntosh, 1996).

1.2.4.5 Improvement in the Nutritional Value and Quality of Food

Milk products that are fermented with LAB (eg. lactobacilli) can decrease the occurrence of food allergies and intolerances by decreasing the levels of food substances that may be harmful to humans (Hull, 1995). Fermentation can also improve the nutritional value of food by increasing the levels of amino acids and B vitamins, and increasing protein and fat digestibility (O'Sullivan *et al*, 1992 and Gorbach, 1990). Acids that are produced during the fermentation process can increase the digestibility of fermented products. Further, fermented products have more of the (+)-L form of lactic acid which is easily metabolised (O'Sullivan *et al*, 1992 and Gorbach, 1990). Fermentation can increase the shelf life of food when the fermentation process removes the sugars and other components in the food that can promote its spoilage. Fermentation can also make food more acceptable in that the texture and flavour of the raw materials are enhanced (Hull, 1995). Several LAB are able to synthesise vitamins such as folic acid, niacin, vitamins B_6 and B_{12} (Shahani & Chandan, 1979). Bifidobacteria can synthesise B vitamins and digestive enzymes like lysozyme (Tannock, 1997). Lactic acid bacteria can thus increase the nutritional value of the food in which they are used.

1.2.4.6 Other Health Benefits of LAB

Some of the other claimed benefits of LAB include prevention of urogenital infections and alleviation of constipation (O'Sullivan *et al*, 1992; Salminen *et al*, 1993 and Fuller, 1989). Prevention of osteoporosis in the elderly (Salminen *et al*, 1993; Marteau & Rambaud, 1993 and Fuller, 1989) by improving calcium bioavailability has also been claimed but there is controversy over whether LAB do increase the bioavailability of calcium (O'Sullivan *et al*, 1992).

1.2.5 Effect of LAB on the Immune System

Lactic acid bacteria have been shown to stimulate both the non-specific and specific immune responses (reviewed by Gill, 1998; Perdigon *et al*, 1995; Marteau & Rambaud, 1993). This has been mostly demonstrated by studies with animals, especially mice. Very few studies have been conducted with humans. The results of studies conducted so far are varied thus making it difficult to draw firm conclusions. Among the reasons that may account for the varied results are the use of poorly designed studies, the use of different strains of LAB which have different efficacies and the use of different doses and duration of LAB feeding. In the following sections, examples of studies that demonstrate the positive effects of LAB on the immune system are discussed.

1.2.5.1 Effect of LAB on the Non-Specific Immune System

The effect of LAB on the non-specific immune response has been the subject of several studies (reviewed by Gill, 1998; Perdigon *et al*, 1995; IDF, 1991).

Several studies have shown that LAB enhance the *in vitro* phagocytic activity of the peritoneal and pulmonary macrophages and the *in vivo* colloidal carbon clearance in animals (Perdigon *et al*, 1988; De Simone *et al*, 1991; Moineau *et al*, 1989; De Petrino *et al*, 1995; Perdigon & Alvarez, 1992; Perdigon *et al*, 1986a & 1986b). In the study by Perdigon *et al* (1988), it was found that there was increased *in vitro* phagocytic activity of the peritoneal macrophages in mice fed a mixture of *L. casei* and *L. acidophilus* fermented milk. There was also an increase in the phagocytic function of the reticuloendothelial (RE) system of mice fed this mixture of fermented milk compared to the control mice. In the study by Moineau *et al* (1989), there was a significantly higher phagocytic index of the pulmonary alveolar macrophages in the *L. acidophilus* and *L. casei* fed mice compared to the control mice fed unfermented milk. In mice fed *L. acidophilus*, *B. longum* and *L. helveticus*, there was also a significantly higher index compared to the control mice.

Studies by Perdigon *et al* (1986a & b) and Paubert-Braquet *et al* (1995) have shown that LAB increase lysosomal enzyme secretions while in a study by Torteuro *et al* (1995),

there was increased lysosomic substances such as lysosomes and phagosomes in piglets fed LAB. In a study by Sato *et al* (1988), there was increased O_2^- production.

In human studies, similar results were found. Schiffrin *et al* (1995) showed that the *in vitro* phagocytic activity towards *E. coli* in the peripheral blood of human subjects fed LA1 and Bb12 was increased.

Very few studies have examined the effect of LAB on NK cell activity. In two studies by De Simone *et al* (1986a & 1988), it was found that there was increased NK cell activity *in vitro* in human peripheral blood lymphocytes (PBL) costimulated with concanavalin A (ConA) and LAB. In another study, De Simone *et al* (1989) found a gradual increase in the percentage of NK cells in human peripheral blood of subjects fed yoghurt for 28 days.

1.2.5.2 Effect of LAB on the Specific Immune System

Lactic acid bacteria have been found in several studies to enhance the specific immune response to enteric pathogens and bacterial vaccines in animals and humans. For example, Perdigon *et al* (1990a) found that orally administered LAB protected mice against infection with *S. typhimurium*, and *E. coli* (Perdigon *et al*, 1991). The protective effects against enteric infection shown in these studies were accompanied by enhanced mucosal and serum immune responses. In these studies it was also observed that not all LAB exhibited protective effects. In other studies, for example, by Nader de Macias *et al* (1992), feeding with LAB fermented milk protected against *Shigella sonnei* infection in mice.

Similar results have been found in children with rotavirus gastroenteritis in that there was increased mucosal and systemic antibody responses (Majamaa *et al*, 1995; Kaila *et al*, 1992). In the study by Majamaa *et al* (1995), it was found that when children suffering from rotavirus gastroenteritis were randomly given LAB, there was increased numbers of rotavirus-specific IgA secretory antibody secreting cells (sASC) and higher levels of serum IgA at the convalescent stage.

Link-Amster *et al* (1994) evaluated whether feeding with *L. acidophilus* LA1 and bifidobacteria fermented milk could improve the immune response of human subjects to the *S. typhi* Ty21a vaccine, which was used to imitate a bacterial infection. It was found

that the LAB treated group had a significantly higher titre of specific serum IgA to *S. typhi* Ty21a and total serum IgA compared to the control group with only milk and without any fermented food intake. This observation was accompanied by an increase in the *L. acidophilus* and bifidobacteria counts in the faecal microflora.

Several other studies with human subjects and mice have also shown the ability of LAB to enhance the humoral and cell-mediated immune responses (De Simone *et al*, 1991; Moineau & Goulet, 1991a; Perdigon *et al*, 1988; Perdigon *et al*, 1987; De Simone *et al*, 1987; Yasui & Ohwaki, 1991; Vesely *et al*, 1985).

Perdigon *et al* (1988) found that the serum antibody titres to sheep red blood cells (SRBC) in all of the LAB fermented milk fed groups were higher than the control group. All treatment groups also showed higher IgM plaque forming cells (PFC) in the spleen cells compared to the control group. In the study by Perdigon *et al* (1987), it was found that mice fed *L. acidophilus* and *S. thermophilus* had a higher anti-SRBC PFC response compared to the control group. De Simone *et al* (1987) found that in mice fed viable lactobacilli-containing yoghurt, there was a significantly higher percentage of B cells in the PP, and increased proliferative responses of PP to phytohaemagglutinin (PHA) (a T lymphocyte mitogen) and lipopolysaccharide (LPS) (a B lymphocyte mitogen) compared to the control group fed cow's milk. Similar results (increased proliferative responses to T and B cell mitogens) were found with murine spleen lymphocytes in a study by Vesely *et al* (1985). In addition, there was also increased serum IgM and IgG_{2a} levels. In these two studies (De Simone *et al*, 1987 and Vesely *et al*, 1985), viable LAB were found to be more effective than non-viable LAB in increasing the proliferative response and for increasing the antibody levels.

Other studies have shown that LAB induce cytokine production by human peripheral blood lymphocytes (Solis Pereyra & Lemonnier, 1993; De Simone *et al*, 1986; De Simone *et al*, 1988). In the study by Solis Pereyra & Lemonnier (1993), it was found that feeding with yoghurt bacteria (*L. bulgaricus* and *S. thermophilus*), and *L. casei*, *L. acidophilus*, *Bifidobacteria* species and *L. helveticus* induced IL-1β, TNF-α and IFN-γ production in blood mononuclear cells.

An *in vitro* study by De Simone *et al* (1988) showed increased IFN-γ release and increased NK cell activity (a non-specific response) in human PBL costimulated with Con-A and LAB. An *in vivo* study by the same authors (De Simone *et al,* 1989), with

yoghurt also showed similar results. Further, this study also showed increased serum IgG levels and frequency of B lymphocytes in the treatment group.

It has also been reported that some LAB strains stimulate specific immune response against themselves (Yasui *et al*, 1989; Yasui & Ohwaki, 1991, Takahashi *et al*, 1993; Vesely *et al*, 1985). If colonisation in the gut epithelium is required for LAB's immunostimulatory effects, then there is a need to select strains that do not stimulate an immune response against themselves; otherwise the mucosal immune response will prevent the colonisation by these LAB. In Yasui and Ohwaki's study, it was found that there was increased anti-LPS antibody production by PP cells, increased anti-SRBC plaque forming cells, and proliferative responses of B cells in the PP of mice fed *B. breve* compared to non-*B. breve* fed mice. In Takahashi's study, it was found that there were increased serum antibody responses, and proliferative responses of the PP cells of mice fed *B. longum* to the cytoplasm of *B. longum* when compared to the control mice, while there were increased serum antibody responses, and proliferative responses of the PP cells of mice fed *L. acidophilus* to both the cell wall and cytoplasm of *L. acidophilus*.

1.3 Summary

In summary, there are two arms to the mammalian immune system, the non-specific and specific immune system. They work together to protect the host against invasion by foreign antigens. The systemic and mucosal immune responses are distinct from each other. The mucosal immune system plays an important part in the defense against antigens encountered on mucosal surfaces. Many claims have been made on the beneficial effects conferred by probiotics. These include prevention of bacterial infection, alleviation of lactose intolerance, reduction of serum cholesterol and stimulation of the immune system.

1.4 Aims of the Research

Sections 1.2.5.1 and 1.2.5.2 have provided many examples of studies on the positive effects of different LAB on the specific and non-specific immune responses. In previous studies (NZDB, 1998), an immunoenhancing probiotic strain, *L. rhamnosus* was identified. There are three aims in this dissertation, i) to examine the effect of dose of *L. rhamnosus* on the mucosal immune response of mice to oral antigens, ovalbumin and cholera toxin; ii) to examine the effect of viability of this probiotic bacteria on the mucosal immune response and iii) to examine the role of the immune system in mediating protection against enteric infection. Studies examining these are discussed in detail in the following sections.

Chapter 2

2.1 Materials and Methods I

2.1.1 Animals

Inbred BALB/c mice were kept at the small animal production unit (SAPU), Massey University, Palmerston North. They were housed in pairs in metal cages at a temperature of 22 ± 2°C, with a 12 hour light / dark cycle. Food and water were available *ad libitum*.

2.1.2 Probiotic Bacteria

Three doses (1×10^7, 1×10^9 and 1×10^{11} cfu) of *L. rhamnosus* HN001 from the New Zealand Dairy Institute of Research (DRI) were used.

2.1.3 Oral Antigens

Ovalbumin (OV) (Grade V) and Cholera toxin (CT), purchased from Sigma Chemical Co., Australia were used. Each dose consisted of 10μg of CT and 1mg of OV in 0.1M sodium bicarbonate.

2.1.4 Preparation of Lymphocytes from Mesenteric Lymph Nodes

This protocol was a modification of that described by Coligan *et al* (1991a). Mesenteric lymph nodes were removed aseptically from the mice and placed in pairs into 2mL of RPMI-1640 media (containing 10% foetal calf serum (FCS), 2mM L-glutamine, 50μM 2-mercaptoethanol, 100U/mL penicillin and 100μg/mL streptomycin sulphate) (purchased from GIBCO Bethesda Research Laboratories (BRL), Life Technologies Ltd., USA) in a 24 well plate (Nunclon Multidishes, Nunc, GIBCO BRL, Life Technologies Ltd., New Zealand). All subsequent steps were also carried aseptically. The lymph nodes were cut into small pieces using scissors / forceps. They were then homogenised by pipetting up and down through a 1mL syringe, after which they were

transferred to 15mL centrifuge tubes containing 5mL RPMI-1640. Samples were centrifuged (Heraeus Sepatech Megafuge 1.0R) for ten minutes at 125 x g. The supernatants were discarded. The pellets were resuspended in 5mL ACK (ammonium chloride / potassium) lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.2 - 7.4) and incubated for five minutes at room temperature with occasional mixing. Following this an additional 5mL of RPMI-1640 was added into each tube. The samples were centrifuged again for 10 minutes at 125 x g. The supernatants were discarded and the pellets were resuspended in RPMI-1640. The samples were centrifuged again and the pellet resuspended in 3mL of RPMI-1640.

2.1.5 Preparation of Lymphocytes from Peyer's Patches

This protocol was a modification of that described by Coligan *et al* (1991b). The faecal material in the small intestine of each mouse was flushed out with 2mL of PBS or 0.66mM CMF/HEPES media (1M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), 10x Hank's Balanced Salt Solution (HBSS), pH 7.2). All subsequent steps were performed aseptically using sterile instruments / equipment. Peyer's Patches (PP) located along the gut were aseptically excised with scissors and placed in pairs into 2mL of the CMF/HEPES media on ice. They were mechanically dissociated with 1mL syringes and the homogenised material placed in 15mL centrifuge tubes containing 5mL of the CMF/HEPES media. The samples were vortexed for 15 seconds at the maximum setting whereupon the contents (minus the debris) were decanted into 50mL centrifuge tubes.

An additional 5 mL of media was added to the 15mL centrifuge tubes and the samples vortexed again. These steps of decanting and vortexing were repeated once more, after which the samples were centrifuged (Heraeus Sepatech Megafuge 1.0R) for 10 minutes at 125 x g. The supernatants were removed and the pellets resuspended in 2mL of CMF/HEPES. The preparation of PP lymphocytes was performed in a sterile environment.

2.1.6 Preparation of Serum

Approximately 1mL of blood was drawn from each mouse and put into 2mL eppendorf tubes. Blood samples were left at room temperature for 2 hours after which the samples were centrifuged (Sorvall MC12V, DuPont) at 1950 x g for 10 minutes. The serum was then carefully removed from the blood clot, placed in another tube and stored frozen until further use.

2.1.7 Preparation of Intestinal Contents

The small intestine (from stomach pylorus (sphincter) to caecum end) of each mouse was removed aseptically and its contents flushed out with 2mL of PBS into 15mL centrifuge tubes. The contents were then centrifuged (Heraeus Sepatech Megafuge 1.0R) at 500 x g for 2 minutes after which the supernatants were removed and placed in another tube. The samples were kept frozen until required for further use.

2.1.8 The Cell Proliferation Assay

The method for the assay was a modification of that found in the Cell Proliferation kit by Boehringer Mannheim, USA (Cell Proliferation ELISA, BrdU (colourimetric), Cat No. 1647229). 50μL of the prepared lymphocytes (from MLN and PP, 2 x 10^6 cells /mL) from each triad of mice were aliquoted into six wells of a 96 well plate (Nunclon MicroWell plate, Nunc, GIBCO BRL, Life Technologies Ltd., NZ). 50μL of RPMI-1640 media was added to three wells to serve as the control (no mitogen wells) while 50μL of mitogen (LPS (final concentration of 5μg/mL) or PHA (15μg/mL)) was added to three wells to act as the 'with mitogen wells'. 100μL of RPMI-1640 media was added to background wells where no lymphocytes were aliquoted. Plates were incubated at 37°C for 72 hours before adding the labelling reagent 5-bromo-2'-deoxyuridine (BrdU). 10μL of BrdU labelling solution was added to each well and the plates incubated at 37°C for an additional 16 hours. The plates were centrifuged at 125 x g for 10 minutes. The labelling medium was removed and the wells were dried with a hairdryer for 15 minutes. 200μL of Fix Denat was added to the wells and incubated for

30 minutes at room temperature (RT) after which the Fix Denat was removed and 100µL of anti-BrdU-POD (peroxidase) working solution was added into each well. The plates were incubated at RT for one hour, after which the antibody conjugate was removed and the plates washed 3x in phosphate buffered saline (PBS) (1.9mM NaH_2PO_4, 8.1mM Na_2HPO_4, 154mM NaCl, pH 7.2 - 7.4). The washing solution was removed and 100µL of substrate solution was added per well and the plates incubated at RT for 20 minutes for LPS and 30 minutes for PHA. Following the incubation period, 25µL of 1M of H_2SO_4 as added into each well and the plates were shaken for about 30 minutes on a shaker. The plates were then read on an ELISA plate reader (KC3 / CERES900C, Bio-Tek Instruments, Inc., USA) at 450nm.

The stimulation index (SI) was used as a measurement of the degree of proliferation of the lymphocytes. The division of the absorbance values of cells incubated with mitogen by that of cells incubated without mitogen gives the stimulation index :

$$SI = \frac{\text{absorbance of cells with mitogen}}{\text{absorbance of cells without mitogen}}$$

2.1.9 The Immunophenotyping Assay

This assay was adapted from Lloyd *et al* (1995) and Nicholson *et al* (1984). 100µL of each cell suspension of PP or MLN were aliquoted into 3 x 2mL eppendorf tubes. Monoclonal antibodies were added in pairs to each tube:

Tube 1: 5µL of rat anti mouse CD4 conjugated to FITC (CD4:FITC) and rat anti mouse CD25 conjugated to R.Phycoerythrin (RPE) (CD25:RPE) (both from Serotec, UK)

Tube 2: 5µL of rat anti mouse CD8:FITC and rat anti mouse CD40:RPE (both from Serotec, UK)

Tube 3: 5µL of rat anti mouse CD45:FITC and CY - chrome rat anti mouse CD3e (145-2C11) (both from PharMingen). Samples were mixed and incubated on ice for 20 minutes after which they were washed twice with 1mL PBS.

