STUDIES ON THE PROTECTIVE ROLE OF PROBIOTICS AND MILK CALCIUM ON *SALMONELLA TYPHIMURIUM* INFECTION IN MICE

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ABSTRACT

The current studies were carried out to evaluate the efficacy of a newly identified LAB strain - DR10™ (*Bifidobacterium lactis*) on host immunity and susceptibility to *Salmonella typhimurium (S. typhimurium)* infection in mice. In addition, the effect of elevated milk calcium levels combined with DR10™ was studied in mice infected with *S. typhimurium*.

After initially establishing a murine infection model, the effect and efficacy of DR10™ in preventing *S. typhimurium* infection and stimulating immunity was examined. The results showed that DR10™ could significantly enhance resistance against *S. typhimurium* infection and stimulate a wide range of immune parameters including non-specific and specific immune responses. In another study the *S. typhimurium* infection model was used to examine the effect of milk calcium and the combination of different amounts of milk calcium with DR10™ on host immunity and prevention of *S. typhimurium* infection in mice. These results demonstrated that milk calcium was very effective in reducing the severity of infection and a high amount of milk calcium combined with DR10™ increased the ability of DR10™ to prevent *S. typhimurium* infection.

The findings of the current study were significant in that they demonstrated the effect and efficacy of DR10™ on promoting enhanced resistance to enteric infection and stimulating immunity, provided additional evidence of the role played by the enhanced immune system in protecting against enteric infection, and ascertained the synergism between milk calcium and LAB in the prevention of *S. typhimurium* infection.

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LIST OF ABBREVIATIONS

ACK: Ammonium chloride/potassium

ALCS: Appearance of lethal clinical symptoms

ANL: ANLENE

AKP: Alkaline phosphatase

CFU: Colony forming unit

ConA: Concanavalin

DTH: Delayed type-hypersensitivity

ELISA: Enzyme-linked Immunosorbent Assay

FITC: Fluorescein isothiocyanate

GALT: Gut-associated lymphoid tissue

GHAS: General Health Appearance Score

GIT: Gastrointestinal tract

HBSS: Hank's Balanced Salt Solution

HLA: Human leukocyte antigens

IBD: Inflammatory bowel disease

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IL: Interleukin

LD_{50}: Mean lethal dose

LFA: Leukocyte function-associated antigen

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

LAB: Lactic acid bacteria

NK: Natural killer

MHC: Major Histocompatibility Complex

MHRC: Milk and Health Research Centre

MLN: Mesenteric lymph nodes

PBL: Peripheral blood lymphocytes

PBS: Phosphate buffered saline

PFC: Plaque forming cell

PP: Peyer's Patches

PHA: Phytohemagglutinin

PMN: Polymorphonuclear

RT: Room temperature

Sal: *Salmonella*

SI: Stimulation Index

SIgA: Secretory IgA

SMP: Skim Milk Powder

SRBC: Sheep red blood cells

S. typhimurium : *Salmonella typhimurium*

T_H: Helper T cell

TNF: Tumor necrosis factor

VLA: Very late antigen

XLD: Xylose-lysine-deoxycholate

Chapter 1. Introduction: Overview of Anti-infection and Immune-stimulating properties of Lactic Acid Bacteria

1.1. An Introduction to the Probiotics

For centuries lactic acid bacteria (LAB) have been used in the preservation of human food for consumption. Consumption of several species of LAB, either through fermented dairy products or as live cells, has been associated with many health benefits in humans, ranging from improving well-being to increasing longevity (Barry, 1998). The observations of Metchnikoff in the early 1900s of long life spans in people who consumed yogurt regularly sparked future research (Trapp *et al.*, 1993). Scientific interest has increased in the past two decades, as researchers have studied the possible probiotic effects related to the consumption of fermented milks containing lactic acid bacteria (LAB).

The term " probiotics" was first used to refer to live microorganisms in supplement form that were fed to farm animals to stimulate growth and to improve resistance to stress (Lilley & Stillwell, 1965). Fuller (1992) defined a probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance". LAB do not necessarily need to adhere to or colonize in the gastrointestinal tract as long as they are regularly consumed (Ray, 1996).

Many species and strains of LAB from several genera have been credited with these health benefits due to their ability to produce different types of antibacterial compounds (Table 1.2). They are consumed through many types of products available commercially (Table1.1). Generally a product, depending upon the type, can contain one or more of the following species: *Streptococcus thermophilus, Lactococcus lactis, Leuconostoc mesenteroides, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus acidophilus*, and *Lactobacillus reuteri* and some *Bifidobacterium* species. In addition, some products can contain other *Lactobacillus*

species, some are even currently not regarded as a species (such as *Lactobacillus caucacicus* in some products sold in health food stores) (Savaiano *et al.*, 1984).

Although many health benefits of LAB have been suggested by many investigators, the results are not always conclusive. Difficulties in research are in part related to the fact that most health effects of LAB are strain-specific, and varying results may occur when using different strains of the same species or even different preparations of the same strain. There is also a large variability in the physiology of individuals, and large populations must be studied to provide valid results.

Table 1.1: Types of probiotics, desirable bacteria in the probiotics and antimicrobial metabolites produced by these bacteria (adapted from Ray, 1996)

Probiotics (predominant)	Bacteria (most commonly used)	Metabolites (in the products)
A. Fermented dairy products (yogurt, buttermilk, *acidophilus* milk, etc.)	*Lab. bulgaricus* *Str. thermophilus* *Lac. lactis* *Leu, mesenteroides* *Lab. acidophilus* *Lab. casei* *Bifidobacteria spp.* *Lab. reuteri*	Lactate, acetate, diacetyl, HCO_3, H_2O_2, bacteriocins, reuterine
B. Supplemented foods (pasteurized milk, drinks)	*Lab. acidophilus* *Bifidobacterium spp.* *Lab. bulgaricus* *Str. thermophilus*	Can supply ß-galactosidase. The indigenous types establish and produce metabolites.
C. Pharmaceuticals (tablets, capsules, granules)	*Lab. acidophilus* *Lab. bulgaricus* *Bifidobacteria spp.*	Can supply ß-galactosidase. The indigenous types establish and produce metabolites.
D. Health food products (liquid, capsules, powders)	*Lab. acidophilus* *Bifidobacterium spp.* (many other *Lactobacilli*, currently not recognized as species, are also used*)*	None, except in liquid fermented products. Can supply ß-galactosidase.

Table 1.2 Health benefits attributed to probiotics (adapted from Ray, 1996)

To control:	growth of indigenous microflora in the intestine
	infections in the intestine by enteric pathogens
	infections in the urinogenital tract
	lactose-intolerance
To reduce:	risk of cancer/tumour in colon (and other organs)
	serum cholesterol and the risk of cardiac heart disease
To stimulate:	immune systems
	bowel movement

This chapter reviews the uses of LAB ingested orally to protect against enteric infection and stimulate immune system.

1.2. LAB in protection against enteric infection and stimulation of immune system

1.2.1. LAB in protection against enteric infection

1.2.1.1. LAB in the prevention and treatment of diarrhoea

Diarrhoea is a common health problem that affects everyone sometime during his or her life. The cause of diarrhoea may vary, and the impact on health and well-being can range from slight discomfort to severe malnutrition and death. Infants and young children are particularly vulnerable, especially in developing countries where up to one billion episodes occur each year, with approximately four million deaths caused by diarrhoea (Brown, 1994).

Long before scientific research was conducted on the subject, empirical evidence suggested that fermented milks could help reduce the incidence and duration of diarrhoea. Fermented milks have been enjoyed for thousands of years, and have been associated with the prevention and treatment of diarrhoea for at least hundreds of years.

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Today, clinical research has provided scientific validation for the use of fermented milks and the LAB commonly used in them to combat diarrhoea. Milk products fermented with LAB may play a role in both the prevention and treatment of diarrhoea. Many studies have been performed to demonstrate their efficacy and determine the mechanisms involved (Table 1.3). Variables such as cause of diarrhoea, population affected, severity of the illness, strain and quantity of LAB used, and other experimental design factors often lead to varying results. The past fifteen years have shown, however, an overall inverse relationship between incidence or duration of certain types of diarrhoea and the intake of fermented milks (Sanders, 1994).