After each wash, samples were centrifuged at 1000 x g for 10 minutes and the supernatants discarded. 100μL of 8% formaldehyde (in PBS) was added to each sample, mixed thoroughly and incubated for one minute. 1mL of PBS was then promptly added and the samples mixed thoroughly. The samples were then analysed by flow cytometry (FACSCalibur ™, Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

2.1.10 The Enzyme-Linked Immunosorbent Assay (ELISA)

The protocol for this assay was a modification of that described by Coligan *et al* (1991c). 96 well plates (Nunc Immuno plates, Nunc, GIBCO BRL, Life Technologies Ltd., NZ) were coated with 100μL/well of 10μg/mL cholera toxin or 138μg/mL ovalbumin antigens in carbonate coating buffer (15mM Na_2CO_3, 34mM $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Plates were removed the following day and washed 3x with PBS/Tween (0.05% Tween 20 in PBS). 100μL of samples (for intestinal fluid, undiluted and for serum, 1/25 dilution in sample buffer (95mL PBS, 5mL of FCS-heat inactivated)) and standards (for intestinal fluid: CT, concentration range of 0.1mg/mL to 0.0016mg/mL, and OV, concentration range of 0.69mg/mL to 0.01mg/mL, and for serum: CT, same concentration range as that of intestinal fluid and OV, concentration range of 1.38mg/mL to 0.022mg/mL) were aliquoted into the wells in triplicate and the plates were covered and incubated overnight at 4°C. Next day, the plates were removed and washed 3x with PBS/Tween. Sheep anti mouse Ig, conjugated to alkaline phosphatase (AKP) (diluted 1/500 in sample buffer (5% FCS/PBS)) (Silenus brand, purchased from Amrad Operations P/L, Australia) was added to the wells and the plates were incubated for 1 hour at 37°C. They were then washed 3x in PBS/Tween and 100μL of alkaline phosphatase substrate (from AKP substrate kit from Bio-Rad Laboratories, CA, USA) was added. The plates were left to incubate for 1 hour at RT before being read on an ELISA plate reader (KC3 / CERES900C, Bio-Tek Instruments, Inc., USA) at 450nm.

2.1.11 The *In Vitro* Antibody Assay

The protocol for this assay was a modification of the antibody ELISA protocol described in Section 2.1.10. Plates were coated with either 500ng/well (100µL) of CT or 6.9µg/well (100µL) of OV in carbonate coating buffer and incubated overnight at 4°C. The plates were washed 3x the following day with PBS/Tween before the addition of 100µl/well of samples (for MLN or PP, undiluted samples at 2 x 10^6 cells/mL). Plates were incubated overnight at 37°C in a 5% CO_2 incubator. Plates were washed 3x the next day with PBS/Tween before adding the alkaline phosphatase conjugated sheep anti-mouse IgA or IgG antibody (diluted 1/500 in sample buffer (5% FCS/PBS)) purchased from Serotec, UK. The plates were then incubated for 1 hour at 37°C. After the incubation period, they were washed 3x with PBS/Tween after which 100µL of alkaline phosphatase (AKP) substrate (AKP substrate kit from Bio-Rad Laboratories, CA, USA) was added. Plates were incubated at RT for 1 hour before being read on the ELISA plate reader (KC3 / CERES900C, Bio-Tek Instruments, Inc., USA) at 450nm.

2.1.12 Statistical Analysis of Results

The results were expressed as means ± standard error of the mean (SE). Students' t test and SAS (SAS® Analysis of Variance Procedures) were used to determine the significance of results. $P<0.05$ (*) is significant and $P<0.01$ (**) is highly significant.

2.2 Materials and Methods II

2.2.1 Animals

Inbred BALB/c mice were kept at SAPU, Massey University, Palmerston North with a 12 hour light / dark cycle and at a temperature of 22 ± 2°C. They were housed in pairs in metal cages. Food and water were available *ad libitum.*

2.2.2 Probiotic Bacteria

Immunoenhancing probiotic strain, *L. rhamnosus* NH001, and its heat killed form HKNH001, from the New Zealand DRI, at a dose level of 1 x 10^9 cfu were used.

2.2.3 Oral Antigens

Refer to Section 2.1.3.

2.2.4 Preparation of Lymphocytes from Mesenteric Lymph Nodes

Method was as described in Section 2.1.4.

2.2.5 Preparation of Lymphocytes from Peyer's Patches

Method was as described in Section 2.1.5.

2.2.6 Preparation of Serum

Method was as described in Section 2.1.6.

2.2.7 Preparation of Intestinal Contents

Method was as described in Section 2.1.7.

2.2.8 The Cell Proliferation Assay

Method was as described in Section 2.1.8.

2.2.9 The Immunophenotyping Assay

Method was as described in Section 2.1.9

2.2.10 The Enzyme-Linked Immunosorbent Assay

Method was as described in Section 2.1.10.

2.2.11 The *In Vitro* Antibody Assay

Method was as described in Section 2.1.11.

2.2.12 Statistical Analysis of Results

Method was as described in Section 2.1.12.

2.3 Materials and Methods III

2.3.1 Animals

As described in Section 2.2.1.

2.3.2 Probiotic Bacteria

Immunoenhancing probiotic strain, *L. rhamnosus* HN001 (1 x 10^9 cfu) from the Dairy Research Institute (DRI), New Zealand was used.

2.3.3 Infection with *S. typhimurium*

Salmonella typhimurium ATCC 1772 (5 x 10^5 cfu) was used.

2.3.4 Preparation of Lymphocytes from Mesenteric Lymph Nodes

Method was as described in Section 2.1.4.

2.3.5 Preparation of Lymphocytes from Peyer's Patches

Method was as described in Section 2.1.5.

2.3.6 Immunophenotyping Assay

Method was as described in Section 2.1.9.

2.3.7 The *In Vitro* Antibody Assay

Method was as described in Section 2.1.11 using lipoteichoic acid (LTA) (10μg/well) isolated from *S. typhimurium* ATCC 1772 (gift from Dr Pramod Gopal, NZDRI) as the antigen.

2.3.8 Bacterial Translocation Assay

Method was as described by Perdigon *et al* (1990b). All steps were carried out aseptically with sterile instruments / equipment. Spleens and livers were aseptically removed from mice and were homogenised in 5mL of 0.1% peptone water (final volume) with a Teflon homogeniser. Cell suspensions were serially diluted in peptone water and were plated on MacConkey agar plates. Bacterial colonies were enumerated after incubation for 24-48 hours at 37°C.

2.3.9 Preparation of Peritoneal Macrophages

Method was an adaptation of that described by Coligan *et al* (1994). Following euthanasia, each mouse was pinned down and its abdomen sprayed with ethanol before its skin was retracted without breaking the peritoneal cavity. 10mL of RPMI-1640 media was injected (10mL syringe with 21G needle) into the cavity and the sides of the cavity were massaged with finger tips. After a break of about 2 minutes, the cavity was massaged again. The peritoneal fluid was withdrawn through the lower side of the cavity and was aspirated into sterile 10mL siliconised glass Vacutainer® tubes in an airflow cabinet. The fluid was kept on ice prior to centrifugation at 125 x g for 10 minutes at 4°C. The supernatant was removed using sterile transfer pipettes. The pellet was resuspended in 10mL of RPMI-1640 after which it was centrifuged for 10 minutes at 4°C.

The supernatant was again removed and the pellet resuspended in 1mL of RPMI-1640. 100µL of the resuspended cells were transferred into FACS tubes containing 400µL of PBS and were counted on the flow cytometer. The samples were adjusted to 1 x 10^6 macrophages/mL with RPMI-1640.

2.3.10 Phagocytosis by Peripheral Blood Leukocytes

Method was an adaptation of that described in the PHAGOTEST® test kit (Orpegen Pharma, Germany). 100µL of whole blood was added to a 2mL glass tube (no EDTA, Vacutainer® blood collection tubes, Becton Dickinson & Co., NJ, USA) which had previously been aliquoted with 10µL of FITC labelled *E coli* (FITC-*E. coli*). The samples were incubated for 30 minutes at 37°C. 100µL of 8% formaldehyde was added to the samples after which they were incubated for 1 minute. 1mL of ice cold water was then added (to lyse the red blood cells) and the samples thoroughly mixed. The samples were incubated for 10 minutes and then centrifuged (Heraeus Instruments Megafuge 1.0R) at 1950 x g for 10 minutes. The supernatants were discarded and 0.5mL of PBS was added to the pellet.

The suspension was thoroughly mixed and transferred into FACS tubes (Falcon® 6mL polystyrene round bottom tubes, Becton Dickinson & Co., NJ., USA) and 50μL of trypan blue (0.4% w/v and filtered) was added. The phagocytic activity of the samples were analysed by flow cytometry.

2.3.11 Phagocytosis by Peritoneal Macrophages

The peritoneal macrophages were prepared as described in Section 2.3.13. The method of measuring the phagocytic activity is adapted from that described in Section 2.3.14. 10μL of FITC-*E. coli* was added to 100μL of macrophages (1 x 10^6 macrophages/mL) in 2mL glass tubes (no EDTA, Vacutainer® blood collection tubes, Becton Dickinson & Co., NJ, USA). The mixture was incubated for 20 minutes at 37°C. The samples were then immediately placed on ice and 0.5mL of ice cold PBS was added to stop the phagocytic activity. 50μL of trypan blue was added to the samples which were then analysed by flow cytometry.

2.3.12 Statistical Analysis of Results

As described in Section 2.1.12.

Chapter 3

3.1 Introduction

As discussed in Chapter 1, various studies have shown the positive effects of LAB on various aspects of the non-specific and specific immune responses (Perdigon *et al*, 1988; De Simone *et al*, 1991; Schiffrin *et al,* 1995; Majamaa *et al*, 1995; Link-Amster *et al,* 1994; Perdigon *et al*, 1987; Perdigon *et al*, 1991a; Solis Pereyra & Lemonnier, 1993). Previous studies conducted by NZDB (1998) have lead to the identification of a probiotic bacteria *L. rhamnosus* that enhances the specific and non-specific immune responses. For example, there was greater phagocytic activity of FITC-*E. coli* exhibited by the peripheral blood leukocytes and peritoneal macrophages from mice treated with *L. rhamnosus* and higher serum and mucosal antibody responses to CT compared to the control mice. There was also increased NK cell activity by splenic lymphocytes and proliferation of these lymphocytes to a T cell mitogen, ConA (an indicator of cellular immunity) in *L. rhamnosus* treated mice compared to the control mice (Gill *et al*, 1998 (NZDB Patent)).

One of the factors that may impact on the immunostimulatory effects of LAB is the effect of dose. However, very few studies have examined the effect of dose on immunostimulation (Kishi *et al*, 1996; Norton *et al*, 1995). In Kishi's study, it was found that the higher the dose of *Lactobacillus brevis* subsp. *coagulans* feeding in human volunteers, the more rapid the increase in IFN-γ production in their blood. In Norton's study, it was shown that the serum antibody responses elicited by LAB was dose-dependent.

The aim of this present study was to determine if the immunostimulatory effects of *L. rhamnosus* as mentioned before are also dose-dependent. The effect of different doses (1×10^7, 1×10^9 and 1×10^{11} cfu) of *L. rhamnosus* on specifically the mucosal immune responses of mice to CT and OV antigens was thus investigated. The most effective / suitable dose for the immunostimulatory effects of *L. rhamnosus* was also determined.

3.2 Experimental Design

Seventy two BALB/c mice were randomly divided into 4 groups of 18 mice each and were allocated to different treatment groups as shown in Table 1. Mice were fed a skim milk powder (SMP) based diet which was introduced during an initial 10 day acclimatisation period. Following acclimatisation, mice were orally administered 1 x 10^7, 1 x 10^9 or 1 x 10^{11} cfu of *L. rhamnosus* in 50μL skim milk daily for 14 days and they were orally vaccinated with 25μL of the CT and OV antigens on days 0 and 7. Control mice were given 50μL of skim milk only. Mice were sacrificed on day 14 and blood, MLN and PP samples, and intestinal contents were collected for different assays.

Table 2 Feeding and Vaccination Schedule

Day	Control	1x 10^7 cfu	1 x 10^9 cfu	1 x 10^{11} cfu
-10	Acclimatisation	Acclimatisation	Acclimatisation	Acclimatisation
0	Skim milk + vaccination 1	*L. rhamnosus* + vaccination 1	*L. rhamnosus* + vaccination 1	*L. rhamnosus* + vaccination 1
7	Skim milk + vaccination 2	*L. rhamnosus* + vaccination 2	*L. rhamnosus* + vaccination 2	*L. rhamnosus* + vaccination 2
14	Skim milk	*L. rhamnosus*	*L. rhamnosus*	*L. rhamnosus*

Various humoral and cell-mediated immune responses in the mucosal tissues were measured (*in vitro* antibody production by PP and MLN lymphocytes; the distribution of lymphocyte subsets of PP and MLN, the proliferative responses of the PP lymphocytes to B and T cell mitogens and the serum and intestinal antibody responses to CT and OV).

3.3 Results

3.3.1 Mucosal and Serum Antibody Responses to CT and OV

All three *L. rhamnosus* treatment groups showed significantly increased mucosal antibody responses to CT when compared to the control group (Figure 7). There was however, no significant differences between the three *L. rhamnosus* treatment groups. All three *L. rhamnosus* treatment groups showed increased mucosal antibody responses to ovalbumin but the increase was only significant for the group treated with 1 x 10^7 cfu of *L. rhamnosus* (Figure 8). There was no significant differences in the mucosal response to OV between the three doses.

All *L. rhamnosus* treatment groups showed significantly increased serum antibody responses to CT (Table 3). There was no significant difference between the different doses. All three *L. rhamnosus* treatment groups showed higher serum antibody responses to OV compared to the control group but only the responses by the 1 x 10^7 and 1 x 10^9 cfu treatment groups were significantly higher. There was no significant difference between the different doses.

Table 3 Effect of Various *L. rhamnosus* Doses on the Serum Antibody Responses to CT and OV

Treatment	Serum antibody response to OV (mean units ± SE)[a]	Serum antibody response to CT (mean units ± SE)[d]
Control	26.33 ± 2.14[b]	87.08 ± 16.84[e]
1 x 10^7	37.98 ± 4.29[c]	321.95 ± 69.88[f]
1 x 10^9	34.83 ± 2.50[c]	271.4 ± 71.88[f]
1 x 10^{11}	34.47 ± 4.21[bc]	223.46 ± 49.78[f]

[a] mean ± SE (n=14-18)

[d] mean ± SE (n=11-18)

[c] Significantly different from value with different superscript

[f] Significantly different from value with different superscript

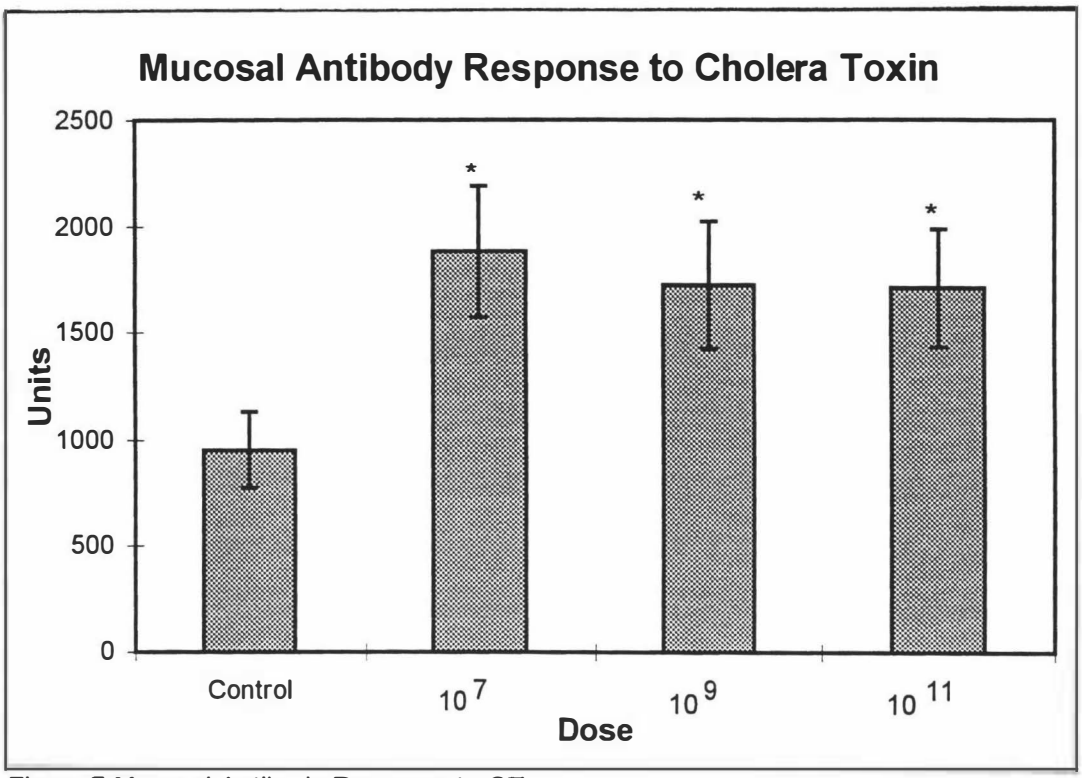

Figure 7 Mucosal Antibody Response to CT.
Each bar represents the mean of 11-18 mice +/- SE.
P<0.05(*) Significantly different from control.

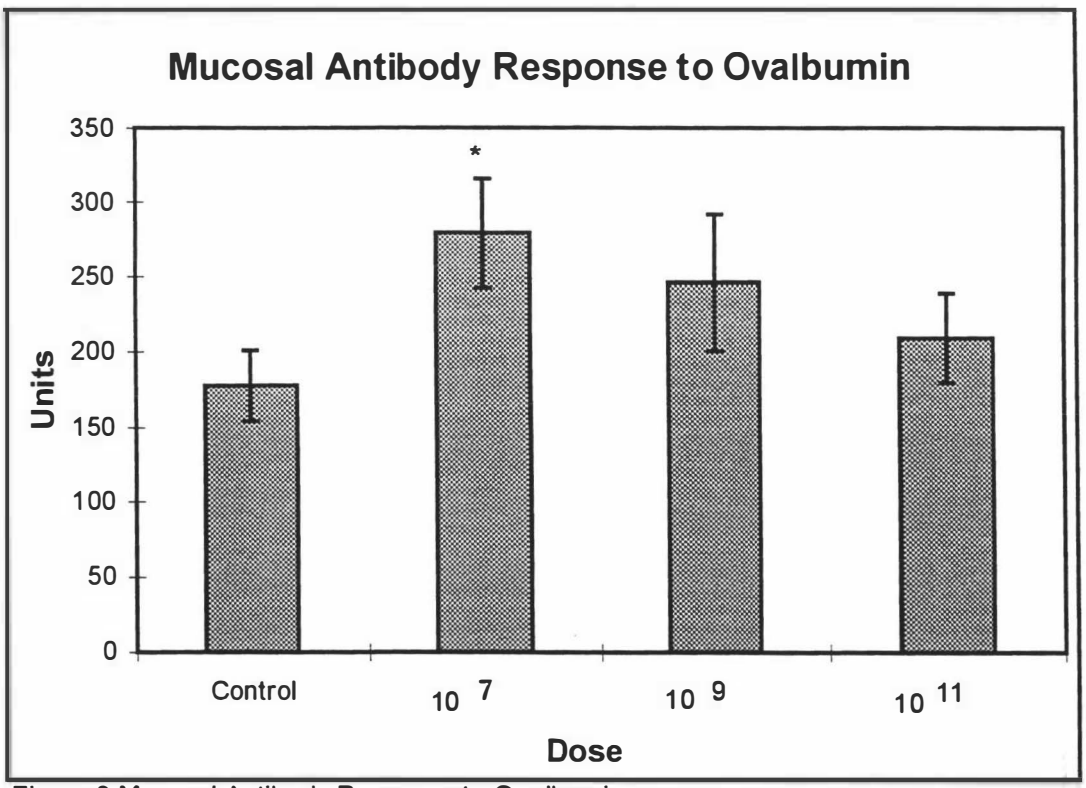

Figure 8 Mucosal Antibody Response to Ovalbumin.
Each bar represents the mean of 16-18 mice +/- SE.
P<0.05(*) Significantly different from control.

3.3.2 *In Vitro* Antibody Production

3.3.2.1 *In Vitro* Antibody Production by PP Lymphocytes in Response to CT and OV

As shown in Table 4, only mice fed with 1 x 10^7 and 1 x 10^9 cfu of *L. rhamnosus* had increased IgA production in response to CT although the increases were not significant. There was no difference in this response between the control mice and those fed 1 x 10^{11} cfu of *L. rhamnosus*. There were no significant differences between the three doses. When compared to the control group, only the 1 x 10^7 and 1 x 10^9 cfu treatment groups had enhanced IgA production in response to OV, but again this was not statistically significant. When compared to the 1 x 10^{11} cfu treatment group, the production of IgA in response to OV was significantly increased in the 1 x 10^7 cfu treatment group. Mice fed with 1 x 10^7 or 1 x 10^9 cfu of *L. rhamnosus* had higher IgG production in response to CT than the control mice but there was no difference between the control mice and mice fed 1 x 10^{11} cfu of *L. rhamnosus*. None of the differences were statistically significant. Only mice treated with 1 x 10^7 cfu of *L. rhamnosus* had higher IgG production (not significant) in response to OV compared to the control group. There was no significant difference between the three *L. rhamnosus* treatment groups.

Table 4 Effect of Various *L. rhamnosus* Doses on the *In Vitro* Antibody Production by PP Lymphocytes in Response to CT and OV

Treatment	*In vitro* antibody production by PP cells to cholera toxin (mean abs ± SE)[a]		*In vitro* antibody production by PP cells to ovalbumin (mean abs ± SE)[a]	
	IgA	IgG	IgA	IgG
Control	0.446 ± 0.069	0.729 ± 0.097	0.292 ± 0.036	0.478 ± 0.069
1 x 10^7	0.502 ± 0.059	0.904 ± 0.094	0.376 ± 0.039	0.536 ± 0.066
1 x 10^9	0.511 ± 0.063	0.840 ± 0.109	0.373 ± 0.051	0.481 ± 0.081
1 x 10^{11}	0.462 ± 0.033	0.765 ± 0.144	0.257 ± 0.034	0.432 ± 0.066

[a] **mean ± SE (n=4-5)**

3.3.2.2 *In Vitro* Antibody Production by MLN Lymphocytes in Response to CT and OV

Table 5 shows the *in vitro* antibody production by MLN lymphocytes in response to CT and OV. There was no difference in the three *L. rhamnosus* treatment groups in the IgA production in response to CT or OV when compared to the control group. There were also no significant differences between the different doses.

No differences were observed in the IgG production in response to CT or OV in the different *L. rhamnosus* treatment groups when compared to the control group except in the group fed 1 x 10^{11} cfu of *L. rhamnosus* where there was a slight decrease in both responses (not statistically significant).

Table 5 Effect of Various *L. rhamnosus* Doses on the *In Vitro* Antibody Production by MLN Lymphocytes in Response to CT and OV

Treatment	Antibody production in vitro by MLN cells to cholera toxin (mean abs ± SE)[a]		Antibody production in vitro by MLN cells to ovalbumin (mean abs ± SE)[a]	
	IgA	IgG	IgA	IgG
Control	0.192 ± 0.020	0.259 ± 0.027	0.153 ± 0.022	0.170 ± 0.019
1 x 10^{7}	0.215 ± 0.023	0.270 ± 0.030	0.160 ± 0.012	0.171 ± 0.011
1 x 10^{9}	0.195 ± 0.020	0.262 ± 0.038	0.163 ± 0.024	0.162 ± 0.020
1 x 10^{11}	0.185 ± 0.021	0.224 ± 0.028	0.140 ± 0.021	0.147 ± 0.019

[a] mean ± SE (n=8-9)

3.3.3 Expression of Lymphocyte Surface Markers

3.3.3.1 Expression of Surface Markers on PP Lymphocytes

Compared to the control mice, mice fed 1 x 10^9 and 1 x 10^{11} cfu of *L. rhamnosus* showed a slight increase in the number of $CD40^+$ lymphocytes. No difference between the control mice and those fed 1 x 10^7 cfu of *L. rhamnosus* was observed.

Only mice fed 1 x 10^7 cfu of *L. rhamnosus* had significantly increased $CD3^+$ lymphocytes compared to the control mice. For other *L. rhamnosus* treatment groups, there was no difference compared to the control group. There was no significant change in the CD4:CD8 ratio of PP lymphocytes in any of the *L. rhamnosus* treatment groups compared to the control group.

Mice treated with different dose levels of *L. rhamnosus* showed an increased number of $CD25^+$ lymphocytes when compared to the control group, however, the increases were only significant for the 1 x 10^7 and 1 x 10^{11} cfu groups (Figure 9). The number of $CD25^+$ lymphocytes in the 1 x 10^{11} cfu treatment group was also significantly higher than that of the 1 x 10^7 cfu treatment group.

No differences were observed in the level of $CD45^+$ lymphocytes between any of the *L. rhamnosus* treatment groups and the control group (data not shown).

Table 6 Effect of Various *L. rhamnosus* Doses on the Expression of Surface Markers on PP Lymphocytes

Treatment	% (mean ± SE) of $CD40^+$ PP lymphocytes[a]	% (mean ± SE) of $CD3^+$ PP lymphocytes[a]	CD4:CD8 of PP lymphocytes (mean ± SE)[b]
Control	69.79 ± 0.69	24.54 ± 1.16	3.94 ± 0.28
1 x 10^7	68.92 ± 1.06	29.11 ± 1.12*	4.16 ± 0.38
1 x 10^9	71.05 ± 1.91	25.76 ± 1.77	4.56 ± 0.83
1 x 10^{11}	72.22 ± 2.11	25.97 ± 1.26	3.59 ± 0.83

P<0.05(*) Significance compared to the control

[a] mean ± SE (n=8-9)

[b] mean ± SE (n=3-8)

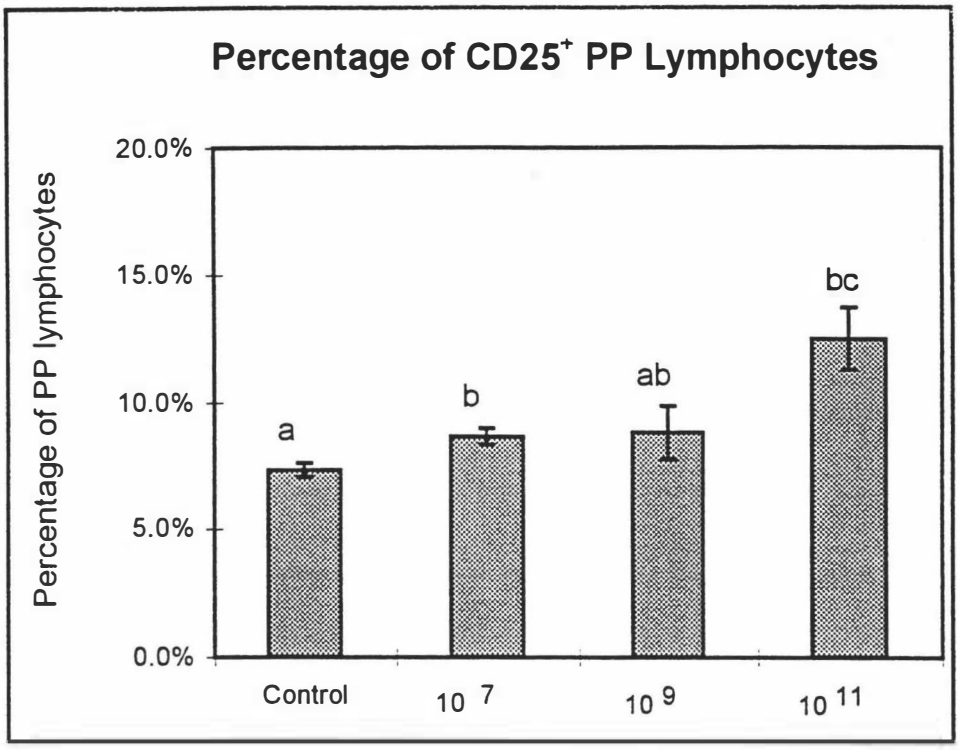

Figure 9 Percentage of $CD25^+$ PP lymphocytes.
Each bar represents the mean of 4-8 mice +/- SE.
a Significantly different from values with different letter.

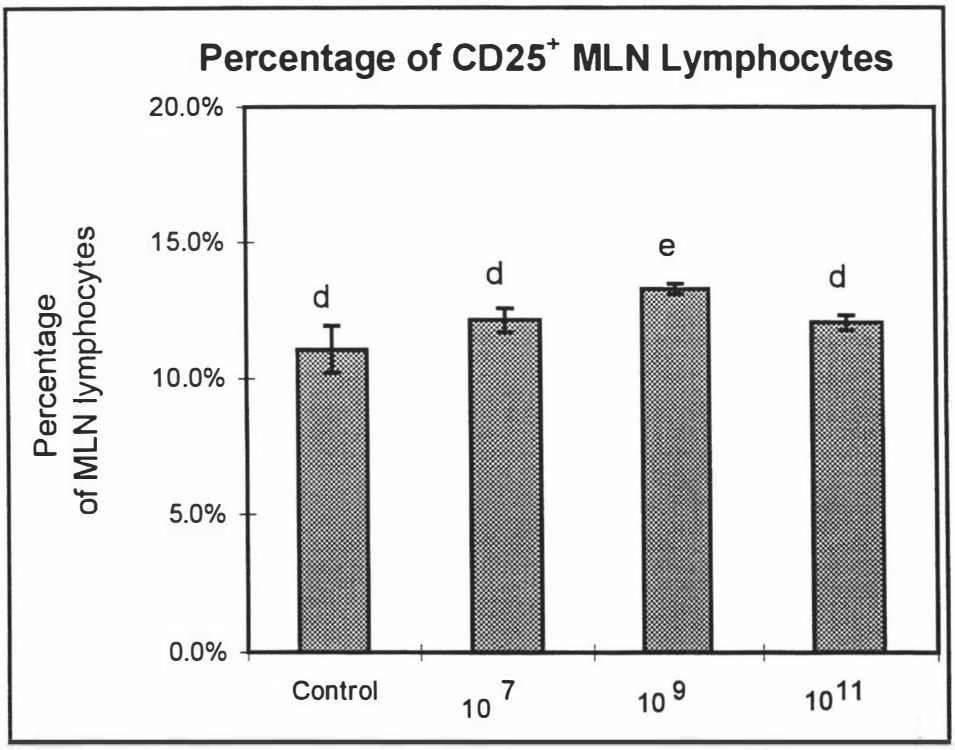

Figure 10 Percentage of $CD25^+$ MLN lymphocytes.
Each bar represents the mean of 7-9 mice +/- SE.
d Significantly different from value of letter e.

3.3.3.2 Expression of Surface Markers on MLN Lymphocytes

There was no difference in the level of expression of the $CD40^+$ or $CD3^+$ markers on MLN lymphocytes of mice fed with the different doses of *L. rhamnosus* when compared to the control mice. There was also no significant change in the CD4:CD8 ratio of MLN lymphocytes in any of the *L. rhamnosus* treatment groups compared to the control group.

Table 7 Effect of Various *L. rhamnosus* Doses on the Expression of Surface Markers on MLN Lymphocytes

Diet	% (mean ± SE) of $CD40^+$ MLN lymphocytes[a]	% (mean ± SE) of $CD3^+$ MLN lymphocytes[b]	CD4:CD8 of MLN lymphocytes (mean ± SE)[b]
Control	25.65 ± 0.99	72.70 ± 1.74	4.378 ± 0.397
1×10^7	24.64 ± 1.11	74.21 ± 0.67	4.564 ± 0.355
1×10^9	26.51 ± 1.35	74.04 ± 1.01	4.679 ± 0.654
1×10^{11}	27.30 ± 1.09	72.80 ± 0.96	4.794 ± 0.285

[a] mean ± SE (n=8-9)

[b] mean ± SE (n=5-8)

All three *L. rhamnosus* treatment groups showed increased $CD25^+$ lymphocytes compared to the control group but this increase was only significant for mice treated with 1×10^9 cfu of *L. rhamnosus*. The 1×10^9 cfu treatment group also had significantly higher numbers of $CD25^+$ lymphocytes when compared to the 1×10^7 and 1×10^{11} cfu treatment groups (Figure 10).

There was no difference in the number of $CD45^+$ lymphocytes in the three *L. rhamnosus* treatment groups compared to the control group (data not shown).

3.3.4 Cell Proliferative Responses to T and B cell Mitogens

3.3.4.1 Cell Proliferative Responses of PP Lymphocytes

There was no difference in the proliferation of PP lymphocytes in response to LPS between the control and the 1 x 10^7 cfu treatment group (Table 8). However, the proliferative responses of the 1 x 10^9 and 1 x 10^{11} cfu treatment groups were lower than that of the control group although this difference was not significant. There was no difference between the responses shown by the mice fed 1 x 10^9 and 1 x 10^{11} cfu of *L. rhamnosus*. When compared to the control mice, the PP lymphocytes of mice fed 1 x 10^7 or 1 x 10^9 cfu of *L.rhamnosus* showed increased proliferation in response to PHA but only the increment in the 1 x 10^7 cfu treatment group was statistically significant (P=0.026) (Table 8). The response of this treatment group (1 x 10^7 cfu) was also significantly higher than that of the 1 x 10^{11} cfu treatment group (P=0.018).

Table 8 Effect of Various *L. rhamnosus* Doses on the Cell Proliferative Responses of PP Lymphocytes to LPS and PHA

Treatment	Stimulation Index of PP to LPS (mean ± SE)[a]	Stimulation Index of PP to PHA (mean ± SE)[a]
Control	8.781 ± 0.259	3.348 ± 0.488
1 x 10^7	8.316 ± 0.271	4.708 ± 0.488*
1 x 10^9	7.882 ± 0.258	4.077 ± 0.581
1 x 10^{11}	7.328 ± 0.943	3.227 ± 0.660

***P<0.05 Significance compared to the control**

[a] mean ± SE (n=3-6)

3.4 Discussion:

A range of factors may impact on the immunostimulatory effects of LAB, such as dose, viability, duration of feeding, medium of administration (eg. in fermented milk), physiological status and dietary intake. However, only a limited number of studies have been conducted to examine the effect of these factors on immunostimulation. In the present study, the effect of different doses of *L. rhamnosus* on the mucosal immune responses to oral antigens was investigated.

Results from this study indicate that several aspects of the mucosal immune responses were enhanced in the different *L. rhamnosus* treatment groups compared to the control group, such as the *in vitro* antibody production by PP lymphocytes; B and T cell numbers in PP and mucosal antibody responses to CT and OV. Furthermore, the immunostimulatory effects of *L. rhamnosus* were dose-dependent. For example, the PP of mice treated with *L. rhamnosus* at the dose level of 1×10^7 and 1×10^9 cfu produced higher levels of antibody in response to CT and OV than the 1×10^{11} cfu dose, while feeding at the dose level of 1×10^9 and 1×10^{11} cfu resulted in a higher number of B cells in PP than the 1×10^7 cfu dose. The higher dose level did not always yield greater responses as observed with the *in vitro* antibody production by PP lymphocytes in response to CT and OV, where the dose level of 1×10^{11} cfu yielded the lowest antibody levels compared to the other dose levels. Feeding at the dose level of 1×10^7 and 1×10^9 cfu resulted in higher mucosal antibody responses to CT and OV than the dose level of 1×10^{11} cfu. The effects observed with the different doses suggest that different LAB concentrations impact on different immune parameters. In our study, the 1×10^7 cfu dose level was found to be more effective in stimulating some of the mucosal immune responses than the the 1×10^9 cfu dose level while in other parameters, the 1×10^9 cfu dose level gave better responses although the differences observed between these were not significant. The 1×10^{11} cfu dose level was the least effective in stimulating the mucosal immune responses and in some cases (eg. proliferative responses of PP cells to LPS), the responses were lower than that observed in the control group.

The *in vitro* antibody responses to CT and OV exhibited by lymphocytes from both PP and MLN in all three of the *L. rhamnosus* treatment groups suggests the presence of cells that are capable of producing antibody in response to CT and OV. Secretory IgAs are the predominant antibodies of the mucosal immune system, therefore, it is likely that these cells in PP and MLN were IgA-committed B cells (the isotype specificity of these cells was not determined). These B cells migrate to the MLN on their way to mucosal effector sites (eg. the lamina propria) where they become IgA-producing cells, producing sIgA into the external secretions at mucosal surfaces. This is consistent with the increased number of B cells and serum antibody responses to CT and OV (when B cells enter the circulation to home to effector sites), and increased mucosal antibody responses (when sIgA are disseminated to other mucosal sites such as the gut) observed in the *L. rhamnosus* treatment groups.

It was found that PP cells were activated as indicated by the increased expression of the $CD25^+$ marker (Abbas *et al*, 1991). However, there was no corresponding increase in B cell proliferation in PP even though there was increased antibody production *in vitro* by these B cells. Whether this was due to the cytokines produced by helper T cells which mediate the proliferation and differentiation of B cells, inhibiting the proliferation instead of inducing it, is unclear. Different cytokines stimulate different stages of B cell proliferation and differentiation and different combinations of cytokines can either work together to stimulate or antagonise the proliferation and differentiation of B cells (Abbas *et al*, 1991). For example, IL-2, IL-4 and IL-5 work together to stimulate the proliferation of B cells. Perhaps other combinations of these cytokines may have antagonistic effects on the proliferation or differentiation of B cells. In this study, cytokine production was not measured so which cytokines were produced is unknown.