1.2.1.2. Possible mechanisms of LAB's anti-diarrhoea action

Besides the effect of the acidic pH in the fermented milk itself, some *in vitro* studies have shown inhibitory effects of some LAB on specific pathogens such as *Escherichia coli* (Black *et al.*, 1991, Coconnier *et al.*, 1993), *Salmonella typhimurium* and *Yersinia pseudotuberculosis* (Coconnier *et al.*, 1993; Bernet-Camard *et al.*, 1994), and *Shigella sonnei* (Apella *et al.*, 1992). Only live bacteria at a pH of around 4.5 were effective (Kotz *et al.*, 1990), but the effect was not caused by low pH alone. The antibacterial effect shown in these studies may prove useful in reducing the incidence of infantile or traveller's diarrhoea (Bernet-Camard *et al.*, 1994), where risk of exposure is high.

There are several possible ways in which fermented milks of LAB may influence the incidence and duration of diarrhoea once they are ingested. LAB can modify the activity or composition of the intestinal microflora and thus limit the invasion of pathogens (the "barrier effect"). In addition, the immune system itself may be reinforced by the action of the LAB, but this remains to be demonstrated (Boudraa *et al.*, 1990) (see the following section).

Not all the parameters responsible for fermented milk's anti-infection / immunomodulatory actions are known. Further research will provide a more precise

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understanding of the mechanisms involved. This will allow LAB to be selected which can perform a specific role within a particular infection regime.

Table 1.3. Principal studies on the efficacy of fermented milks or LAB used in fermented milks in the prevention and treatment of infantile diarrhoea.

SUBJECTS	EXPERIMENTAL DESIGN	RESULTS	REF.
49 healthy children at risk of diarrhoea aged 5-25 months (Argentina)	Ingestion of skim milk supplemented with unfermented milk (control) or milk fermented with *Lactobacillus casei* and *L. acidophilus*	Decrease in incidence of diarrhoea Increase in weight gain ($p<0.05$)	Gonzalez *et al.*, 1990
71 children aged 4-4.5 months recovering from acute diarrhoea (Finland)	Ingestion of fermented milk or freeze-dried powder; compared to heat-treated fermented milk (placebo)	Decrease in duration of acute *rotavirus* diarrhoea in fermented milk and freeze-dried powder groups	Isolari *et al.*, 1991
39 children aged 6-39 months with rotavirus gastroenteritis (Finland)	Ingestion of milk fermented with *L. casei*; compared to heat-treated fermented milk (placebo)	Decrease in duration of acute *rotavirus* diarrhoea ($p<0.05$)	Kaila *et al.*, 1992
55 infants aged 5-24 months (USA)	Ingestion of infant formula supplemented with *Bifidobacterium bifidum* and *Streptococcus thermophilus* for duration of hospital stay; compared to standard formula (control)	Decrease of incidence of acute diarrhoea ($p<0.05$)	Saavedra *et al.*, 1994
9 boys aged 7-29 months (Algeria)	Breath hydrogen measurement after lactose load in water, in milk, and in yogurt	Decrease of faecal output in 8 of 9 subjects with lactose intolerance	Dewit *et al.*, 1987
52 children aged 3-36 months (Algeria)	Ingestion of yogurt; compared to milk formula (control)	Decrease of clinical failure	Boudraa, *et al.*, 1990
78 infants aged 3-36 months (Algeria)	Ingestion of yogurt; compared to milk formula (control)	Increase of recovering % within 48 hours	Touhami, *et al.*, 1992

1.2.2. LAB in stimulation of the immune system

The effect of LAB on the modulation of the immune system is the centre of much ongoing research. Health benefits are associated with consumption of fermented milks: LAB and fermented milks have been found to modulate certain parameters of both the non-specific and specific immune responses. However, the link between these benefits and immune modulation has not been fully identified. In addition, the mechanisms involved remain to be further elucidated.

1.2.2.1. Overview of the immune system

The body has a number of defences against the massive invasion of foreign antigens. The nature of the body's response depends on both the type of foreign particles (e.g. viruses, bacteria, parasites, fungi, pollens, certain food proteins), and the route of entry (e.g. the skin, blood, the lungs, and the epithelium of the gastrointestinal tract). The first line of defence involves physical-chemical barriers such as the skin and the mucus layers, e.g., in the nose and intestines, which prevent some infectious diseases from occurring (Roitt, 1994).

The immune system represents the second line of defence against microorganisms, and its response involves a complex interaction of its various components. There are essentially three main parts involved in this response: recognition of the foreignness of the molecule, destruction of the foreign matter, and regulation of the response through multiple feedback controls (Roitt, 1994).

1.2.2.1.1. Non-specific immune system

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 are long-lived cells with significant rough-surfaced endoplasmic reticulum and mitochondria.

Polymorphonuclear phagocytes are short-lived cells with a multilobed nucleus and an array of granules and they include neutrophils, basophils and eosinophils. In addition, complement, a complex series of proteins in plasma, is also involved in the non-specific immune response. The non-specific immune system may act in the following ways (Roitt, 1994): 1) physiological barriers against infection, 2) phagocytic cells that kill microorganisms, 3) complements that facilitate phagocytosis, 4) the complement-mediated acute inflammatory reaction, 5) humoral factors as a second defensive strategy, and 6) extracellular killing.

1.2.2.1.2. Specific immune system

The specific immune response is mediated by lymphocytes. There are two subsets of lymphocytes, T and B cells. 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 (adult mammals) (Roitt, 1994). The T cells are differentiated in the thymus into different subpopulations, the T helper (T_H) or T cytotoxic-suppressor cells, with different markers to identify them: $CD4^+$ for T_H and $CD8^+$ for T cytotoxic or suppressor cells (Shaw, 1987). T_H can provide help to B cells for the production of T-dependent antibodies and plays a pivotal role in most types of immunity – especially in inflammatory cell-mediated responses. T_H can be loosely divided into two broad subsets, T_H1 and T_H2 based on the types of cytokines they secrete. T_H1 cells produce interferon γ (IFN-γ), interleukin 2 (IL-2) and lymphotoxin (tumor necrosis factor β, TNFβ), whereas T_H2 cells produce IL-4, IL-5, IL-6, IL-9 and IL-10. $CD4^+$ T lymphocytes can also be cytotoxic, but this function is primarily the responsibility of $CD8^+$ T lymphocytes. $CD8^+$ T lymphocytes also secrete various cytokines and have been loosely divided into T_C1 and T_C2 cells on the same basis as described for T_H1 and T_H2 cells. Induction of effective cytotoxic T lymphocyte responses may require "help" from $CD4^+$ T lymphocytes. (Heath, 1998).

B cells are differentiated in the bone marrow and consist of two populations; the first constitutes 15% of the population, is identified by a characteristic marker, $CD5^+$, and is found in the peritoneal cavity. The other population constitutes 85% of B cells and

is found in the blood and all organs of the immune system (Uytdehaag *et al.*, 1991). There is also a population of "null" cells, not-T non-B cells, the killer (K) and natural killer (NK) cells, have important functions in host defence against tumours (Roitt, 1994).

When a foreign antigen penetrates the body, the cellular interaction of the immune system is produced. This interaction can induce an immune response that is specific, nonspecific, or both. The first response against the antigen is the nonspecific immune response through an inflammatory response in which the phagocytic cells actively participate.

The inflammatory response is characterized by great cellular infiltration, which releases inflammatory mediators (Snyderman *et al.*, 1981). If prolonged, this response can damage the tissue. The first step in the inflammatory response is characterized by the appearance of neutrophils at the lesion site that fulfill their phagocytic function and release chemotactic substances to attract the macrophages that participate in the second step. Macrophages have a phagocytic function similar to that of neutrophils, but in addition antigen can be expressed on the macrophage membrane. Thus, the macrophage can present the antigen to T lymphocytes, which participate in the specific immune response. This presentation is realized through the association of the antigen with the special molecules that are present on their surface: histocompatibility antigens (Babbit *et al.*, 1985). There are two kinds to human leukocyte antigens (HLA): HLA class I are expressed in all cells of the body, and HLA class II are expressed on antigen presenting cells including epithelial cells (reviewed by Pan-yunting and Baldwin, 1993).