Antigen-sensitised T cells, like B cells, also home to the effector sites from the PP (Husband *et al*, 1990) and the results in this study also suggest that T cells were activated and were proliferating *in vivo* in PP. For T_H cells activated by antigen, cytokines would be produced and for CTL, they act to destroy antigens in effector sites (Abbas *et al*, 1991). Cytokines mediate the proliferation of T cells through an autocrine growth pathway (Abbas *et al*, 1991) and also influence effector functions of B cells. This may occur in the PP and MLN as well as the effector sites as the T cells home to effector sites.

It was noted in some parameters that *L. rhamnosus* feeding of mice did not affect their responses. For example, the *in vitro* antibody production by MLN lymphocytes and the number of B and T cells in MLN. This could be due to several reasons. One of the reasons could be the vaccination schedule, or the *L. rhamnosus* feeding schedule used in our studies. It is known that much higher doses and more frequently administered antigens are required to induce a mucosal immune response than to induce a systemic immune response (McGhee *et al*, 1992; Walker, 1994). For example, Solbreux *et al* (1990) used four enteral doses of CT (10mg) at 12 day intervals and found that this regime yielded the highest IgA, IgG and IgM SFC responses in various lymphoid tissues. Therefore, a much higher and more frequently administered dose of OV and CT than what was used in the present study might have yielded better responses in the *in vitro* antibody production than what was observed here. The duration of feeding of LAB is also known to affect the serum and mucosal immune responses (Perdigon *et al*, 1991a; Takahashi *et al*, 1993). In the study by Takahashi *et al* (1993), it was found that the serum antibody responses to *B. longum* and *L acidophilus* were only detected in mice administered each of these LAB for at least 8 or 6 weeks respectively. Thus it may be that a longer duration of feeding *L. rhamnosus* is required for a greater and significant effect on the serum and mucosal immune responses.

The age and strain of experimental animals is also known to influence immune responses (Norton *et al*, 1995). However, age may not be a factor in this study because the mice used were between 6-8 weeks old, which falls within the limit of 6-12 weeks suggested by Norton *et al* (1995) for optimal serum antibody responses.

The BALB/c strain of mice was used in our study and this again is probably not a factor here because according to Norton *et al* (1995), BALB/c mice showed serum antibody responses with even a low dose of LAB of 5 x 10^6 cfu. In our study, a higher dose than this (1 x 10^9 cfu) was used. An additional factor that could impact on the lack of statistically significant immune responses is the number of experimental animals used. Some of the results of this study were based on a very small number of animals. By using a larger number of animals, significant differences may become more evident.

Another reason that could account for the lack of increase in certain immune responses could be the fact that the responses were not measured at the appropriate time. It is known that antigen-sensitised lymphocytes proliferate and undergo partial differentiation (Brandtzaeg, 1998) in inductive sites such as the PP and home to various effector sites when they leave the inductive sites such as PP. This means that the lymphocytes are constantly on the move once they encounter antigens, and the appropriate time to measure the immune responses elicited by these lymphocytes in different mucosal or lymphoid organs may not have been chosen in this study. The importance of the timing of measuring immune responses at mucosal sites and other lymphoid organs was shown in a study by van der Heijden *et al* (1995). They observed that after intraperitoneal immunisation and an oral booster immunisation with OV, the OV-specific IgA antibody responses in the small intestine of mice varied at different days after the booster immunisation. For example, the response was detected from day 4 and the first peak response was on day 7. From day 7, the response decreased until day 11 and a second peak response was obtained on day 14. From this, it is possible to see that measuring the IgA responses on different days after the final immunisation yielded different responses and this could be due to the movement of the antigen-sensitised lymphocytes through the common mucosal immune system as mentioned before. Therefore it is crucial to measure the immune responses at the optimum time.
By designing future studies to take into account these factors, this may result in more significant increases in immune responses than what was observed in the present study.

3.5 Summary

In summary, the results of the present study showed that the immunostimulatory effects of *L. rhamnosus* were dose-dependent. Some immune parameters were increased by feeding at the dose level of 1×10^7 cfu while for other parameters, feeding at the dose level of 1×10^9 cfu was more effective. The higher dose level of 1×10^{11} cfu was the least effective in stimulation of mucosal immune responses. Several factors such as the vaccination schedule, the timing of measuring the responses and the duration of feeding *L. rhamnosus* may account for the lack of significant responses observed in some immune parameters.

Chapter 4

4.1 Introduction

In order for LAB to exert their beneficial effects on the intestinal tract, they need to be able to adhere and colonise the gut (Salminen *et al*, 1993). One of the ways LAB stimulates the immune system is by inducing cytokine production by immunocompetent cells. It has been suggested that LAB may interact with immunocompetent cells because peptidoglycan (a LAB cell wall component) is present on lymphocytes and macrophages (Dziarski, 1991). This interaction may induce cytokine production as was demonstrated by the ability of peptidoglycan to induce IFN-γ production by lymphocytes (Tufano *et al*, 1991) and IL-1, IL-6 and TNF-α production by monocytes (Bhakdi *et al*, 1991; Heumann *et al*, 1994).

Several studies have shown the higher efficacy of viable LAB in stimulating the immune response than non-viable bacteria (De Simone *et al*, 1987; Kishi *et al*, 1996; Vesely *et al*, 1985; Portier *et al*, 1993). For example, in Kishi's study, oral administration of live *L. brevis* subsp. *coagulans* (Labre) to human volunteers resulted in increased (not significant) IFN-γ production in the blood of these subjects compared to those given heat-killed Labre. In Vesely's study, there was increased serum antibody responses and a change in the expression of the cell surface markers of the splenic T lymphocytes of mice fed yoghurt with live lactobacilli compared to the control mice (given normal feed), while there was no significant differences between mice fed yoghurt with heat-killed lactobacilli and the control mice in some of these immune parameters.). From the above, it appears that the viability of LAB is important for their positive impact on the immune system.

The aim of the present study was to examine if the viability of *L. rhamnosus* affects its immunostimulatory properties. Therefore the effect of viability of *L. rhamnosus* on the mucosal immune responses to CT and OV antigens was investigated. From the dose-response study (Chapter 3), it was concluded that the dose level of 1 x 10^9 cfu would be used in this present study, as the immunostimulatory effects displayed by this dose were optimal over the range of immune functions measured. The selected dose is similar to that (1.2 x 10^9 cfu daily) used by other investigators in their studies on LAB's stimulation of the immune system (Perdigon *et al*, 1988; Perdigon *et al*, 1986a; Perdigon *et al*, 1991a).

4.2 Experimental Design

Sixty BALB/c mice were randomly allocated to different treatment groups (20 mice / group) (Table 9). Mice were fed a SMP based diet which was introduced during an initial 7 day acclimatisation period. Following acclimatisation, mice were orally administered (day 0) 1 x 10^9 cfu of *L. rhamnosus* or its heat-killed form in 50μL skim milk daily for 14 days. The mice were orally vaccinated with 25μL of the CT and OV antigens, with the primary vaccination being administered on day 0 while the second vaccination was given 7 days later. Control mice were given 50μL of skim milk only. Mice were sacrificed on day 14 and blood, MLN and PP samples, and intestinal contents were collected for various assays.

Table 9 Feeding and Vaccination Schedule

Day	Control	*L. rhamnosus* HN001	Heat-killed *L. rhamnosus* HKHN001
-7	Acclimatisation	Acclimatisation	Acclimatisation
0	Skim milk + vaccination 1	*L. rhamnosus* + vaccination 1	*L. rhamnosus* (heat-killed) + vaccination 1
7	Skim milk + vaccination 2	*L. rhamnosus* + vaccination 2	*L. rhamnosus* (heat-killed) + vaccination 2
14	Skim milk	*L. rhamnosus*	*L. rhamnosus* (heat-killed)

Various humoral and cellular immune parameters were measured using mucosal tissues. These were *in vitro* antibody production by PP and MLN lymphocytes; the level of lymphocyte subsets of PP and MLN, the serum and intestinal antibody responses to CT and OV.

4.3 Results

4.3.1 Mucosal Antibody Responses to CT and OV

As shown in Figure 11, the mucosal antibody response to CT was significantly higher in the live ($P<0.01$) and dead ($P<0.05$) *L. rhamnosus* treatment groups compared to the control group. There was however, no significant difference between the live and dead *L. rhamnosus* treatment groups. Only the mucosal antibody response to OV in the live *L. rhamnosus* treatment group was higher (not significant) than the control group; there was no difference between the dead *L. rhamnosus* treatment group and the control group (Figure 12).

4.3.2 Serum Antibody Responses to OV and CT

Mice fed live or dead *L. rhamnosus* had significantly higher serum antibody responses to CT when compared to the control mice (Table 10). There was however, no significant difference between the live and dead *L. rhamnosus* treatment groups in the serum response to CT.

Both *L. rhamnosus* treatment groups had higher serum antibody responses to OV but the increases were not significant. The response to OV by the dead *L. rhamnosus* treatment group was also higher than that of the live *L. rhamnosus* treatment group (not significant).

Table 10 Serum Antibody Responses to CT and OV

Treatment	Serum antibody response to cholera toxin (mean units ± SE)[a]	Serum antibody response to ovalbumin (mean units ± SE)[a]
Control	88.69 ± 18.52	27.77 ± 3.24
L. rhamnosus (live)	258.45 ± 74.28*	32.68 ± 3.24
L. rhamnosus (dead)	217.65 ± 55.70*	37.00 ± 6.00

P<0.05(*) Significance compared to the control

[a] mean ± SE (n=14-20)

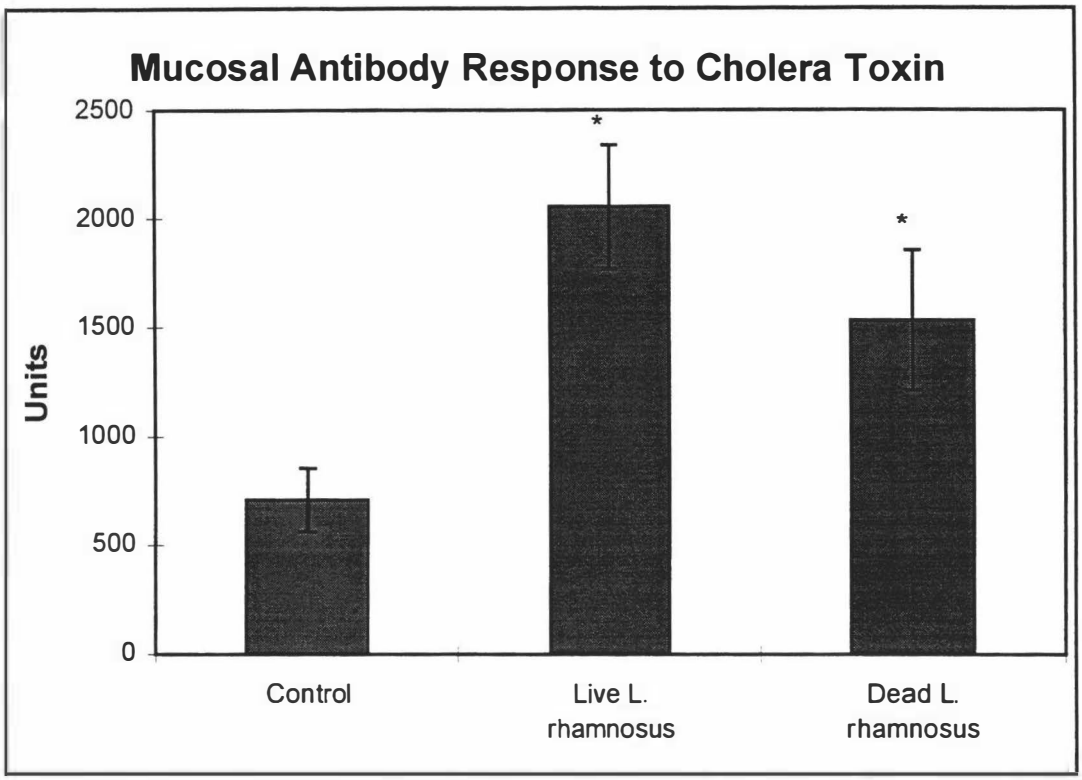

Figure 11 Mucosal Antibody Response to CT.
Each bar represents the mean of 14-20 mice +/- SE.
* Significantly different from control.

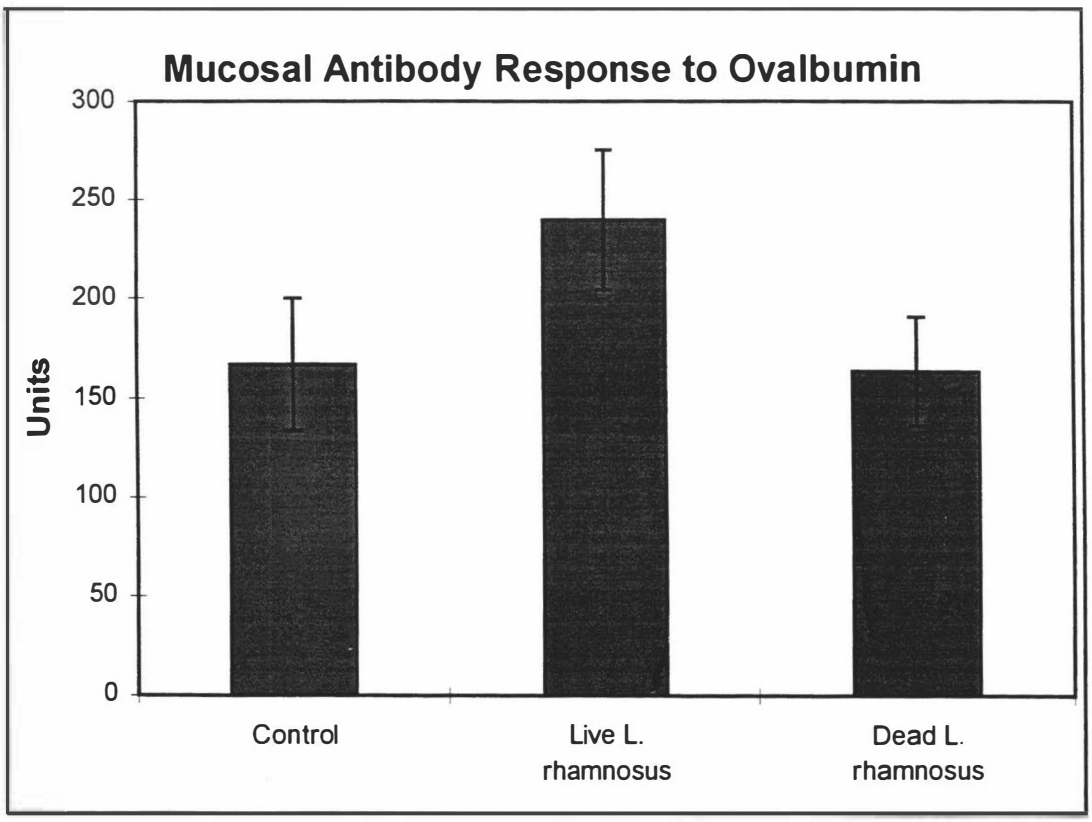

Figure 12 Mucosal Antibody Response to OV.
Each bar represents the mean of 15-20 mice +/- SE.

4.3.3 *In Vitro* Antibody Production

4.3.3.1 *In Vitro* Antibody Production by PP Lymphocytes in Response to CT and OV

Table 11 shows the *in vitro* antibody production by PP lymphocytes in response to CT. It can be observed that the PP from the live and dead *L. rhamnosus* treated mice produced higher amounts of IgA to CT compared to the control mice (not statistically significant). There was no difference in the IgA levels in response to CT in mice fed dead *L. rhamnosus* compared to mice fed live *L. rhamnosus*. The IgG levels in response to CT were also increased in the live and dead *L. rhamnosus* treatment groups compared to the control group but the increase was only significant for the dead *L. rhamnosus* treatment group. When compared to the live *L. rhamnosus* treatment group, the IgG response to CT was also significantly higher in the dead *L. rhamnosus* treatment group. IgA levels against OV in the live and dead *L. rhamnosus* treatment groups were higher than the control mice but the differences were not statistically significant (Figure 13). There was also no significant difference between the live and dead *L. rhamnosus* treatment groups. There was no difference in the level of IgG produced in response to OV by the live *L. rhamnosus* treatment group when compared to the control group (Figure 14). The levels of IgG produced in response to OV was however, slightly increased (not significant) in the dead *L. rhamnosus* treatment group when compared to the control group.

Table 11 Effect of Live or Dead *L. rhamnosus* on the *In Vitro* Antibody Production by PP Lymphocytes in Response to CT

Treatment	*In vitro* IgA production by PP cells to cholera toxin (mean abs ± SE)[c]	*In vitro* IgG production by PP cells to cholera toxin (mean abs ± SE)[c]
Control	0.553 ± 0.046	0.778 ± 0.073[a]
L. rhamnosus (live)	0.629 ± 0.080	0.856 ± 0.041 [a]
L. rhamnosus (killed)	0.600 ± 0.05	1.083 ± 0.053 [b]

[a] Significantly different from value with superscript [b]

[c] mean ± SE (n=2-4)

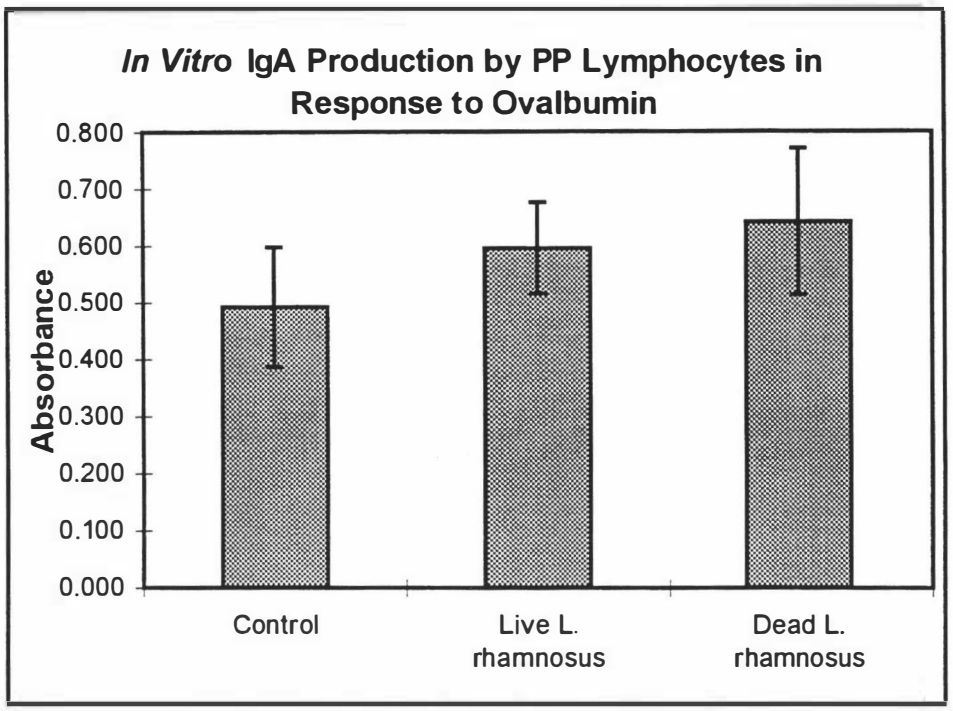

Figure 13 *In Vitro* IgA Production by PP lymphocytes in Response to OV. Each bar represents the mean of 4 mice +/- SE.

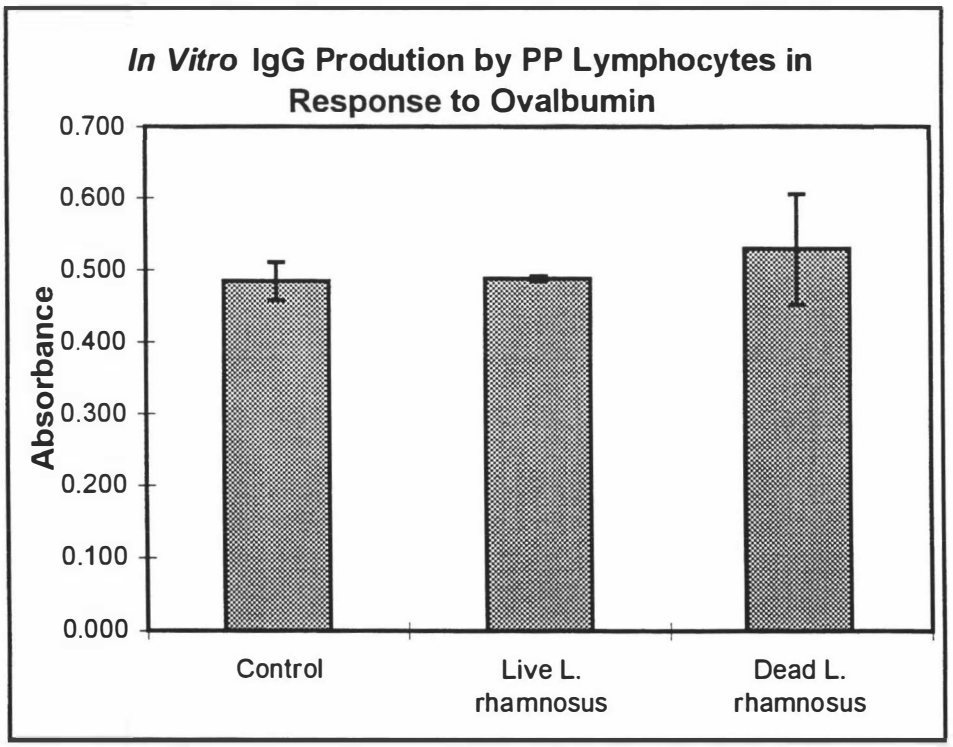

Figure 14 *In Vitro* IgG Production by PP lymphocytes in Response to OV. Each bar represents the mean of 3 mice +/- SE.

4.3.3.2 *In Vitro* Antibody Production by MLN Lymphocytes in Response to CT and OV

There was no difference in the IgA levels produced by MLN lymphocytes in response to CT between the control group and the live *L. rhamnosus* treatment group (Table 12). The IgA levels in the dead *L. rhamnosus* treatment groups were however, lower than that of the control group although this difference was not statistically significant. The IgG levels produced in response to CT by the live and dead *L. rhamnosus* treatment groups were slightly lower than that of the control group. There was no difference in this response between the live and dead *L. rhamnosus* treatment groups.

When compared to the control group, only the live *L. rhamnosus* treatment group had slightly higher IgA levels in response to OV (Figure 15). The IgA levels from the dead *L. rhamnosus* treatment group were however, slightly lower than that of the control. There was no significant difference in this response between the live and dead *L. rhamnosus* treatment groups. There was a lower level of IgG produced in response to OV from both of the *L. rhamnosus* treatment groups compared to the control group although the difference was not statistically significant (Figures 16). There was no difference between the live and dead *L. rhamnosus* treatment groups in this response.

Table 12 Effect of Live or Dead *L. rhamnosus* on the *In Vitro* Antibody Production by MLN Lymphocytes in Response to CT

Treatment	*In vitro* IgA production by MLN cells in response to cholera toxin (mean abs ± SE)[a]	*In vitro* IgG production by MLN cells in response to cholera toxin (mean abs ± SE)[a]
Control	0.254 ± 0.031	0.279 ± 0.036
L. rhamnosus (live)	0.244 ± 0.011	0.263 ± 0.025
L. rhamnosus (killed)	0.197 ± 0.034	0.248 ± 0.015

[a] mean ± SE (n=5-7)

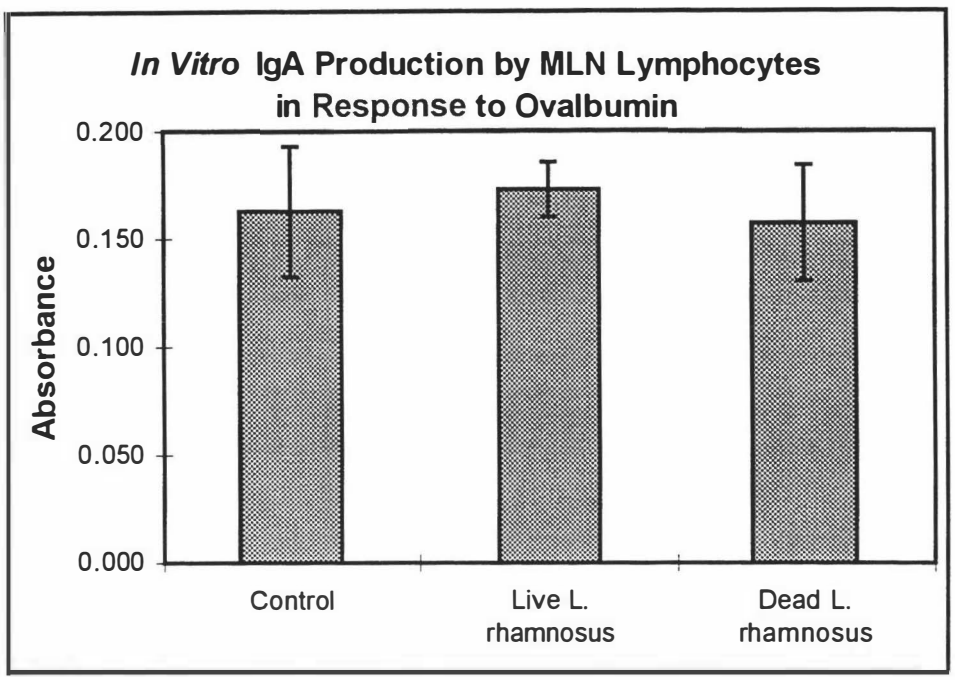

Figure 15 *In Vitro* IgA Production by MLN lymphocytes in Response to OV. Each bar represents the mean of 5-7 mice +/- SE.

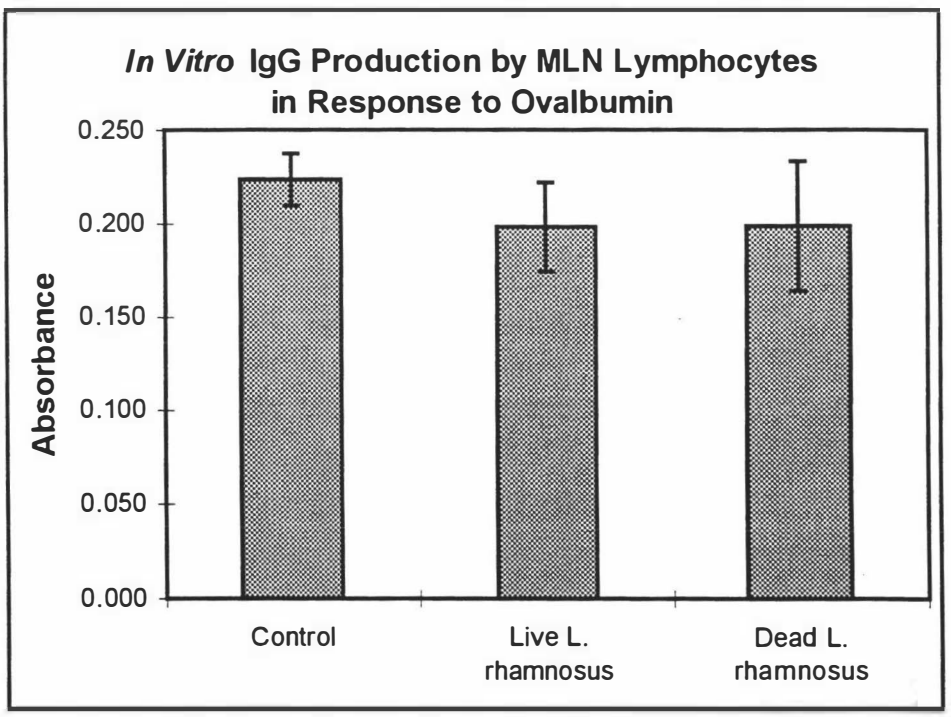

Figure16 *In Vitro* IgG Production by MLN lymphocytes in Response to OV. Each bar represents the mean of 5-7 mice +/- SE.

4.3.4 Expression of Lymphocyte Surface Markers

4.3.4.1 Expression of Surface Markers on PP Lymphocytes

There was a greater number of $CD40^+$ PP lymphocytes in both the live and dead *L. rhamnosus* treatment groups compared to the control group (Table 13). However, the increase was only significant for the live *L. rhamnosus* treatment group. The number of $CD40^+$ lymphocytes in the dead *L. rhamnosus* treatment group was slightly lower than that of the live *L. rhamnosus* treatment group.

Table 13 Immunophenotyping of PP lymphocytes

Treatment	% (mean ± SE) of $CD40^+$ PP lymphocytes[a]	% (mean ± SE) of $CD3^+$ PP lymphocytes[b]	CD4:CD8 of PP lymphocytes (mean ± SE)[b]
Control	65.74 ± 1.69	28.77 ± 1.77	3.49 ± 0.40
L. rhamnosus (live)	71.90 ± 0.89**	28.55 ± 1.30	3.50 ± 0.27
L. rhamnosus (killed)	69.69 ± 1.94	29.24 ± 1.65	4.22 ± 0.34

P<0.01(**) Significance compared to the controls

[a] mean ± SE (n=4-9)

[b] mean ± SE (n=6-10)

There was no difference in the number of $CD3^+$ PP lymphocytes in mice fed live or dead *L. rhamnosus* when compared to the control mice. There was also no difference in the CD4:CD8 ratio of the PP lymphocytes of the live *L. rhamnosus* treatment group when compared to the control group. The ratio was however, higher (not statistically significant) in the dead *L. rhamnosus* treatment group than the control group. There was no difference in the number of $CD4^+$ lymphocytes between any of the treatment groups compared to the control group (data not shown), but there was a lower number of cytotoxic T cells ($CD8^+$ phenotype) in both of the *L. rhamnosus* treatment groups compared to the control group (data not shown). Compared to the control and the dead *L. rhamnosus* treatment groups, significantly more of the PP lymphocytes of the live *L. rhamnosus* treatment group were $CD25^+$ (Figure 17). The number of $CD25^+$

lymphocytes in the dead *L. rhamnosus* treatment group was only slightly higher than the control group.

No changes were observed for the number of $CD45^+$ (leukocyte common antigen) lymphocytes in either of the treatment groups compared to the control group (data not shown).

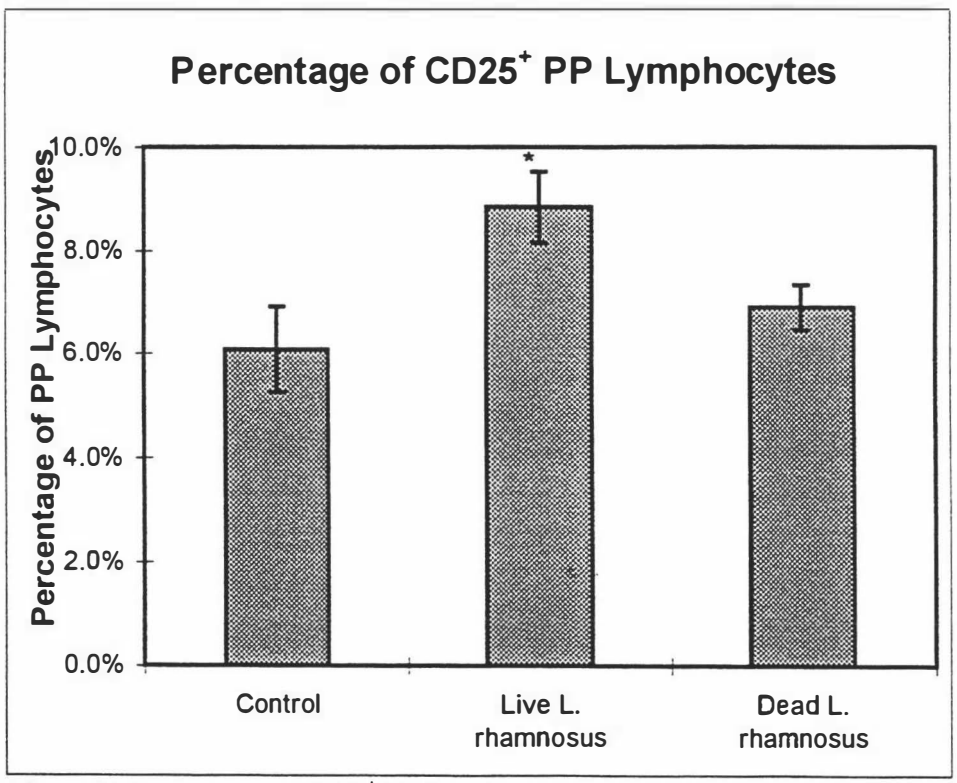

Figure17 Percentage of $CD25^+$ PP lymphocytes.
Each bar represents the mean of 5-8 mice +/- SE.
P<0.05(*) Significantly different from control group.

4.3.4.2 Expression of Surface Markers on MLN Lymphocytes

There was no difference in the numbers of $CD40^+$ MLN lymphocytes in mice fed live or dead *L. rhamnosus* when compared to the control mice. There was also no difference between the live and dead *L. rhamnosus* treatment groups in this response (Table 14).

Table 14 Immunophenotyping of MLN lymphocytes

Treatment	% (mean ± SE) of $CD40^+$ MLN lymphocytes[a]	% (mean ± SE) of $CD3^+$ MLN lymphocytes[b]	CD4:CD8 of MLN lymphocytes (mean ± SE)[a]
Control	22.03 ± 0.92	74.28 ± 2.16	3.99 ± 0.18
L. rhamnosus (live)	24.09 ± 0.94	75.49 ± 1.96	4.21 ± 0.26
L. rhamnosus (killed)	23.07 ± 0.64	71.34 ± 6.84	4.22 ± 0.22

[a] mean ± SE (n=8-10)

[b] mean ± SE (n=2-3)

There was no difference between the control and the live *L. rhamnosus* treatment groups in the number of T cells ($CD3^+$ phenotype). However, there was a lower number of T cells in the MLN of mice fed dead *L. rhamnosus* than the control mice, although this was not significant. No significant differences were also observed in this response between the live and dead *L. rhamnosus* treatment groups. There was no difference between the live or dead *L. rhamnosus* treatment groups and the control group in the CD4:CD8 ratio. There was also no difference in the CD4:CD8 ratio between the live and dead *L. rhamnosus* treatment groups.

The number of $CD25^+$ MLN lymphocytes in the live *L. rhamnosus* treatment group was slightly higher than that of the control group (Figure 18). There was no difference between the control mice and those fed dead *L. rhamnosus* in this response.

No significant differences were observed between the control and the *L. rhamnosus* treatment groups in the $CD45^+$ MLN lymphocytes (data not shown).

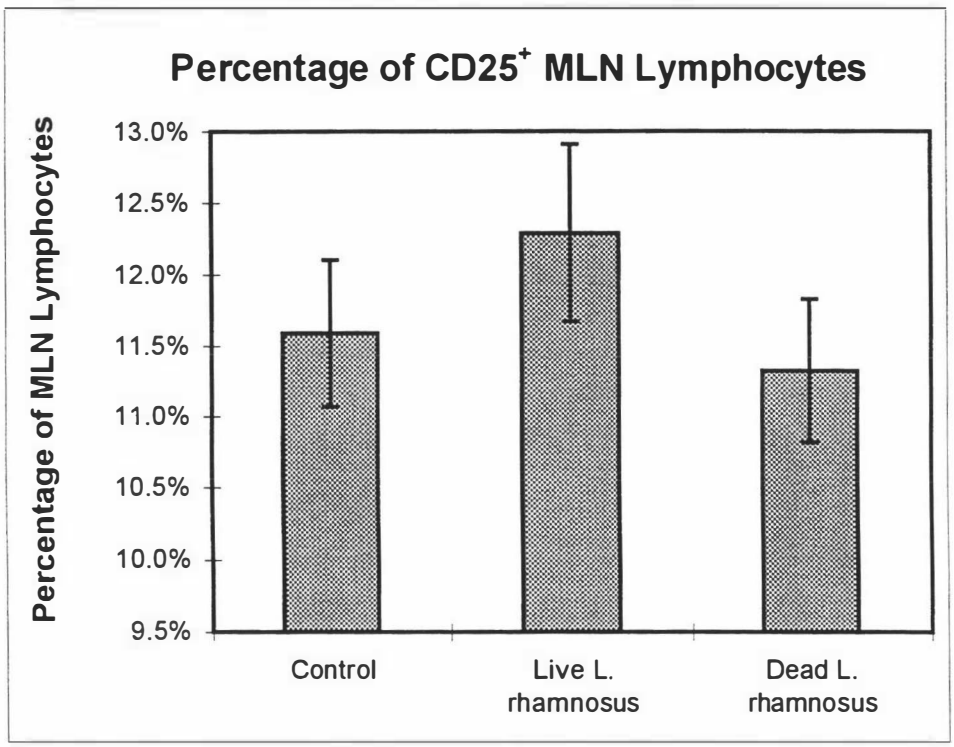

Figure 18 Percentage of $CD25^+$ MLN lymphocytes.
Each bar represents the mean of 8-9 mice +/- SE.