There is a complex interaction between cell-mediated and humoral responses. The specific immune response involves cellular immunity mediated by specifically sensitive immune cells and the humoral immune response mediated by antibody production. Cellular immunity has great importance in the host defence against all pathogens. Induction of an immune response needs not only cellular interaction but also cytokines and adhesion molecules. Cytokines are substances produced by

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immune or nonimmune cells when activated (e.g. by the antigen stimulation) (Miyajima *et al.*, 1992). Cytokines are known as the interleukins (ILs). Currently, more than 18 ILs are known; these IL are produced by different subsets of T_H lymphocytes, NK cells, macrophages, endothelial cells, epithelial cells, and fibroblast cells (Roitt, 1994). Other cytokines are the growth factors, such as granulocyte colony-stimulating factor; macrophage colony-stimulating factor; fibroblast growth factor; substances that promote the cellular differentiation of transforming growth factor; interferon-α, -β, or -γ; tumour necrosis factor (Phipps *et al.*, 1991).

Most cytokines carry out multiple functions, and immune cells selectively express specific membrane receptors for some of them, allowing the precise and intimate interaction of various cell types in a process that is now recognized as the " cytokine network" (Matsuura *et al.*, 1993).

The adhesion molecules on the surface of immune cells form connections between cells (van Seventer *et al.*, 1991) and are the second signals to obtain an immune response. Termed integrins or selectins, these molecules include intercellular adhesion molecule, leukocyte function-associated antigen (LFA1 and LFA2), and very late antigen (VLA1 and VLA2).

1.2.2.1.3. The mucosal Immune System

Unencapsulated mucosa-associated lymphoid tissues include lingual, palatine and pharyngeal tonsils, Peyer's patches, and the appendix. It contains many diffuse collections of lymphocytes, plasma cells and phagocytes which are not constrained by a connective tissue capsule (Roitt, 1994). It is generally agreed that it forms a separate interconnected secretory system within which cells committed to IgA or IgE synthesis may circulate (McGhee *et al.*, 1992). Many of these lymphocytes have large granular lymphocytes (LGL). The gut is also well endowed with its own variant of mast cell i.e. the mucosal mast cell.

In the gut, antigen uptake by M cells occurs in gut-associated lymphoid tissue and results in the initial activation of the antigen-sensitive lymphocytes. These cells leave via efferent lymphatics, migrate to mesenteric lymph nodes (MLN) and into the thoracic duct to the bloodstream. These migrating cells enter the IgA effector sites (lamina propria) where terminal differentiation, synthesis and transport of S-IgA occur, and protect wide areas of the bowel with S-IgA.

In addition to the organized lymphoid tissue that forms the mucosa-associated lymphoid tissue system, a large number of lymphocytes are found in the connective tissue of the lamina propria and within the epithelial layer of the gastrointestinal tract. Lamina propria lymphocytes (LPLs) are mainly activated T cells. However, large numbers of plasma cells and activated B cells also occur under normal physiological conditions. These plasma cells secrete predominantly IgA, which is transported across the epithelial cells and released into the lumen. Intraepithelial lymphocytes (IELs) reside between intestinal epithelial cells and the first cells to contact luminal antigen that cross the mucosa in an M cell-independent manner. Like LPLs, IELs are predominantly T cells. However, they display phenotypic features different from those of LPLs (Lydyard and Grossi, 1996). A large number of IELs are $\gamma\delta$ TCR$^+$ cells and most of them express CD8, which is rarely found on the majority of $\gamma\delta$ TCR$^+$ in the peripheral circulation. The expression of CD8 on $\gamma\delta$ TCR$^+$ has been related to their state of activation (Stites *et al.*, 1994).

T cells in LPL and IEL have almost exclusively the CD45R0 phenotypes that are typically found on memory cells. It is difficult to activate IEL and LPL with anti-CD3, but they may be stimulated via other activation pathways (e.g. via CD2 or CD28) (Lydyard and Grossi, 1996). IELs can produce cytokines including IFNγ and IL-5. They may also perform immune surveillance against mutated or virus-infected host cell (Lydyard and Grossi, 1996).

1.2.2.2. Immune Responses

1.2.2.2.1. Systemic Immune Response

When the antigen penetrates the body parenterally, a systemic immune response is produced:

1. The nonspecific immune response occurs through the inflammatory response with the active participation of the phagocytic cells [polymorphonuclear (**PMN**) and macrophages].

2. The processed antigen is expressed in the membrane of the antigen-presenting cell and shown to the lymphocytes through the HLA class I or class II pathway. The HLA class I molecules generally present cytoplasmic antigens (endogenous antigens) that are primarily synthesized by the infected cell, for example, viral proteins. In contrast, general HLA class II molecules primarily present peptides derived from exogenous antigens, for example, those internalized by the antigen-presenting cells from the extracellular medium [e.g., soluble protein antigen, or antigen derived from microorganisms] (Neefjes *et al.* 1993).

3. In the specific immune response, if the antigen is associated to HLA class I, a cytotoxic cellular response is obtained through cytotoxic T lymphocyte (TL), which has the marker $CD8^+$. If the antigen is associated to HLA class II, a humoral response with antibodies is obtained through the helper T lymphocyte, which has the marker $CD4^+$. This last population cooperates with B lymphocytes (BL), which become plasma cells, producing any of the five classes of Ig (IgM, IgG, IgA, IgE, and IgD). There is a complex interaction between cell-mediated and humoral responses.

1.2.2.2.2. Secretory Immune Response

The gastrointestinal tract encounters a myriad of microorganisms that enter through the mouth. A first non-specific defence to pathogens is provided by the barrier effect of the gastrointestinal digestive juices, the intestinal flora, and the intestinal mucus layers, all of which prevent microorganisms from entering the body (Roitt, 1994).

In addition, the gut has a specialized mucosa-associated lymphoid system called the gut-associated lymphoid tissue (GALT) which interacts continually with the general immune system. It is composed of organized lymphoid tissue in the ileum (Peyer's patches) as well as a large variety of immune cells (B and T lymphocytes, plasma cells, macrophages, mast cells, eosinophils, and basophils) which infiltrate the gastrointestinal mucosa. The principal antibody synthesized by local plasma cells is secretory IgA (sIgA) which is found in other body secretions as well, such as in saliva, mucus, colostrum, and tears (Kraehenbuhl *et al.*, 1992).

If the antigen penetrates by oral route, the first immune response that occurs is oral tolerance, through the intraepithelial lymphocytes that carry the $CD8^+$ marker. However, oral tolerance can be abrogated, and then an immune response is produced. The stimulation of one response or the other depends on the physical state of the antigen; thus, in general, soluble antigens induce oral tolerance, and particulated antigens produce an immune response that is mainly a humoral response; the number of IgA-producing cells and the synthesis of secretory IgA (sIgA) increase. Although all of the immune cells are present at the mucosa, the cytotoxic cellular response at the mucosa is limited by immunoregulatory mechanisms to avoid intestinal damage.

The events of the secretory immune response are the following. Luminal antigen is transported into Peyer's patch through M cells of the follicle-associated epithelium and presented to T cells associated with HLA class II molecules on dendritic cells or macrophages. Antigen can also be presented by B cells to T cells. Primed T and B cells migrate through to the peripheral blood circulation and extravasation mainly

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occurs in the gut lamina propria and in other exocrine tissue. Intestinal B cells differentiate to plasma cells producing IgA and CD8$^+$ T cells migrate into the epithelium to mediate oral tolerance to food antigens (McGhee *et al.*, 1992).

1.2.2.3 Effect of lactic acid bacteria (LAB) on the immune response

1.2.2.3.1. Effect of LAB on the non-specific immune response

The effect of LAB consumption on non-specific immune response has been the subject of many experimental studies. The non-specific immune response can be measured via phagocyte function and NK cell activity. Phagocyte functions include phagocytosis of microorganisms or foreign particles, production of reactive oxygen and nitrogen species, lysosomal enzymes and monokines, and *in vivo* clearance of colloidal carbon in experimental animals or humans (Gill, 1998).

Several studies have shown that LAB enhance the *in vitro* phagocytic activity of the peritoneal, blood and pulmonary macrophages and the *in vivo* colloidal carbon clearance in animals (Perdigon *et al.*, 1986a & 1986b; 1988; 1992; de Simone *et al.*, 1991; Moineau *et al.*, 1989; de Petrino *et al.*, 1995; Paubert-Braquet *et al.*, 1995; Gill, 1998). In the study by Perdigon *et al.*, (1988), it was found that *in vitro* phagocytosis of peritoneal macrophages from mice treated with milk fermented with *Lactobacillus casei* or *Lactobacillus acidophilus* and a mixture of both bacteria showed a peak on the 2nd day of administration, with values three to four-fold higher than controls, and maintained values of 50% macrophage-phagocytosing bacteria until the 8th day (in mice fed with *L. casei*-fermented milk, a peak was reached on the 3rd day, with values three-fold higher than controls up to the 8th day; in the feeding with *L. acidophilus*-fermented milk, the values obtained were three times higher than controls from the 2nd day onwards). They also found that there was an increase in the phagocytic function of reticuloendothelial (RE) system cells of mice fed these fermented milks compared to the control mice by the method of *in vivo* clearance of colloidal carbon. Using same method, Moineau *et al.*, (1989) also found that there was a significantly higher phagocytic index of the pulmonary alveolar

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macrophages in the *L. acidophilus* and *L. casei* fed mice compared to the control mice fed unfermented milk.

Similar results have been found in human subjects. In the study by Schiffrin *et al.* (1995), 28 healthy adults aged 23 to 62 years drank milk fermented with either *B. bifidum* (1×10^{10} cfu/d) or *L. acidophilus* (7×10^{10} cfu/d). The increase of phagocytic activity of blood leukocytes was found and this was coincidental with colonisation by LAB. Granulocytes showed a greater increment in phagocytosis than monocytes. The increase in phagocytic activity persisted for 6 weeks after the cessation of consumption of fermented milk.

The peritoneal macrophage activity was quantified by assaying lysosomal and non-lysosomal enzymes: β-Glucuronidase lactate dehydrogenase, and β-Galactosidase activity in Perdigon *et al.*, (1988)'s study. In mice fed with *L. casei*-fermented milk, β-Galactosidase enzymatic activity was two to three-fold higher than in the controls, and lactate dehydrogenase three to six times higher. But β-Glucuronidase remained at values close to those controls. In addition, LAB has been found to be able to enhance the reactive oxygen production (Sato *et al.*, 1988), nitrogen species and monokines by phagocytic cells (Gill, 1998). Mikes (1995) also found a significant increase in the ability of neutrophils from human subjects given *Enterococcus faecium* for 6 weeks, to produce oxygen radicals following incubation with zymosan or phorbol myristate acetate.

The effect of LAB consumption on NK cell activity has been studied in only a few cases. There is some evidence to indicate that some strains of LAB are also able to influence the activities of NK cells. De Simone *et al.*, (1993) found increase of NK cell activity in the peripheral blood of healthy adults aged 20-47 years ingesting lyophilized dietary *lactobacilli* (3×10^{12} cfu/d) and 200 g of plain yogurt at 24 h intervals for 28 days. Similar results have been found in another study (De Simone *et al.*, 1986). It has been found that there was increased NK cell activity *in vitro* in human peripheral blood lymphocytes (PBL) costimulated with concanavalin (ConA) and LAB. The potentiating effect was attributed to the interferon-γ present in the

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culture supernatants. In addition, oral administration of milk-based diets containing some LAB strains has been found to be able to enhance NK cell activity of mouse spleen cells against tumour cells (Gill, 1998).

The effect of LAB on the non-specific immune response is strain-dependent and may be dose-dependent. *Lactobacillius casei* were more effective at enhancing the secretion of lysosomal enzymes in macrophages than *L acidophilus* and *Streptococcus thermophilus* (Perdigon *et al.*, 1992). Mice fed with *S. thermophilus* also had a lower ability to clear colloidal carbon than mice receiving *L. acidophilus*, *L. casei* and *Lactobacillus delbrukii* subsp. *bulgaricus* (Perdigon *et al.*, 1986; Perdigon and Alvarez, 1992). The different efficacy may be due to structural differences in the cell wall composition of different LAB strains (Gill, 1998). Furthermore, strains that are able to survive in the gastrointestinal tract, adhere to the gut mucosa and persist above a critical level are more efficient at stimulating immune responses (Perdigon and Alvarez 1992; Schiffirn *et al.*, 1995). Gill (1998) also found the magnitude of response depends on the dose of LAB. Mice receiving a milk-based diet containing 10^{11} cfu/day of *L. rhamnosus HN001* for 10 days showed significantly greater phagocytic activity than mice receiving 10^9 or 10^7 LAB.

1.2.2.3.2. Effect on Specific Immune Response

Many studies have demonstrated that LAB are able to enhance the several aspects of humoral and cell-mediated immunity (Table 1.4 & 1.5). For example, Perdigon *et al.*, (1988) found that the serum antibody titre to sheep red blood cells (SRBC) in the entire LAB fermented milk fed groups were higher than the control group. All treatment groups also showed higher IgM plaque forming cell (PFC) numbers in the spleen compared to the control group. In the study by De Simone *et al.*, (1987), there was a significantly higher percentage of B cells in the Peyer's Patches (PP), and increased proliferative responses of PP to phytohaemagglutinin (PHA) (a T lymphocyte mitogen) and lipopolysaccharide (LPS) (a B lymphocyte mitogen) in mice fed with viable *lactobacilli*-containing yogurt compared to the control group. Vesely *et al.*, (1985) also found similar results.

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In some human studies, LAB have also been demonstrated to be able to induce cytokine production by human peripheral blood lymphocytes (Solis *et al.*, 1993; De Simone *et al.*, 1986 & 1988), and IL-1β, TNF-α and IFN-γ production in blood mononuclear cells (Solis *et al.*, 1993). In addition, an *in vitro* study by De Simone *et al.*, (1988) found increased IFN-γ release and increased NK cell activity in human PBL costimulated with Con-A and LAB. An *in vivo* study by the same authors (De Simone *et al.*, 1989) with yogurt also showed similar results.

Table 1.4. Examples of Studies on the Effect of LAB ingestion on Specific Immune Responses in Experimental Animals (Adapted partially from Gill, 1998).

Dietary treatment	Effect	Reference
Mice fed with *L. casei*, then challenge with *S. typhimurium*.	↑ IgA-producing cells. ↑ in phagocytic activity of macrophages.	Perdigon *et al.*, (1995a)
Mice fed conventional diet plus yogurt supplements.	↑ IgA-secreting B cells.	Perdigon *et al.*, (1994)
Mice fed with viable LAB or yogurt, then implanted with fibrosarcoma.	↑ some kinds of immune cells	Perdigon *et al.*, (1993)
Mice fed with *L. casei* and injected with tumor cells (before or after *L. casei* feeding)	↑ phagocytosis. ↑ inhibition of tumour growth, both preventively and therapeutically.	Perdigon *et al.*, (1993)
Mice fed milk fermented with LAB and challenged with *S. typhimurium*	↑ survival ↑anti-*S.typhimurium* antibody response in serum and intestinal fluid.	Perdigon *et al.*, (1990)
Mice fed milk fermented with LAB and inoculated with sheep red blood cells (SRBC)	↑ IgM plaque-forming cells against SRBC ↑ anti-SRBC circulating antibodies	Perdigon *et al.*, (1988)
Mice fed with *L. casei* fermented milk, then vaccinated against *Vibrio cholerae*.	↑ specific antibody responses in the serum.	Portier *et al.*, (1993)
Mice fed with a lyzozyme lysate from *L. bulgaricus*, then injected with *Klebsiella pneumoniae* or *Listeria monocytogenes*.	↑ spreading ability of macrophages. ↑ phagocytosis. ↑ IL-1 production. ↓ mortality.	Popova *et al.*, (1993)
Normal and immunosuppressed mice given several strains of LAB and infected with *Candida albicans*	↑ PFC and DTH responses and protection in immunosuppressed mice	De. Petrion *et al.*, (1995)
Mice fed with *B. longum* or *L. acidophilus*.	↑ lymphocyte proliferative response.	Takahasi *et al.*, (1993)
Mice fed live cultures or heat-treated LAB.	↑lymphocyte proliferation ↑% of Thy 1.2 cells in spleen	Vesely *et al.*, (1985)
Mice fed *B. breve*	↑ anti-*B. breve* antibody levels	Yasui *et al.*, (1989)
Mice fed *B. breve* and immunized with cholera toxin	↑ IgA production, ↑ anti-*cholera* IgA antibody levels.	Yasui & Ohwaki (1991)
Mice fed yogurt and *Kefir* and inoculated with tumour cells	↑ delayed-type hypersensitivity (DTH) ↑ in plaque-forming cells (PFC) in the spleen	Furukaa *et al.*, (1991)
Mice fed milk fermented with LAB and challenged with *Shigella sonnei*	↑ serum anti-*Sh. sonnei* antibody concentration in serum and intestinal fluid	Nader de Macias, *et al.*, (1992)

Table 1.5. Examples of Studies on the Effect of LAB Ingestion on Specific Immune Responses in Humans (Adapted partially from Gill, 1998).