4.4 Discussion

In this study, the effect of viability of *L. rhamnosus* on the mucosal and serum immune responses of mice to CT and OV antigens was examined.

The results of this study indicate that not all mucosal immune parameters in mice fed with *L. rhamnosus* (either live or dead) were enhanced when compared to the control mice, and that viability of *L. rhamnosus* does affect the mucosal and serum immune responses to CT and OV. Mice treated with live *L. rhamnosus* generally exhibited greater mucosal immune responses than those treated with dead *L. rhamnosus*. For example, the MLN of mice fed with live *L. rhamnosus* produced higher levels of both IgA and IgG *in vitro* in response to CT than the MLN of mice fed dead *L. rhamnosus*, although this difference was not significant. The number of T cells in the MLN of mice treated with live *L. rhamnosus* was higher than that of mice fed dead *L. rhamnosus* but this was also not significant. The mucosal antibody responses of mice treated with live *L. rhamnosus* in response to CT and OV were higher than that of mice treated with dead *L. rhamnosus*.

The finding that there was slightly increased B cell numbers in the PP of mice treated with live *L. rhamnosus* compared to that in mice fed dead *L. rhamnosus* was consistent with the result found by De Simone *et al* (1987). In their study, there was significantly increased B cell numbers in the PP of mice fed yoghurt with live lactobacilli for 7 days compared to that in mice fed heat-treated yoghurt. When mice were fed yoghurt with live lactobacilli for 14 days, there was only a slight increase in the number of B cells in this treatment group when compared to the group fed heat-treated yoghurt and this difference was not significant. There was no difference between the number of T cells in the PP of mice fed live *L. rhamnosus* and those fed dead *L. rhamnosus* and this was also found by De Simone.

In a similar study to that by De Simone *et al* (1987) using spleen lymphocytes, Vesely *et al* (1985) found that feeding mice yoghurt with live lactobacilli resulted in increased (but not significant) T cell numbers and proliferative responses of splenic lymphocytes compared to that of mice fed heat-treated yoghurt. These findings and that of the present study suggest that dead LAB are not as effective as live LAB in stimulating several aspects of the humoral and cellular immune responses. However, the *in vitro* IgG production by PP lymphocytes from mice treated with dead *L. rhamnosus* was greater than that of the live *L. rhamnosus* treated mice. The reason(s) for this is not clear.

The precise mechanisms by which LAB gain access to the immune system and stimulate the immune response are unknown. However, several mechanisms have been suggested as to how LAB stimulate the immune system (Gill, 1998). Link-Amster *et al* (1994) suggested that LAB can translocate via the M cells to the PP and other GALT where they will be processed immunologically. Lactic acid bacteria or their products could be taken up by the M cells of PP and delivered to the underlying lymphoid cells that will then induce B and T cell responses (Schiffrin *et al*, 1997 and Kitazawa *et al*, 1996). Lactic acid bacteria can also gain entry into the host via non-specific uptake via epithelial cells or receptor-ligand mediated uptake (Claassen *et al*, 1995). Lactic acid bacteria or their products are also transported through the circulation to the systemic lymphoid tissues such as spleen and MLN to stimulate their immune response (Steffen & Berg, 1983). Lactic acid bacteria have been shown to be transported from the gut to lymphoid tissues by Claassen *et al* (1995). They found that lactobacilli were taken up by M cells from the PP and after 6 to 12 hours, they were seen in the dome area and then in MLN after 48 hours.

One of the ways LAB stimulate the immune system is by inducing cytokine production by immunocompetent cells. Cell wall components of LAB such as capsular polysaccharides, peptidoglycans and LTA are able to induce cytokine production (Miettinen *et al*, 1996; Bhakdi *et al*, 1991; Heumann *et al*, 1994). In studies using mice and humans, lactic acid bacteria and their products have been shown to stimulate cytokine production both *in vitro* and *in vivo* (De Simone *et al*, 1987; De Simone *et al,* 1986; Hatcher & Lambrecht, 1993; Sato *et al*, 1988; Kishi et al, 1996; Aattouri & Lemonnier, 1997).

Muramyl dipeptide which is present in peptidoglycan can also induce cytokine production *in vitro* (Aattouri & Lemonnier, 1997). LAB cell wall components may induce cytokine production by binding to epithelial cells; lymphocytes and polymorphonuclear cells (Bhakdi *et al*, 1991; Beachey *et al*, 1979; Courtney *et al*, 1981; Levy *et al*, 1990; Dziarski, 1991).

Intestinal epithelial cells (IEC) can produce cytokines when challenged with pathogenic bacteria *in vitro* (Jung *et al,* 1995) and are activated by IFN-γ or TNF-α and cell surface molecules (Delneste *et al*, 1998). Lactic acid bacteria have been shown to adhere to the IEC *in vitro* and to the intestinal mucosa *in vivo* (Schriffrin *et al*, 1997 and Johansson *et al*, 1993). Whether LAB can directly act on IEC to produce cytokines is unclear. In a recent study, it was shown that LAB do not directly activate intestinal epithelial lymphocytes (IEL) but potentiate the IFN-γ dependent activation of IEL (Delneste *et al*, 1998). The colonisation of germ free mice with live LAB was shown in one study to increase the proportion of intraepithelial cells in the proximal small intestine by Schiffrin (1992) (unpublished data), cited from Schiffrin *et al* (1997). The author suggests that either a signal generated by LAB, or LAB themselves can adhere and act on the epithelial cells or intraepithelial lymphoid compartment of the proximal small intestine. Activated lymphocytes can then produce cytokines that will exert a variety of effects on immune and non-immune cells (Ebert, 1989 and Ebert, 1990).

Cytokine production can also be induced when LAB antigens are processed and presented by APC to T cells. In the study by Aattouri & Lemmonier (1997), it was shown that both the MHC class I and II pathways are involved in processing *S. thermophilus* and thus stimulate the production of IFN-γ by $CD4^+$ and $CD8^+$ T cells. This mechanism could also apply for other LAB. The authors also found that the MHC class II pathway could be involved in the production of IFN-γ in blood mononuclear cells (BMC) enriched in lymphocytes (EBMC) that are stimulated by MDP.

Cytokines can also influence the production of other cytokines. For example, IL-2 and IL-4 have been shown to play a role in IFN-γ secretion (Nussler & Thomson, 1992).

4.5 Summary

In summary, the results of the present study demonstrated that feeding mice with either live or dead *L. rhamnosus* stimulated several aspects of their mucosal immune responses, such as the *in vitro* antibody responses in PP, B cell and activated T cell numbers, and mucosal antibody responses, and serum immune responses to CT and OV. The responses increased to a greater degree in mice fed live *L. rhamnosus* compared to mice fed dead *L. rhamnosus*. It is unclear why dead *L. rhamnosus* was found to enhance certain aspects of immune function more efficiently than the live bacteria. Cell traffic from the mucosal inductive sites (eg. PP) to the effector sites (eg. intestinal contents) was also demonstrated in this study. Several mechanisms by which LAB gains access to the immune system and stimulates the immune responses, for example, the ability of LAB to translocate to the PP and other GALT for immunological processing and cytokine production are suggested.

Chapter 5

5.1 Introduction

Diarrhoea due to enteric infection is a common occurrence for people of all ages, but especially the elderly and children. In developing countries, diarrhoeal diseases are one of the main causes of morbidity and mortality in children (up to four million yearly) (Katelaris, 1996). Travellers to developing countries are also faced with the risk of developing traveller's diarrhoea. Diarrhoeal diseases are common even in developed countries (Katelaris, 1996) although they may not occur as frequently. There are many causes for infectious diarrhoea; including bacteria, viruses and parasites (Katelaris, 1996).

The most common method employed for treating diarrhoeal infections that are already established is to replace fluid and electrolyte losses (Katelaris, 1996). Specific antimicrobial treatment (eg. antibiotics) are also used although it has been shown that they are only effective in some bacterial infections (Katelaris, 1996).

Consumption of LAB has been shown to provide protection against diarrhoeal diseases. Several studies have shown the inhibitory effects of *L. acidophilus* and *B. bifidus* on many common pathogens such as *E. coli*, *Staphylococcus aureus* and *S. typhi* (Gilliland, 1979; Gilliland & Speck, 1977a; Vincent *et al*, 1959). *L. acidophilus* and *B. bifidum* cultured milks have also been shown to have preventive effects against intestinal infections (Nader de Macias *et al*, 1992; Perdigon *et al*, 1990b; Saavedra *et al*, 1994; Watkins *et al*, 1982, cited by Gilliland, 1989; Perdigon *et al*, 1990a). Other LAB have also been shown to have protective effects against intestinal infections (Majamaa *et al*, 1995; Kaila *et al*, 1992; Miake *et al*, 1985; Nader de Macias *et al*, 1993; Siitonen *et al*, 1990; Hitchins *et al*, 1985).

The results from studies on the ability of LAB to prevent travellor's diarrhoea are variable. Some studies did not find any difference between the treatment groups and the control (placebo) while others did find differences but they were not statistically significant (de dios Pozo-Olano *et al*, 1978; Katelaris *et al*, 1995; Oksanen *et al*, 1990). Whether this was due to the use of different LAB or whether different bacteria were causing the diarrhoea, is not clear.

L. acidophilus and *L. bulgaricus* have been successfully used to prevent antibiotic-associated diarrhoea. For example, Gotz *et al* (1979) (cited by Salminen *et al*, 1993) found that *L. acidophilus* and *L. bulgaricus* can prevent ampicillin associated diarrhoea in hospital patients. However, Tankanow *et al* (1990) (cited by Salminen *et al*, 1993) did not find any significant preventive effects on diarrhoea caused by amoxycillin treatment in paediatric patients fed with the same probiotic mixture.

In vitro studies have also shown the antagonistic activity of LAB against bacterial invasion of the cultured enterocyte-like Caco-2 cells (Hudault *et al*, 1997). Other *in vitro* studies have shown that lactobacilli can prevent the adhesion and cell invasion of Caco-2 cells by pathogenic bacteria such as *E. coli* and *S. typhi* (Bernet *et al*, 1994).

Several mechanisms by which LAB mediate the protection against enteric infection have been suggested, for example, production of antibacterial substances (Mishra & Lambert, 1996), competition with pathogenic bacteria for adhesion receptors (Bernet *et al*, 1994), competition for nutrients for growth (Reddy *et al*, 1988) and stimulation of the immune system. However, the relative importance of each of these mechanisms for the protection against enteric infection is not clear.

The aim of this present study was to examine the role of immune enhancement in the prevention of *S. typhimurium* infection in mice. The severity of the infection was assessed by monitoring the liveweight change and measuring the number of *S. typhimurium* in systemic tissues such as liver and spleen. The immune responses of the mice following infection were assessed by measuring various mucosal immune parameters such as the *in vitro* antibody production by PP and MLN lymphocytes. Some systemic immune parameters (eg. phagocytic activity of peritoneal macrophages) were also measured.

In previous studies, *L. rhamnosus* was found to enhance a range of specific and non-specific immune responses such as the phagocytic activity of the peripheral blood leukocytes and peritoneal macrophages, NK cell activity, and serum and mucosal antibody responses (NZDB, 1998). In the present study, mice were fed with *L. rhamnosus* and challenged with *S. typhimurium* and their responses monitored.

5.2 Experimental Design:

Fifty three BALB/c mice were randomly divided into 3 groups, with 2 treatment groups having 21 animals in each and a control group with 11 animals. They were allocated to different treatments as shown in Table 15. Mice were fed a SMP based diet which was introduced during the initial 5 day acclimatisation period. Following this, mice were orally administered *L. rhamnosus* (1 x 10^9 cfu) in 50μL of skim milk daily for 14 days on day -7 and were infected with *S. typhimurium* (ATCC 1772) (5 x 10^5 cfu) at a dose rate of 50μL/mouse of bacteria on day 0. The *Salmonella* only treated mice were fed skim milk and infected with *Salmonella* on day 0 while the control mice were fed skim milk only. The resistance of mice to infection was measured by monitoring the liveweight change and translocation of *Salmonella* to the liver and spleen. Six mice from each group were randomly chosen to be killed on day 7 for analysis. Blood, PP and MLN samples, peritoneal macrophages, spleen and liver were collected for assays. Various immune parameters such as *in vitro* antibody production, cell proliferation, and phagocytic activity of peritoneal macrophages were measured. The results are presented in the following sections.

Table 15 Treatment Schedule

Day	*Salmonella*	*L. rhamnosus*	Control
-12-6	Acclimatisation	Acclimatisation	Acclimatisation
-7	Skim milk powder	Skim milk + *L. rhamnosus*	Skim milk powder
0	Skim milk powder + *S. typhimurium*	Skim milk + *S. typhimurium* + *L. rhamnosus*	Skim milk powder
7	Skim milk powder	Skim milk + *L. rhamnosus*	Skim milk powder

5.3 Results

5.3.1 Liveweight Change

As shown in Figure 19, there was a positive weight change (weight gain) in the control mice while in mice fed *L. rhamnosus* + *Salmonella,* there was no weight change (maintenance of liveweight). The weight change differences observed between both the control and the *L. rhamnosus* + *Salmonella* treatment groups were significantly different when compared to the *Salmonella* only treatment group. Mice fed *Salmonella* only had a negative weight change (lost weight).

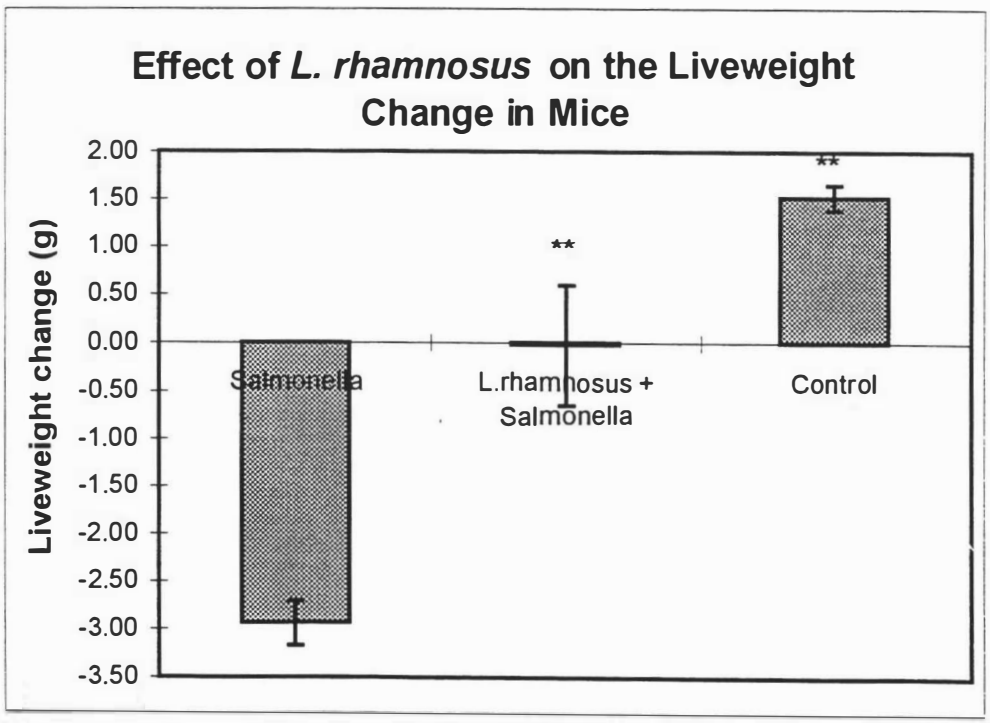

Figure 19 Effect of *L. rhamnosus* on the Liveweight Change in Mice.
Each bar represents the mean of 6 mice +/- SE.
P<0.01(**) Significantly different from *Salmonella* only treatment group

5.3.2 Number of Viable *S. typhimurium* in the Liver and Spleen of Mice

As shown in Table 16, no bacteria were found in the liver or spleen of control mice. This was highly significant when compared to the *Salmonella* only treated mice. The number of bacteria in the liver and spleen of mice fed *L. rhamnosus* + *Salmonella* was significantly lower than that of mice fed *Salmonella.* The *Salmonella* only treated mice had the highest translocation rate.

Table 16 Log Number of Viable *S. typhimurium* in Liver and Spleen of Mice Challenged with *S. typhimurium*

Treatment	Log no of viable bacteria in liver / organ[a]	Log no of viable bacteria in spleen / organ[a]
Salmonella	6.342 ± 0.396	6.068 ± 0.434
L. rhamnosus + *Salmonella*	4.302 ± 0.394*	4.172 ± 0.510*
Control	0**	0**

P<0.05 (*), P<0.01(**). Significance of values compared to the *Salmonella* only treatment group.

[a] mean ± SE (n=5-6)

5.3.3 Effect of Feeding *L. rhamnosus* on the Phagocytic Activity of Peritoneal Macrophages

The phagocytic activity of peritoneal macrophages to FITC-*E. coli* from both the control group and the *L. rhamnosus* + *Salmonella* treated group were significantly higher than that of the *Salmonella* only treated group (Figure 20). The phagocytic activity of the control mice was higher than that of the *L. rhamnosus* + *Salmonella* treated mice but the difference was not statistically significant.