Dietary treatment	Effect	Reference
Ex vivo incubation of human blood mononuclear cells with several LAB	↑ production of IL-1, α, β TNF, IFN-γ	Solis *et al.*, (1993)
Adults with bladder cancer ingested viable *L. casei*.	↑ 50 % recurrence-free ↑ production of TNF-α.	Aso *et al.*, (1992)
Children with gastroenteritis ingested milk fermented with *L. casei*.	↑ IgA, IgG, IgM	Kaila *et al.*, (1992)
Infants ingested of freeze-dried *L. casei* and given rotavirus vaccine.	↑ *rotavirus*-specific IgM-secreting cells ↑ *rotavirus* IgA seroconversion	Isolauri *et al.*, (1995)
Healthy adults ingested milk fermented with LAB then administered with *S. typhimurium* oral vaccine.	↑ specific serum IgA antibody ↑ total serum IgA.	Link-Amster *et al.*, (1994)
Healthy adults ingested of lyophilized dietary *lactobacilli*.	↑ IFN-γ, B lymphocytes, and natural killer cells.	De Simone *et al.*, (1993)
Healthy adults ingested lyophilized *B. bifidum* and *L. acidophilus*.	↑ colonic inflammatory infiltration. ↑ B cell frequency in the peripheral blood. ↑ TNF-α	De Simone, *et al.*, (1992)
Healthy elderly subjects given LAB.	↑ Frequency of B cells in the peripheral blood. ↑ Colonic inflammatory infiltration	De Simone *et al.*, (1991)
Patients with Dukes A colorectal cancer given *L. casei shirota*	↑ % Helper T cells. ↓ % Suppressor T cells	Sawamura *et al.*, (1994)
Children with acute rotavirus gastroenteritis given LAB.	↓ Duration of diarrhoea ↑ *Rotavirus*-specific IgA secreting cells ↑ Serum IgA antibody	Majamaa, *et al.*, (1995)

1.2.2.3.3. Effect of LAB on immune-mediated anti-infection properties

Consumption of LAB cultures has been shown to augment specific immune responses to enteric pathogens and bacterial, tumour and viral vaccines given orally (Table 1.4 & 1.5). Perdigon *et al.,* (1990) fed Swiss albino mice with milk fermented with *L. casei* and *L. acidophilus* separately or in combination (2.4×10^9 cfu/d for 8 days), then challenged with *Salmonella typhimurium*. They found the highest level of anti-*S. typhimurium* antibodies in intestinal fluid for the *L. casei* group, the highest circulating antibodies in serum and 100% survival for *L. casei* + *L. acidophilus* group compared to 20% survival in the control group. In another study by Perdigon, *et al.* (1990), similar results have been found. Nader de Macias *et al.* (1992) also found feeding mice with *L. casei* and *L. acidophilus* could increase anti-*Sh. sonnei* antibody concentration in serum and intestinal fluid and survival (100% vs 40% in 3 weeks). In a study by Popova *et al.,* (1993), mice fed with a lyzozyme lysate isolated from *L. bulgaricus* then challenged with *Klebsiella pneumoniae* or *Listeria monocytogenes* have been found to have lower mortality and higher spreading ability of macrophages, phagocytosis of *E. coli,* and IL-1 production compared to the control mice. An increase in specific mucosal and serum antibody responses in children with acute *rotavirus* diarrhoea following administration of *Lactobacillus* GG has also been observed (Kaila *et al.*, 1992, Majamaa *et al.*, 1995). Feeding mice with *L. casei* fermented milk then vaccinating those mice against *Vibrio cholerae* increased specific antibody responses in the serum (Portier *et al.*, 1993). In humans, ingestion of *L. acidophilus* has been found to be able to significantly enhance the effectiveness of oral *Salmonella typhimurium* and *rotavirus* vaccines (Link-Amster *et al.*, 1994, Isolauri *et al.*, 1995). In a study by Perdigon *et al.,* (1993), mice were fed with viable *L. casei*, *L. acidophilus* at 1.2×10^9 cfu/d for 2, 5, or 7 days, then implanted with tumour cells (using sterile nonfat milk as control). They found that *L. casei* could increase the number of plasma cells, lymphocytes, and macrophages; *L. acidophilus* could increase the number of plasma cells, lymphocytes, IgA-producing cells, IgG-producing cells.

LAB have also been found to enhance immunity in animal models of immunosuppression and subjects with tumours. Perdigon *et al.,* (1995b) used *L. casei* to treat malnourished mice, which are very vulnerable to enteric infections. They found that *L. casei* could increase the number of IgA-producing cells, circulating leukocytes, IgA, and IgM in the intestinal fluid of malnourished mice. Administration of *L.casei* and *L. bulgaricus* to corticoid-treated (immunosuppressed) mice increased delayed type-hypersensitivity responses to higher levels than those observed in non-immunosuppressed controls, which demonstrated that LAB feeding could restore cellular immune function in immunocompromised hosts. Perdigon *et al.,* (1995b) fed mice with yogurt then induced an intestinal tumour with 1-2-dimethylhydrazine. They found that yogurt feeding inhibited the development of the intestinal carcinoma with an increase of IgA-producing cells and T lymphocytes in the large intestine. Sawamura *et al.,* (1994) also demonstrated that ingestion of *L. casei* in patients with Dukes A colorectal cancer could increase the percentage of Helper T cells and decrease the percentage of suppressor T cells.

1.2.2.4. The mechanisms by which LAB stimulate the immune system and protect against enteric pathogens

So far, the mechanisms by which LAB are able to gain access to the immune system and exert their immunostimulatory effects are not clear (Gill, 1998). LAB or their products may be taken up by M cells and transported to the underlying lymphoid follicles where they are subjected to immunological scrutiny by immunocompetent cells, or are transported to the systemic lymphoid tissues such as mesenteric lymph nodes or the spleen for immunological analysis (Gill, 1998). The results of some studies support these mechanisms. Adherence and uptake of LAB by M cells of the PP in mice orally fed with LAB has been found in a study by Claassen *et al.,* (1995). LAB were observed in the dome area of PP after 6-12 h and in the mesenteric lymph nodes 48 h after ingestion. In addition, Steffen and Berg (1983) also reported that bacterial translocation can occur in situations where certain indigenous bacteria exceed a particular threshold.

LAB may act directly on the intestinal epithelium which contains a large number of lymphocytes that are able to secrete a variety of cytokines and influence the local immunoregulatory environment to stimulate an immune reaction (Gill, 1998). Furthermore, LAB or their products may access the body via non-specific and receptor-mediated mechanisms. It has been reported that some LAB are able to adhere to intestinal epithelial cells *in vitro* and to the intestinal mucosa *in vivo* (Johansson *et al.*, 1993, Schiffrin *et al.*, 1997). Thus, the interactions between LAB or their products and the immunocompetent cells, such as macrophages and T cells, may result in the secretion of a variety of cytokines that are known to have a multitude of effects on both immune and non-immune cells (Nussler & Thomson, 1992). The effects may include: activation of T and B lymphocytes, phagocytes, complements and cytokine production (Table 1.4 & 1.5). The mechanisms by which LAB induce cytokine secretion are not yet clear. There may be two possible mechanisms (Gill, 1998). First, cytokine secretion may be triggered following antigen (derived from LAB) presentation by antigen presenting cells to T cells. Second, it may result from a direct interaction between LAB or their cell wall components and the immunocompetent cells. The receptors for a cell wall component of LAB wall (peptidoglycan) have been found on lymphocytes and macrophages (Dziarski, 1991). The ability of peptidoglycan to induce secretion of IL-1, IL-6 and TNF-α by monocytes and IFN-γ by lymphocytes has been reported (Bhakadi *et al.,* 1991; Tufano *et al.*, 1991; Heumanm *et al.*, 1994). Although the physiological significance of LAB- induced cytokine secretion to human health remains unclear (Gill, 1998), cytokines have been demonstrated to be able to directly or indirectly augment a range of host protective responses (Nussler & Thomson, 1992). For example, IFNs may inhibit viral replication, induce expression of class I and II MHC antigens, stimulate helper-T cell function, activate macrophages, and augment the immunogenicity of vaccines (Murray, 1988). IL-1 stimulates activation of T and B cells. IL-6 induces differentiation of B cells to antibody-secreting plasma cells and TNF-α exerts cytotoxic effects on tumour cells (Gill, 1998).

In addition to the mechanisms by which LAB enhance host immunity previously discussed, secretion of antibacterial or inhibitory substances may also contribute to

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preventing bacterial infection. Mishra and Lambert (1996) suggested that the production of antimicrobial substances such as lactic and acetic acids, H_2O_2, diacytyl and CO_2, and bacteriocins such as nisin, lactocidin and reuterin, might play some role in this process. These substances employ several mechanisms to destroy pathogens and their toxins. For instance, H_2O_2 may inhibit pathogens by its production of the toxic reactive O_2 species such as O_2^- and (hydroxyl radical) OH^- (Hollang *et al*, 1987). Bernet-Camard *et al*. (1997) also demonstrated that *L. acidophilus* Strain LA1 developed an antibacterial activity in germ-free mouse models orally infected by *S. typhimurium*. The culture supernatant of this strain contained antibacterial components against a wide range of gram-negative and gram-positive pathogens. Other proposed mechanisms include; the ability of LAB to adhere to the intestinal wall thus, competing with enteric pathogens for adhesion receptors (Bernet *et al*., 1994), the ability of LAB to compete with pathogenic bacteria for nutrients for growth and survival in the intestine (Reddy *et al*., 1988), and the production of antitoxins (Mitchell & Kenworthy, 1976).

1.3. The Future directions in research

From the discussion presented above, based on experimental evidence, there are many strong reasons that suggest that some species and strains of LAB may have beneficial health effects in humans and animals. These include: improving lactase deficiency, reducing serum cholesterol level, combating chronic kidney diseases, improving the nutritional value and quality of food, reducing risk of colon cancer, protecting against enteric infection and diarrhoea, and stimulating the immune system. This highlights the opportunities for dairy and health food industries to develop novel, value-added probiotic products. However, there are some inconsistencies in the results that could be due to the methods used in the studies. Thus, the proper designing of experiments and analysis and interpretation of results is essential in the future research. In addition, it has been found that the LAB differ greatly in their ability to produce a specific benefit in the intestine of humans and animals, especially for the stimulation of the immune system. This may be due to the following factors (Gill, 1998):

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- A large variation exists in the ability of LAB to impact on the immune system.
- The effect of LAB on the immune system is dose-dependent with a higher intake of LAB resulting is a superior response compared with a lower intake.
- Live cultures are more efficient at enhancing certain aspects of immune function than killed cultures.
- LAB delivered in fermented products induce a superior response compared to cultures given in unfermented products. Thus, the species and strains of LAB, the dose, preparation of cultures, and the routines of delivery must be considered in the future study. Furthermore, there are many significant gaps remaining in our knowledge, especially for the mechanisms by which LAB work to produce beneficial effects in our body. Therefore, Gill (1998) suggested that future studies should be directed at:
- Demonstrating the efficacy of LAB in target population groups using randomized, double-blind, placebo-controlled studies.
- Demonstrating the relevance of immunomodulation to better health.
- Defining the effective dose for each strain.
- Defining situations in which consumption of LAB would result in the desired health benefit.
- Elucidating the mechanisms by which LAB act on the immune system.
- Studying the effectiveness and safety of long-term consumption of LAB.
- Identifying new strains that are able to inhabit desired anatomical sites in the gut and modulate desired immune functions.

1.4 Aims of the Research

The efficacy of LAB in stimulating the immune system and exerting anti-infection differs greatly among different species and strains. In order to evaluate this efficacy, a proper animal model to study infectious processes will be required. In addition, there is some evidence that milk calcium is able to increase host defence against bacterial infections. In rats, milk calcium inhibited the gastrointestinal survival, colonisation, and translocation to extra-intestinal organs of some pathogens. The

probable mechanisms for this action could be that milk calcium stimulates protective bacteria such as LAB, and strengthens the mucosal barrier in the small intestine (Van der Meer, *et al.*, 1998). Thus, an interesting question arises: is there any synergism between those two in protecting against enteric infection and stimulating immunity?

In our laboratory, a newly identified strain of LAB, DR10™ (*Bifidobacterium lactis* HN019, has been found to be able to enhance natural and acquired immunity in both human and animal subjects (Gill, *et al* 2000; Arunachalam, *et al*, 2000). However, the anti-infection and immunity enhancement properties of the strain have not been fully studied so far. The aims of this thesis are to answer the above question and to evaluate the effect and efficacy of DR10™ on stimulating immunity and anti-infection. Three trials will be carried out. The first trial is going to establish a bacteria-infected murine model. That is: to determine the suitable challenge dose of *Salmonella typhimurium* which can cause appearance of clinical symptoms of infection in mice but let mice live long enough to study immune status. The second trial will evaluate the efficacy of DR10™ in stimulating immunity and resisting enteric infection. The last one is going to study the possible synergism between DR10™ and milk calcium in protecting against *Salmonella* infection.

Chapter 2. Materials and Methods

2.1. Animals and Animal Ethics Approval

Male BALB/c mice aged 6-7 weeks and weighing 15-20 g were obtained and kept at the small animal production unit (SAPU), Massey University, Palmerston North. They were housed individually in metal cages at temperature of 22± 2 °C, with a 12 hour light/dark cycle. Food and water were available *ad libitum*. The animal ethics consent of this study was obtained from the Massey University Animal Ethics Committee (Protocol No: 98/74).

2.2. *Salmonella typhimurium*

Salmonella typhimurium ATCC 1772 was used.

2.3. Preparation and enumeration of *Salmonella typhimurium*

Salmonella typhimurium was subcultured from an original plate into a blood agar plate and incubated (37°C) for 24 hours. This procedure was repeated twice. The *Salmonella typhimurium* was then subcultured from blood agar into 5ml BHI broth and incubated (37°C) for 18 hours. The BHI broth was transferred into 2ml eppendorf tubes and centrifuged (Sorvall MC 12V, Dupont) at 2650g for 10 minutes. The supernatant was discarded and the cell pellets were resuspended in 0.5ml PBS. All the suspended culture in the eppendorf tubes was then transferred into a 10ml tube. 0.4ml of the suspended culture was placed in a spectrophotometer (U-2001 UV/VIS, HITACHI) to measure the absorbance at 686nm wavelength, using PBS as the blank solution. The concentration of suspended culture was established using a standard curve. PBS was used to adjust the concentration of suspended culture to the desired concentration. (The dose rate should be 25µl bacteria suspension/mouse. If 1×10^7 cfu of bacteria was needed, the desired concentration should be: 1×10^7 cfu/25 µl = 4×10^8/ml).

2.4. Determination of Standard Curve

A *S. typhimurium* culture suspension was made using 10 serial 1:2 dilutions (the suspension was prepared by the method stated above). The absorbance of each dilution was measured by spectrophotometer (U-2001 UV/VIS, HITACHI) at 686nm wavelength. Meanwhile, the bacteria count of each dilution was determined by the plate count method. A standard curve was obtained by plotting the absorbance of each dilution against its corresponding plate count.