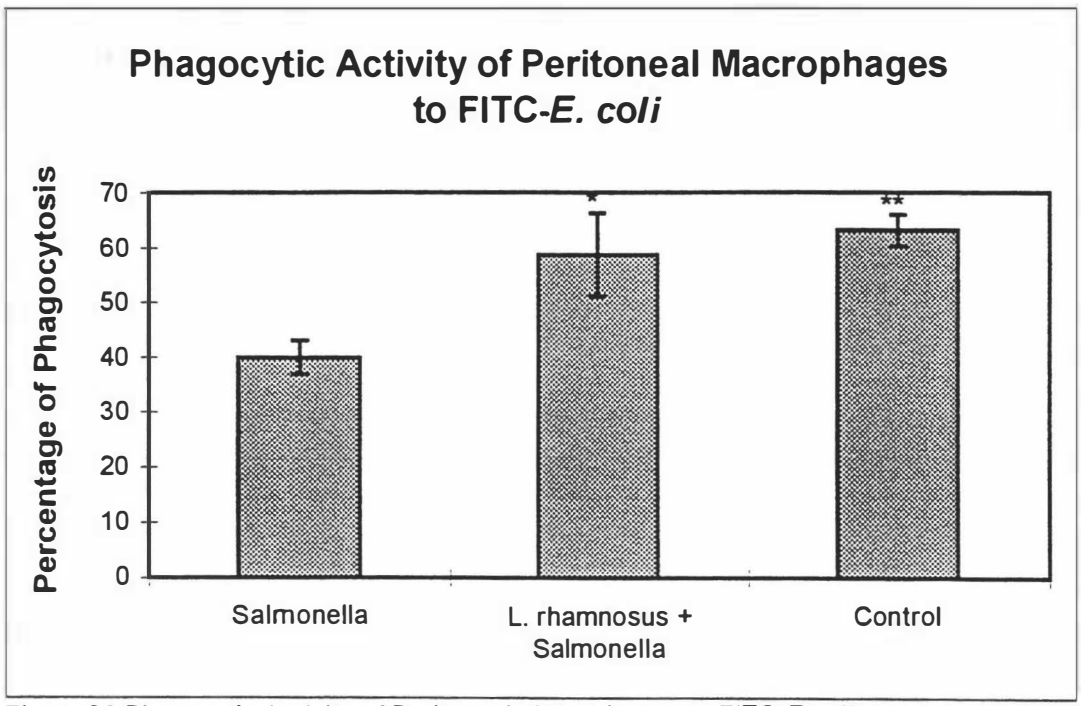

Figure 20 Phagocytic Activity of Peritoneal Macrophages to FITC-*E.coli*. Each bar represents the mean of 6 mice +/- SE. P<0.05(*), P<0.01(**).

5.3.4 Effect of Feeding *L. rhamnosus* on the Phagocytic Activity of Peripheral Blood Leukocytes

The phagocytic activity of peripheral blood leukocytes to FITC-*E. coli* from the control group and the *L. rhamnosus* + *Salmonella* treated group was significantly higher than that of the *Salmonella* only treatment group. The phagocytic activity of the control mice was slightly lower than that of the *L. rhamnosus* + *Salmonella* treated mice but this was not statistically significant (Figure 21).

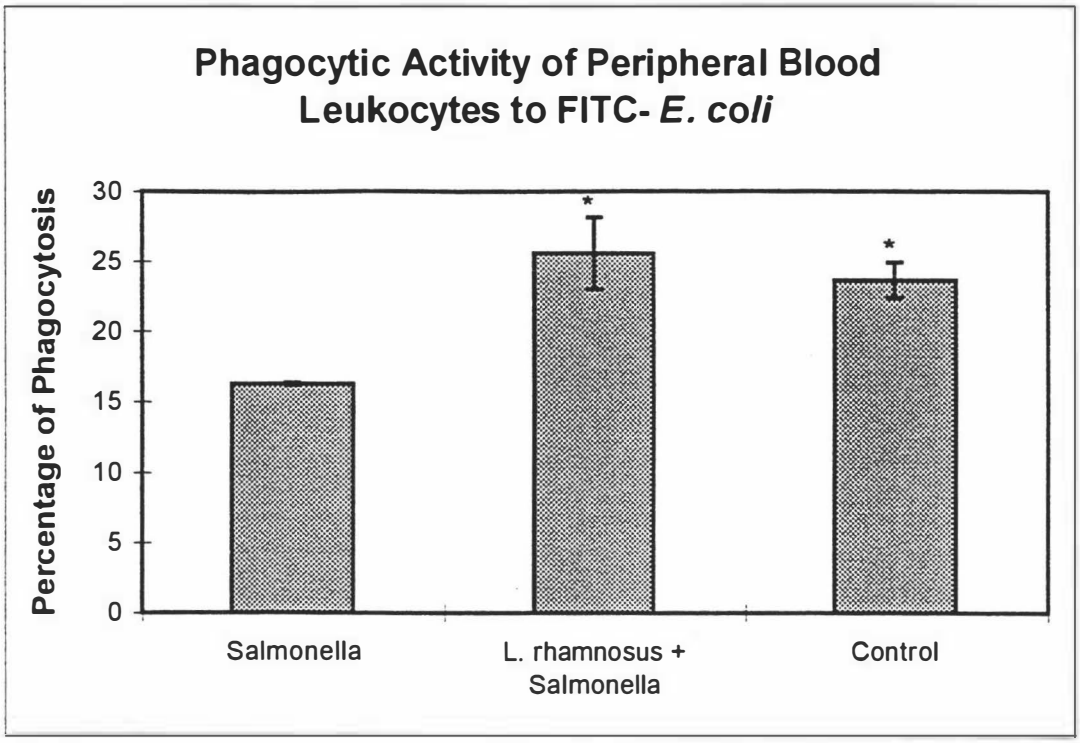

Figure 21 Phagocytic Activity of Peripheral Blood Leukocytes to FITC-*E.coli*. Each bar represents the mean of 6 mice +/- SE. P<0.05(*).

5.3.5 Effect of Feeding *L. rhamnosus* on the *In Vitro* Antibody Production by PP and MLN Lymphocytes in Response to LTA from *S. typhimurium*

As shown in Figure 22, the *in vitro* IgA production in response to LTA by PP lymphocytes from the control group was higher than that of the *Salmonella* treatment group. The production of antibodies by the PP of the *L. rhamnosus* + *Salmonella* treated mice was also increased compared to the *Salmonella* treated mice. The IgA production in response to LTA by the *L. rhamnosus* + *Salmonella* treated mice were higher than that of the control mice. It was not possible to determine the significance of the differences between the treatment groups as the PP of all 6 mice in each group were pooled and thus there was only one data point for each treatment group. This had to be done due to the lack of adequate number of PP lymphocytes from each mouse. It was observed that there were fewer PP along the small intestine of mice infected with *Salmonella* than the control mice.

There was increased IgG production in response to LTA by the PP lymphocytes of both the control mice and *L. rhamnosus* + *Salmonella* treated mice compared to the *Salmonella* treated group (Figure 23). There was no difference in this response between the control mice and those treated with *L. rhamnosus* + *Salmonella.*

Compared to the control mice, there was lower IgA production in response to LTA by the MLN lymphocytes of mice treated with *Salmonella.* Compared to the *Salmonella* treated mice, mice treated with *L. rhamnosus* + *Salmonella* showed significantly higher IgA production (P=0.024) (Table 17). There was no difference in the IgG production between the MLN lymphocytes of control mice or those treated with *L. rhamnosus* + *Salmonella* and mice treated with *Salmonella* only.

Table 17 *In Vitro* IgA and IgG Production by MLN Lymphocytes in Response to LTA

Treatment	IgA production by MLN lymphocytes (mean abs ± SE)[a]	IgG production by MLN lymphocytes (mean abs ± SE)[a]
Salmonella	0.318 ± 0.020	0.341 ± 0.022
L. rhamnosus + *Salmonella*	0.577 ± 0.035*	0.343 ± 0.108
Control	0.408 ± 0.040	0.368 ± 0.042

P<0.05(*) Significance of value compared to the *Salmonella* group.

[a] mean ± SE (n=2-3)

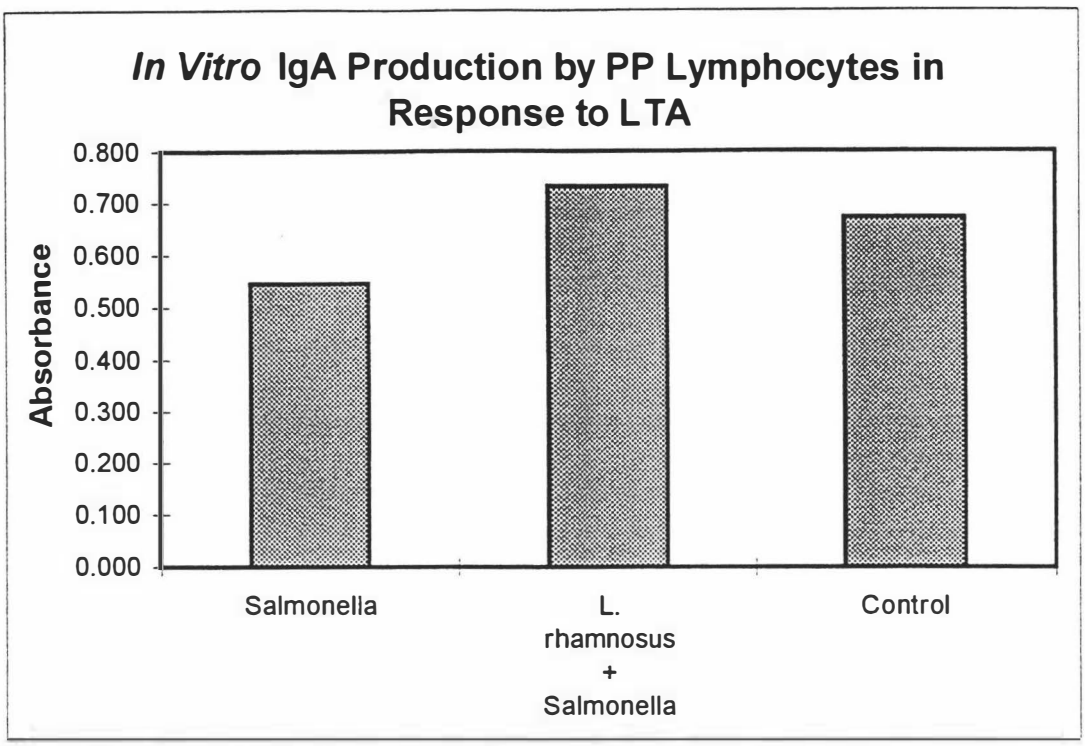

Figure 22 *In Vitro* IgA Production by PP Lymphocytes in Response to LTA

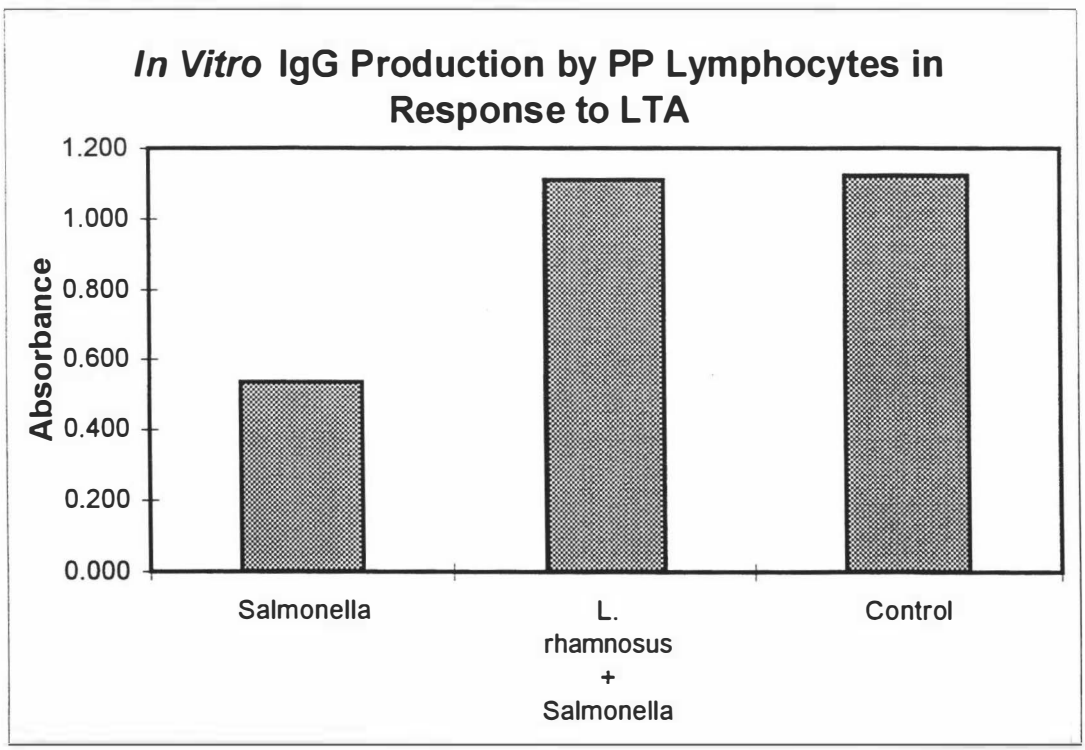

Figure 23 *In Vitro* IgG Production by PP Lymphocytes in Response to LTA

5.3.6 Effect of Feeding *L. rhamnosus* on the Expression of Surface Markers on PP Lymphocytes

The control mice and the *L. rhamnosus* + *Salmonella* treated mice had higher levels of $CD40^+$ lymphocytes than the *Salmonella* treated mice (Table 18). The CD4:CD8 ratio of the *L. rhamnosus* + *Salmonella* treatment group was higher than that of the *Salmonella* treatment group. This increase was due to the increase in helper T cells ($CD4^+$) as shown in Figure 24. The control group also had a higher ratio than the *Salmonella* treatment group. Immunophenotyping of the $CD3^+$ lymphocytes in PP could not be conducted due to the lack of adequate number of PP lymphocytes. It was also not possible to determine the significance of the differences between the treatment groups as the PP of all 6 mice were pooled and therefore there was only one data point for each treatment group. This had to be done due to the lack of adequate PP cells from each mouse.

Table 18 Immunophenotyping of PP lymphocytes

Treatment	% of $CD40^+$ PP lymphocytes	% of CD4:CD8 of PP lymphocytes
Salmonella	71.63	2.19
L. rhamnosus + *Salmonella*	76.66	3.72
Control	74.76	3.12

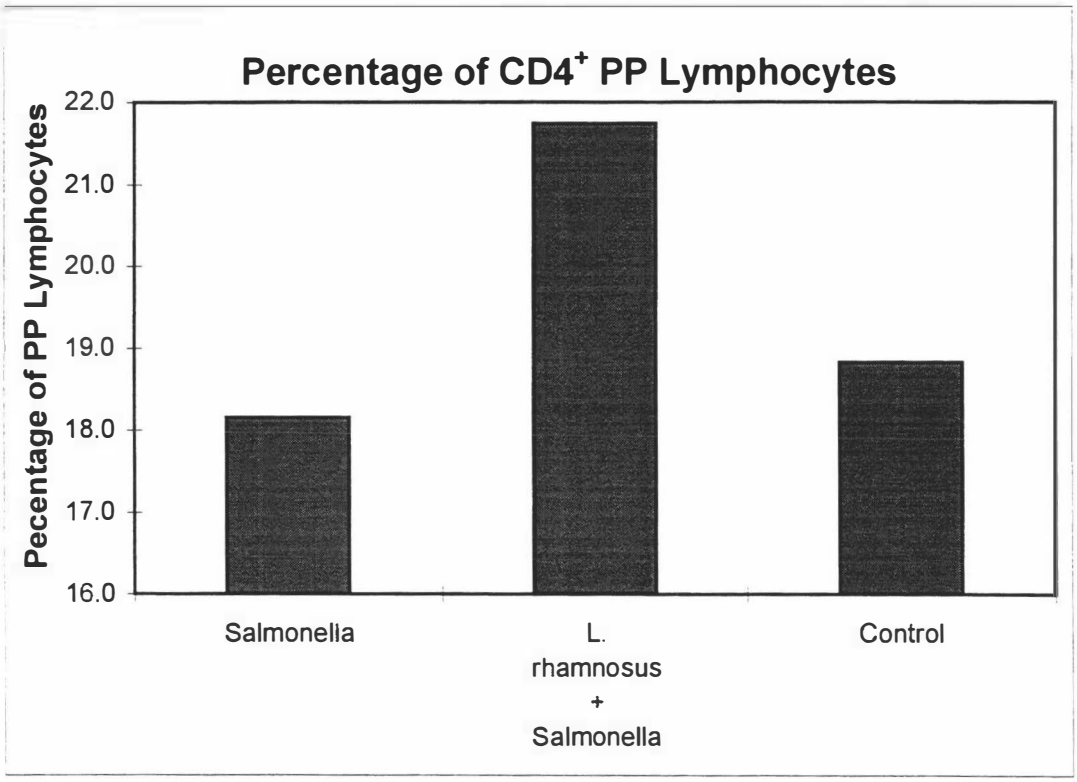

Figure 24 Percentage of $CD4^+$ PP lymphocytes

5.3.7 Effect of Feeding *L. rhamnosus* on the Expression of Surface Markers on MLN Lymphocytes

As shown in Table 19, the number of $CD40^+$ MLN lymphocytes in both the control and the *L. rhamnosus* + *Salmonella* treatment groups were significantly lower than that of the *Salmonella* only treatment group. Compared to the *Salmonella* treated mice, the control mice and those fed *L. rhamnosus* + *Salmonella* showed a significant increase in the number of $CD3^+$ lymphocytes. Compared to mice fed *Salmonella* only, there was no significant difference in the CD4:CD8 ratio of the MLN lymphocytes of control mice and those fed *L. rhamnosus* + *Salmonella*.

Table 19 Immunophenotyping of MLN lymphocytes

Treatment	mean % of $CD40^+$ MLN lymphocytes ± SE[g]	mean % of $CD3^+$ MLN lymphocytes ± SE[g]	mean CD4:CD8 ratio of MLN lymphocytes ± SE[g]
Salmonella	53.59 ± 2.72 [a]	40.74 ± 2.16 [d]	3.59 ± 0.26
L. rhamnosus + *Salmonella*	44.20 ± 0.26 [b]	51.29 ± 0.20 [e]	3.89 ± 0.12
Control	24.94 ± 0.867 [c]	68.42 ± 0.59 [f]	3.808 ± 0.31

[a] Significantly different from value with different superscript

[d] Significantly different from values with a different superscript

[g] mean ± SE (n=3)

5.4 Discussion

Salmonella are invasive pathogenic microorganisms and are able to invade the small intestinal mucosa and cause systemic infection (Drasar, 1989). In this study, the role of immune enhancement in the prevention of *S. typhimurium* infection in mice was investigated.

Results indicate that *L. rhamnosus* provided significant protection against *S. typhimurium* infection. This was shown by significantly lower numbers of *Salmonella* in the liver and spleen of infected mice, and a maintenance of their liveweight. The enhanced resistance of *L. rhamnosus* to *S. typhimurium* was accompanied by increased cellular immune responses to LTA, a cell surface component of *Salmonella*, and specific and non-specific immune responses.

The observation that there was significantly lower bacteria numbers in the liver and spleen of the *L. rhamnosus* + *Salmonella* treated mice suggests that *L. rhamnosus* feeding prevented *Salmonella* from translocating to other organs. This is consistent with the observations of other investigators in similar experiments (Nader de Macias *et al*, 1993; Perdigon *et al*, 1990b). For example, in Perdigon's study, there were lower numbers of *S. typhimurium* in the spleen and liver of mice treated with *L. casei* and *L. acidophilus* when compared to the control mice (infected but not given probiotics) at 7 days post-challenge. The maintenance of liveweight observed in the *L. rhamnosus* fed mice suggests that feeding with *L. rhamnosus* enhanced the resistance of these mice to the infection. This was also demonstrated by the better general health appearance and lower occurrence of diarrhoea in these mice (personal communication with Dr Q.Shu, Milk and Health Research Centre).

Increased resistance of *L. rhamnosus* fed mice to *S. typhimurium* was accompanied by enhanced immune responses to *Salmonella* as mentioned earlier. It was found that there was increased *in vitro* antibody production by the PP and MLN lymphocytes of mice fed *L. rhamnosus*; increased B cell numbers and T helper cells in PP and T cell numbers in MLN. There was also significantly higher *in vitro* phagocytic activity by the peripheral blood leukocytes and peritoneal macrophages in the *L. rhamnosus* fed mice. The significantly enhanced phagocytic activity of the peripheral blood leukocytes and peritoneal macrophages observed in the *L. rhamnosus* fed mice was consistent with the observations of other investigators (Perdigon *et al,* 1986b; Perdigon *et al,* 1988; Perdigon *et al,* 1986a; Moineau *et al*, 1989; Paubert-Braquet *et al*, 1995). For example, Perdigon *et al* (1988) found that the *in vitro* phagocytic activity of peritoneal macrophages to *S. typhi* in mice fed LAB fermented milk was higher than that of the control mice. The increased specific and non-specific immune responses observed in these mice suggest that immunostimulation may play an important role in increasing the resistance of mice to *Salmonella* infection. Several studies have shown that increased specific and non-specific immune responses may play a role in protecting against bacterial infection (Nader de Macias *et al*, 1992; Perdigon *et al*, 1991a; Paubert-Braquet *et al*, 1995; Link-Amster *et al*, 1994). Further examples of the importance of the immune system in enhancing the resistance to *S. typhimurium* was provided by Carter & Collins (1974) when they found that there were low numbers of *Salmonella* in the liver and spleen of infected mice and suggested that some bacteria could be destroyed in the PP (Carter & Collins, 1974) and MLN by immunocompetent cells, before they could translocate to other organs. Marneerushapisal & Rowley (1981) suggested that in mice orally infected with *Salmonella* over a period of about 10 days that the Salmonella bacteria were destroyed in the PP because none were found in the spleen. If the bacteria were not destroyed in the PP, then it possible that immunocompetent cells such as phagocytes present in the spleen and liver could eliminate them.

The increased antibody production by PP lymphocytes in the *L. rhamnosus* + *Salmonella* treatment group was accompanied by an increased number of B cells in PP. The increased antibody production by MLN was however, not accompanied by increased B cell numbers. Rather, there was a lower number of B cells. The reason(s) for this is unclear but one possibility that could account for this is that there was an increased frequency of antigen-specific B cells which differentiate into plasma cells in the effector sites thus producing antibodies without a corresponding increase in cell numbers.

It was observed in this study that there were fewer PP along the intestine of the *Salmonella* infected mice compared to the control mice. It is likely that this could account for the low number of cells obtained from the PP of these mice. Another possibility would be that the antigen-sensitised lymphocytes had already left the PP and had entered the circulation to home to the LP and so not many remained in the PP at the time of sampling. The reduction in size of PP in the *Salmonella* treated mice would suggest that the infection caused the PP to somehow reduce their size so that they were not visible to the naked eye.

The reason(s) for this reduction in size is unclear and so far this observation has not been reported by other investigators. In a study by Jones *et al* (1994), they have shown that *Salmonella* initiates infection by penetrating the M cells of PP and destroying them. Figure 25 illustrates this process of how *Salmonella* invade the PP as observed by these authors. The bacteria ruffles the membrane of an M cell at its apical surface and was then taken up by the M cell (Figure 25a).

The M cells with the bacteria die thus forming a gap in the follicle-associated epithelium (FAE) (Figure 25b). Bacteria can now move freely into the basement membrane of the epithelium (Figure 25c). Once they are under the epithelial surface, they induce sloughing of the enterocytes and invade the lymphoid cells within the follicle dome. Perhaps this process contributed in some way(s) to the reduction in size of PP observed in this study.

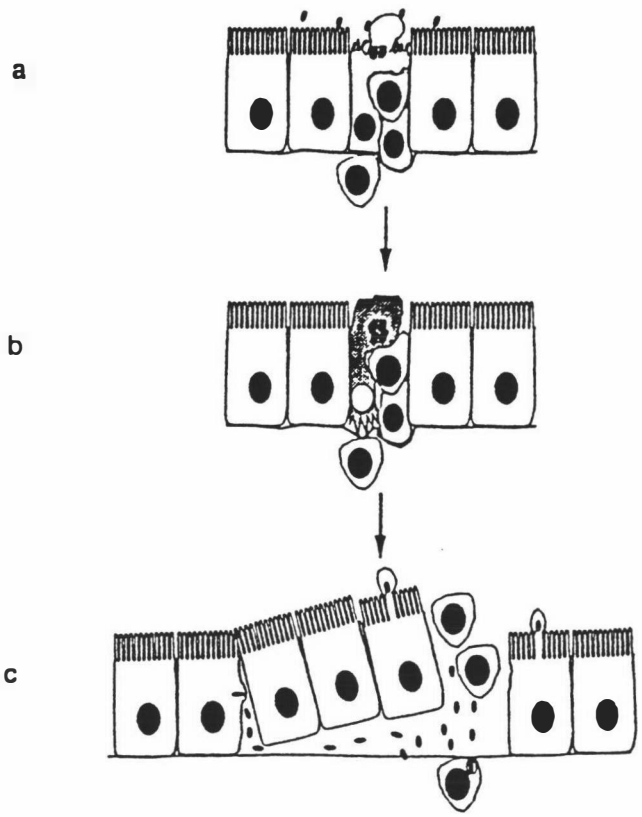

Figure 25 Diagram Illustrating the Process of Invasion of the PP of Mice by *S. typhimurium* (adapted from Jones *et al*, 1994)

Several mechanisms other than the stimulation of the immune system may also protect against enteric infection and these have already been mentioned in Chapter 1 and will only be briefly mentioned here.

One of the mechanisms is the production of antimicrobial substances such as lactic and acetic acids; H_2O_2, diacetyl and CO_2, and bacteriocins such as nisin, lactocidin, acidolin and reuterin (Mishra & Lambert, 1996). Other suggested mechanisms include the ability of LAB to adhere to the intestinal wall thus competing with pathogenic bacteria for adhesion receptors (Bernet *et al*, 1994), ability of LAB to compete with pathogenic bacteria for nutrients for growth and survival in the intestine (Reddy *et al*, 1988) and production of antitoxins (Mitchell & Kenworthy, 1976). However, the precise role and relative importance of these mechanisms (including immunostimulation) in mediating protection against enteric infection is still unknown and remains to be elucidated.

5.5 Summary

In summary, feeding with *L. rhamnosus* was found to enhance the resistance of mice to *S. typhimurium* infection as indicated by a lower number of bacteria translocating to the systemic organs and a maintenance of liveweight. The increased resistance was also accompanied by an increase in several aspects of the mucosal and systemic immune responses. These results suggest that the enhanced resistance to *S. typhimurium* by *L. rhamnosus* feeding could be due to the increased specific and non-specific immune responses.

Chapter 6

6.1 General Discussion and Conclusions

Probiotic bacteria refer to micro-organisms that exert beneficial effects on the host by improving the indigenous intestinal microbial balance (Richardson, 1996; Salminen *et al*, 1993; Havenaar & Huis In'T Veld, 1992). Lactic acid bacteria (LAB) are a group of Gram-positive anaerobic bacteria that have been used as probiotic bacteria. Various species of LAB have been used, for example, strains of *L. acidophilus*, *L. casei* strain GG, *L. rhamnosus*, *B. bifidum*, *S. thermophilus* and *E. faecium* (Tannock, 1997). Several beneficial claims have been made about LAB. These include protection against enteric infection, alleviation of lactose intolerance, lowering serum cholesterol, anticarcinogenic effects, improvement in the nutritional value of food and stimulation of the immune system (Salminen *et al*, 1993; Gorbach, 1990; Marteau & Rambaud, 1993; IDF, 1991; Hull, 1995; O'Sullivan *et al*, 1992). LAB's beneficial effect on lactose intolerance appears to be one of the best established effects of LAB. For other effects, there have been a mixture of positive and negative results and therefore further studies are required before any firm conclusions can be drawn (Sanders, 1993).

Many studies have been conducted over the last decade to examine the effect of LAB on the immune system (IDF, 1991 and Marteau & Rambaud, 1993). However, variations exist in the design of these studies thus making it difficult to compare the results, and to draw conclusions from them.

Studies that have shown positive results were discussed previously in Chapter 1. In relation to specific immune responses, LAB were found to stimulate the mucosal and systemic antibody responses in human subjects and mice (De Simone *et al*, 1991; Moineau & Goulet, 1991a; Perdigon *et al*, 1988; Yasui *et al*, 1989; Perdigon *et al*, 1990; Perdigon *et al*, 1991; Nader de Macias *et al*, 1992; Saucier *et al*, 1992; Majamaa *et al*, 1995; Kaila *et al*, 1992 and Link-Amster *et al*, 1994).

Studies by Solis Pereyra & Lemonnier (1993); De Simone *et al* (1986); De Simone *et al* (1988) and De Simone *et al* (1989) have shown that consumption of LAB enhanced cytokine production by human PBL's.

Of the non-specific immune responses, LAB have been found to increase the *in vitro* phagocytic activity of the peritoneal and pulmonary macrophages of mice and humans (Perdigon *et al*, 1988; De Simone *et al*, 1991; Perdigon *et al*, 1986; De Petrino *et al*, 1995; Moineau *et al*, 1989; Schiffrin *et al*, 1997; Moineau & Goulet, 1991b); increase lysosomal enzyme secretions, and the presence of lysosomic substances such as phagosomes and lysosomes (Perdigon *et al*, 1986b; Paubert-Braquet *et al*, 1995 and Torteuro *et al*, 1995); increase the *in vivo* phagocytic activity of the RE system by increasing the clearance of colloidal carbon (Perdigon *et al*, 1988; Perdigon & Alvarez, 1992; Perdigon *et al*, 1986a), and increase the NK cell activity *in vivo* and *in vitro* in human PBL's (De Simone *et al*, 1986; De Simone *et al*, 1988 and De Simone *et al*, 1989).

However, many questions / gaps still remain in our knowledge of LAB's effect on the immune system. For example, not all LAB have immunoenhancing abilities and different strains and species of LAB have different abilities to stimulate the immune system, as was shown by several investigators (Majamaa *et al,* 1995; De Simone *et al,* 1987; Perdigon *et al,* 1986; Schiffrin *et al,* 1995; Tortuero *et al*, 1995; Paubert-Braquet *et al,* 1995; Perdigon *et al,* 1990). Hence, there is a need to determine what strains or species of LAB have immunomodulatory properties and what contributes to the different immunoenhancing abilities of different strains and species of LAB so that we may better understand the mechanism(s) whereby different LAB enhance the immune system.

Other questions remaining to be answered include what is the most effective dose for different LAB in their immunostimulatory functions; the mechanism(s) by which LAB stimulates the immune system; what is the effect of fermentation on immunoenhancement by LAB; why and how do dead LAB stimulate the immune system; what is the host specificity of LAB, i.e. which LAB works best in which host (humans or animals), and in humans, what conditions (eg. healthy versus non-healthy) will result in immune enhancement by LAB feeding; what is the effect of LAB on the immune system of adults, the elderly and children and what is the effect of long-term

LAB consumption on the immune system of both the healthy and unhealthy (eg. immunocompromised), and the effect of dietary intake on immunoenhancement by LAB? (Richardson, 1996; Hamilton-Miller, 1996 and Perdigon & Alvarez, 1992).

Previous studies have identified an immunoenhancing LAB strain, *L. rhamnosus*. There were three aims in this dissertation. The first aim was to examine if the immunostimulatory effects of *L. rhamnosus* were dose-dependent (Chapter 3). It was found in this study that the enhancement of mucosal immune responses to the CT and OV antigens elicited by *L. rhamnosus* were dose-dependent and that not all immune parameters were enhanced when given *L. rhamnosus*, regardless of the dose. Mice fed at the dose level of 1×10^7 cfu showed increased *in vitro* antibody production and T cell numbers in PP to a greater degree than feeding at the dose level of 1×10^9 cfu. Feeding at the dose level of 1×10^9 cfu generated a greater number of B cells in PP and MLN than feeding at the dose level of 1×10^7 cfu. There was no increased proliferation of B cells in the PP of mice fed *L. rhamnosus* regardless of the dose but there was increased proliferation of T cells in the PP of mice fed with 1×10^7 or 1×10^9 cfu dose levels. Feeding at the dose level of 1×10^{11} cfu elicited the least response in almost all parameters. The responses elicited by feeding at the dose level of 1×10^9 cfu was found to be intermediate between that of the 1×10^7 and 1×10^{11} cfu dose levels. This study provides an additional example of the dose-dependency of LAB in immunostimulation and demonstrates that different doses of *L. rhamnosus* exert different degrees of immune enhancement. This may also apply for other LAB, thus it is important to determine the effective dose for immune enhancement for each LAB, as they may differ. The findings of this study are significant in that they have shown that feeding at different dose levels elicited different degrees of immunostimulation. Thus, if probiotic products with *L. rhamnosus* or other LAB are to be marketed to consumers, the appropriate dose for the immunostimulatory benefits of different LAB to humans must be ascertained. Consumers will need to be advised on the amount of the product to eat daily so that they are taking in the appropriate dose of bacteria for immunoenhancement. Furthermore, the effect of storage of probiotic products must be considered in relation to the dose of LAB in the product because with increasing shelf life of the product, the dose of viable LAB may decrease (Saxelin, 1996). The dose and efficacy of LAB in

different types of product, for example, in yoghurt, whey-based drink or sweet milk may also be different (Saxelin, 1996). Thus, choosing the right medium for administration of LAB is crucial in order to obtain the appropriate dose of LAB in these products to elicit immune enhancing effects.

The second aim was to examine the effect of viability on the immunostimulatory effects elicited by *L. rhamnosus* (Chapter 4). It was found that in most immune parameters except one, live *L. rhamnosus* showed a higher efficacy than the heat-killed form. The reason(s) for a higher efficacy of dead *L. rhamnosus* than the live form in the IgG production by PP lymphocytes in response to CT and the serum antibody response to OV is not known. Compared to mice fed heat-killed *L. rhamnosus*, there was increased B cell numbers in the PP and increased B and T cell numbers in the MLN, and mucosal antibody responses to CT and OV in mice fed live *L. rhamnosus*. It was also found that not all parameters were enhanced by *L. rhamnosus* feeding, regardless of whether it was the live or dead form. These results suggest that live *L. rhamnosus* was more effective than its dead form in stimulating certain components of the mucosal immune system. This finding is also significant because it shows that viability of LAB is important to the stimulation of the immune system. Thus in the production and marketing of probiotic products, viability of LAB in the products must be considered so that the benefits of consuming these products can be optimally obtained. However, it was also found in this second study that dead *L. rhamnosus* can stimulate certain immune parameters more effectively than the live bacteria. It is not known why and how this occurred, but this observation suggests that perhaps viability is not always required for LAB to elicit positive effects on the immune system. Hence, maintaining the viability of LAB in probiotic products may not be of prime importance as long as beneficial effects are still obtained. Furthermore, if viability of LAB is not necessarily needed for their beneficial effects, then the use of dead LAB would eliminate some of the problems encountered during storage and transport of probiotic products with live LAB, such as the need for refrigeration, and a shorter shelf-life of the product.

The third aim (Chapter 5) was to examine what role immunoenhancement played in the prevention of *S. typhimurium* infection. It was found that *L. rhamnosus* enhanced the resistance of mice to *S. typhimurium*, as shown by the inhibition of *Salmonella* from translocating to the liver and spleen, and the maintenance of liveweight. There were also increases in certain aspects of the mucosal and systemic immune responses to *S. typhimurium* such as the phagocytic activity of the peritoneal macrophages and peripheral blood leukocytes; *in vitro* antibody production by PP and MLN lymphocytes and the B cell numbers in PP. This suggests that the immune system may play an important role in increasing the resistance of these mice to *S. typhimurium.*

This study provides additional evidence of the role played by the enhanced immune system in protecting against enteric infection. Further studies with LAB that have positive effects on the immune system, using human subjects and farm animals could perhaps lead to these LAB being marketed as natural prophylactics against enteric infection, thus reducing the need for antibiotics. The use of antibiotics is the second most common method for treating enteric infections.

The results of the three studies presented here suggest that a range of immune parameters were enhanced by feeding with *L. rhamnosus.* Further, the immunostimulatory effects of *L. rhamnosus* were dose-dependent and the live form was more effective than the dead form. *Lactobacillus rhamnosus* was also effective in protecting against *S. typhimurium* infection and this might be due to the increased specific and non-specific immune responses observed. However, there are still gaps in our studies that warrant further investigations. For example, the effect of long-term feeding of *L. rhamnosus* on the immune system is not known. This information would aid in determining an appropriate duration of feeding for optimal stimulation of the immune system. This knowledge can also determine if long-term feeding of *L. rhamnosus* is safe for the host. It is also not known what the effect of *L. rhamnosus* feeding is on the immune system of humans, as human clinical trials have not yet been conducted.

The precise role and significance of the immune system and other antibacterial mechanisms (such as competition for adherence to the gut wall and production of antitoxins) in mediating the protective effects of *L. rhamnosus* against *S. typhimurium* infection is unknown, and needs to be examined in future studies. The protective effects of *L. rhamnosus* against other pathogenic bacteria, and viruses also needs to be examined to see if the anti-infection properties are effective for other microorganisms. We also need to examine why certain parameters were enhanced by *L. rhamnosus* feeding and not others, and why certain immune parameters were enhanced by dead *L. rhamnosus* more effectively than the live form. The answers to the above mentioned questions will be provided as future studies are conducted.

In conclusion, the present study showed the dose and viability-dependency of an immunoenhancing probiotic strain, *L. rhamnosus*. Live *L. rhamnosus* was found to be more effective than its dead form in enhancing certain aspects of the immune response. *Lactobacillus rhamnosus* can also protect against *S. typhimurium* infection and this was associated with the increased immune responses elicited by *L. rhamnosus*. These findings are important in that they help identify factors that can affect the immunostimulatory effects of LAB, thus providing information on immune enhancing LAB strains to the sectors involved in production and marketing of probiotic products, enabling specific product benefit claims to be made.

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