2.5. Identification of *Salmonella typhimurium*

Salmonella typhimurium was grown in MacConkey Agar (pale/opaque colonies) then identified by xylose-lysine-deoxycholate agar (XLD) and modified Brilliant Green Agar (personal communication with Dr. Stan Fenwick). MacConkey Agar is a selective and differential medium containing lactose, bile salts and neutral red indicator. Lactose – fermenting bacteria produce acids, which by reacting with the indicator result in the colonies being coloured red or pink. Other bacteria either do not ferment lactose, and therefore produce pale, non-pink colonies, or do not grow on the medium at all because of the presence of bile salts. The inhibitory action of crystal violet in MacConkey Agar on the growth of gram-positive organisms allows for the isolation of gram-negative bacteria. Incorporation of the carbohydrate lactose, bile salts, and the pH indicator neutral red permits differentiation of enteric bacteria on the basis of their ability to ferment lactose. *Salmonella typhimurium* are not lactose fermenters and therefore do not produce acid. XLD agar is a selective and differential medium designed for the isolation and presumptive identification of both *Salmonella* and *Shigella*. The medium relies on xylose fermentation, lysine decarboxylation and the production of hydrogen sulphide for the differentiation of these genera from non-pathogenic bacteria. Phenol red indicator is incorporated in the medium to detect acid production. Rapid xylose fermentation is a feature of most enteric bacteria, however *Shigella* is an exception, and can be identified by a negative reaction i.e. red (alkaline) colonies. *Salmonella* species are differentiated from xylose fermenters by the incorporation of lysine into the medium. *Salmonellae*

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exhaust xylose and decarboxylate lysine, altering the pH to alkaline and mimicking the *Shigella* reaction. Lactose and sucrose, added to the medium in excess, prevent the lysine-positive coliform from similarly altering the pH to alkaline. Modified brilliant green agar has some inhibitory substances and thus usually only support growth of *S. typhimurium*. The colonies of *Salmonella typhimurium* were colorless (pale/opaque) in MacConkey Agar, pink to red with black center on XLD, and pink-white opaque surrounded by brilliant red medium in modified brilliant green agar.

2.6. The Scoring System of General Health Appearance

Score 1(healthy): Mouse bright-eyed and alert, has a smooth coat with sheen, responds to stimulus, shows interest in its environment.

Score 2 (slightly sick): Fur slightly ruffled a loss of sheen to the coat, mouse remains alert and active.

Score 3 (sick): Fur noticeably ruffed, parts of coat forms clumps, mouse not as alert or active, less interested in its environment outside of cage, signs of hyperventilating when handled.

Score 4 (very sick): Mouse hunched over and sleepy, little interest shown in environment, fur clumped.

Score 5 (dying): Mouse non-reactive to stimulus, fur has a 'bottle brush' appearance, i.e., standing on end, mouse hunched over preferring to sleep than react to environment, mouse cold to touch, paws are cold to touch.

2.7. Challenge of *Salmonella typhimurium*

Mice were orally challenged with *S. typhimurium* ATCC 1772 suspension in 25µl PBS.

2.8. Feeding of Probiotic Bacteria and Measuring the Feed and Water Intake

Freeze dried powder of DR10™ (*Bifidobacterium lactis*) from the New Zealand Dairy Research Institute (NZDRI) was ground with a mortar and pestle. It was then

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well mixed into either Skim Milk Powder (SMP) or ANLENE (high calcium milk powder) to a concentration of 2×10^8 cfu/gram. The diets were then left in a large metal diet mixer for 20 minutes to obtain even distribution of DR10™. These diets were kept in airtight containers in the freezer. Each DR10™ treatment group was fed *ad libitum* with either a SMP or ANLENE based diet. Feed intake and water intake were recorded and calculated daily.

2.9. Preparation of lipoteichoic acid (LTA) from crude *S. typhimurium* LPS.

This protocol was adapted from Cappuccino *et al.*, (1996). Freshly harvested cells from an autoclaved culture of *S. typhimurium* were suspended in 0.1M sodium acetate buffer (pH=4.5) (Thick slurry ~ 700 mg wet weight/ml buffer). Then a 2:1 volume mixture of methanol and chloroform was added into this suspended bacterial culture and left at room temperature for 3 hours. Bacteria were recovered by filtration or centrifugation. This procedure was repeated twice. Recovered cells were washed with methanol (about two times the cell volume) and resuspended in 0.1 M acetate buffer (pH=5.0). The suspension of the cells was mixed with an equal volume of hot 80% w/v aqueous phenol. The phenol solution was stirred constantly for 45 minutes in water bath at 65 °C. On cooling an emulsion formed, which was broken into two phases by centrifugation at 4130g (Heraeus Sepatech Megafuge 1.0R) for 30 minutes (4 °C). The upper aqueous phase (containing LTA) was collected. Phenol was removed from aqueous phase by dialysis against 0.1M acetate buffer at pH 5.0. Six changes of buffer were needed for total of 24h.

2.10. Preparation of Spleen Lymphocytes

This protocol was a modification of that described by Coligan *et al.*, (1994a). Spleens were removed aseptically from the mice and placed in a 24 well plate (Nunc, USA) containing 2 ml GI media (containing 2% Fetal Bovine Serum, 2mM L-glutamine, 50µM 2-ME, 100u/ml penicillin, 100µg/ml streptomycin sulfate, purchased from GIBCO Bethesda Research Laboratories, Life Technologies Ltd.,

USA) per well. The spleens then were transferred into another 24 well plate and washed. The spleens were cut or pulled into small pieces using scissors or forceps (forceps were wiped with alcohol wipes between each sample). The resulting suspensions were then sucked up and down in a 1ml syringe to break up the tissue, and transferred to labeled 15ml tubes containing 5ml GI. The tubes were centrifuged for 10 minutes at 325g (Heraeus Sepatech Megafuge 1.0R), and the supernatant was discarded by tipping. The cell pellets were then resuspended in 5ml ACK lysis buffer (ammonium chloride/potassium, containing 0.15M NH_4CL, 1mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.3~7.4) using a transfer pipette and incubated for 5 minutes at room temperature (RT) with occasional mixing. Another 5ml GI was added into the tubes. The tubes were centrifuged for 10 minutes at 325g (Heraeus Sepatech Megafuge 1.0R), and the supernatant discarded by tipping. The pellets were resuspended in 5ml GI again and centrifuged for another 10 minutes at 325g (Heraeus Sepatech Megafuge 1.0R). Again, the supernatant was discarded and the pellets were resuspended in 5 ml GI. Then 1ml of cell suspension was taken and placed into tubes containing 4 ml GI (made 1/5 dilution). The small amount of cell suspension from the 1/5 dilution tubes was transferred into labeled flow cytometer tubes and counted on the flow cytometer (FACSCalibur™, Becton Dickinson Immunocytometry Systems, San Jose, California, USA). The cell concentration was adjusted to 2×10^6/ml.

2.11. Preparation of Lymphocytes from Mesenteric Lymph Nodes (MLN)

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, 2mM L-glutamine, 50μM 2-mercaptoethanol, 100U/ml penicillin and 100μg/ml streptomycin sulphate, purchased from GIBCO Bethesda Research Laboratories, Life Technologies Ltd., USA) in a 24 well plate (Nunc, USA). All subsequent steps were also carried aseptically. The lymph nodes were cut into small pieces using scissors/forceps. They were then mechanically dissociated by being sucked 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 325g, and the supernatants discarded. The pellets were resuspended in 5ml ACK lysis buffer and incubated for five minutes at room temperature with occasional mixing. Following this step an additional 5ml of RPMI-1640 was added into each tube. The samples were centrifuged again for 10 minutes at 325g. 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.

2.12. Preparation of Lymphocytes from Peyer's Patches (PP)

This protocol was a modification of that described by Coligan *et al.*, (1991b). The digesta in the small intestine of each mouse was flushed out with 2ml of PBS or 0.66 mM 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 by being sucked up and down with 1ml syringes and then the homogenized 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 15ml centrifuge tubes.

An additional 5ml of media was added to the 15ml centrifuge tubes and the samples were 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 325g. The supernatants were removed and the pellets resuspended in 2ml of CFM/HEPES. The preparation of PP lymphocytes was performed in a sterile environment.

2.13. Preparation of Serum

Approximately 1ml of blood was drawn from each mouse by cardiac puncture and put into 2ml eppendorf tubes. Blood samples were left at room temperature for 2 hours after which the samples were centrifuged (Sorvall MC 12V, Dupont) at 1950g for 10 minutes. The serum was then carefully removed from the blood clot, placed in another tube and stored at -20°C for ELISA antibody assay.

2.14. Preparation of Intestinal Contents

The small intestine (from stomach pylorus 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 1295g for 10 minutes after which the supernatants were removed and placed in another tube. The samples were kept at -20°C for ELISA antibody assay.

2.15. 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: 5-bromo-2'-deoxyuridine [colourimetric], Cat No: 1647229). 50 μl of the prepared lymphocytes (from MLN and PP, 2×10^6 cells/ml) from each pair of mice were aliquoted into six wells of a 96 well plate (Nunclon MicroWell plate). 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 well". 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 labeling reagent 5-bromo-2'-deoxyuridine (BrdU). 10μl of BrdU labeling solution was added to each well and the plates incubated at 37°C for an additional 16 hours. The plates were centrifuged at 325g (Heraeus Sepatech Megafuge 1.0R) for 10 minutes. The labeling medium was then

removed and the wells dried with a hairdryer for 15 minutes. 200μl of Fix Denat was added to the wells and incubated for 30 minutes at the room temperature (RT) after which Fix Denat was removed and 100μl 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 were washed 3 times in phosphate buffered saline (PBS). The washing solution was removed and 100μl of substrate solution was added to each 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 was added into each well and the plates were shaken for about 30 seconds on a shaker. The plates were then read on an ELISA plate reader (KC3/CERES 900C, Bio-Tek Instruments, Inc., USA) at 450nm. Stimulation index (SI) was used as 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= absorbance of cells with mitogen/absorbance of cells without mitogen.

2.16. Immunophenotyping Assay

This assay was adapted from Lloyd *et al.*, (1995) and Nicholson *et al.*, (1984). The methods for preparation of MLN and PP lymphocytes were as described in Section 2.11. and 2.12. 100μl of each cell suspension of PP or MLN was aliquoted into two 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). 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 980g (Sorvall MC 12V, Dupont) 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 sample

mixed thoroughly. The samples were then analyzed by flow cytometry (FACSCalibur™, Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

2.17. Enzyme-linked Immunosorbent Assay (ELISA) for detecting both mucosal antibody (IgA) and serum whole antibodies

This protocol for this assay was a modification of that described by Coligan *et al.* (1991c). Both standard curve and antibody titre methods were used to detect antibody levels. For the standard curve method, 96 well plates (Nunc Immuno plates, Nunc, GIBCO BRL, Life Technologies LTD, NZ) were coated with 100μl/well of 1μg/ml lipoteichoic acid (LTA, derived from crude *S. typhimurium* LPS) in carbonate coating buffer (1.59g Na_2CO_3 and 2.93g $NaHCO_3$ in 1 liter MQ water, pH=9.6) and incubated overnight at 4°C. Plates were removed the following day and washed three times with PBS/Tween. 100μl of samples (for intestinal fluid, undiluted and for serum, 1/25 dilution in sample buffer [95ml PBS, 5 ml Fetal Calf Serum]) and standard serum or intestinal fluid (serum detected with whole antibodies or intestinal fluid detected with mucosal antibody [IgA] from previous trials were used as standards, eight serial doubling dilutions of standards were used) were aliquoted into wells in pairs, and the plates were covered and incubated overnight at 4°C. Next day, the plates were removed and washed three times with PBS/Tween. Sheep anti- mouse whole Ig conjugated to alkaline phosphatase (AKP) (for serum antibody assay, diluted 1/500 in sample buffer) (Silenus brand, purchased from Amrad Operations P/L, Australia. Catalogue No: 985034010) or sheep anti-mouse IgA conjugated to AKP (for mucosal antibody assay, diluted 1/500 in sample buffer, purchased from Serotec, UK. Catalogue No: 138660-17) was added to the wells and the plates were incubated for 1 hour at 37 °C. They were then washed three times 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.

The steps in the antibody titre method were almost the same as these in standard curve method, except that the serum and gut samples were diluted two-fold sequentially in the sample buffer down the plate, giving eight dilutions. Each dilution of the samples was set up in triplicate on the plates with each well having 100μl diluted sample. The ELISA end point (titre) was defined as the highest dilution that provided OD value approximately 0.25.

2.18. Preparation of Peritoneal Macrophages

This method was an adaptation of that described by Coligan *et al.* (1994b). Following euthanasia, each mouse was pinned down and its abdomen sprayed with ethanol before its skin was retracted without breaking the peritoneal cavity. 8-10ml 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 approximately 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 325g (Heraeus Sepatech Megafuge 1.0R) for 10 minutes at 4°C. The supernatant was removed using sterile transfer pipettes. The pellet was resuspended in 10 ml of RPMI-1640 after which it was centrifuged at 325g 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 1×10^6 macrophages/ml with RPMI-1640.

2.19. Phagocytosis by Peripheral Blood Leukocytes

This method was an adaptation of that described in the PHAGOTEST® test kit (Orpegen Pharma, Germany). 100μl of whole blood which had been collected into

Vacutainers containing EDTA was transferred to a 2ml glass tube (no anticoagulant, VACUTAINER® blood collection tubes, Becton Dickinson & Co., NJ, USA) which had previously been aliquoted with 10µl of FITC (Fluorescein iothiocyanate) labeled *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 1950g for 10 minutes. The supernatants were discarded and 0.5ml of PBS was added to the pellet.

The resuspension was thoroughly mixed and transferred into FACS tubes (Falcon® 6ml 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 was analyzed by flow cytometer (FACSCalibur™, Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

The principle underlying this technique is that bacteria (*E. coli*) are labelled with fluorescein isothiocyanate (FITC) and then phagocytosed by macrophages (personal communication with Dr. Kay Rutherfurd). Following this, a second dye (trypan blue) which binds DNA, is added. It only stains dead cells. Thus, only the *E. coli that* are dead are stained with trypan blue, and this quenches the fluorescence of the unphagocytosed particles. Fluorescence from *E. coli* which have been phagocytosed by macrophage, are not quenched because the macrophage is alive and therefore the trypan blue can not get inside the cell. On the flow cytometer, light scatter can be used to isolate macrophage populations. In the assay, the macrophage population is gated and the level of fluorescence coming from this population is detected. Thus, phagocytic activity of macrophages is obtained by measuring the percentage of the cells that are gated. This principle also applies to the technique for assaying phagocytosis by peritoneal macrophages (see Section 2.20 below).

2.20. Phagocytosis by Peritoneal Macrophages

The peritoneal macrophages were prepared as described in Section 2.18. The method measuring the phagocytic activity is adapted from that described in Section 2.19. 10μl of FITC-*E. coli* was added to 100μl of macrophages (1×10^6 macrophages/ml) to 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 analyzed by flow cytometer (FACSCalibur™, Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

2.21. Bacterial Translocation Assay

The method was as described by Perdigon *et al.,* (1990). All steps were carried out aseptically with sterile instruments and equipment. Spleens and livers were aseptically removed from mice and were homogenized in 5 ml of 0.1% peptone water (final volume) with a Teflon homogeniser. Each sample was made 3 serial 1:10 dilutions. Each dilution was made in triplicate and then plated on MacConkey agar plates. Bacterial colonies were enumerated after incubation for 24-48 hours at 37°C. The plates with 10 to 60 colonies were selected for enumeration. *Salmonella typhimurium* was identified as described in Section 2.5.

2.22. NK Cell Activity Assay

The flow cytometry method of Johann *et al.,* (1995) was used to assess NK cell activity. YAC-1 cells (target cells) were labeled at 1×10^6 cells/ml with the labeling reagent D275 (3,3'-Dioctadecyloxacarbocyanine Perchilorate) (1μl of D275 for every ml of cells) overnight in the 37°C incubator. Spleen lymphocytes were processed as for the cell proliferation assay (as described in Section 2.10.). At the step where diluted samples of cells were added to flow cytometry tubes, 1μl of

5mg/ml propidium iodide was also added and incubated at 37 °C for 10 minutes. The spleen lymphocytes were counted on a flow cytometer to find their concentrations. The YAC-1 target cells were washed twice in RPMI (325g for 10min) (Heraeus Sepatech Megafuge 1.0R) and the pellet was resuspended to give a cell concentration of 1×10^6 cells/ml. Samples of spleen lymphocytes were set up in duplicate on a 96 well cytotoxicity plate (N163320, purchased from Nunc, GIBCO BRL, Life Technologies LTD, NZ) with each well having 1×10^6 cells. Then 25 μl (containing 25000 cells) of target cells were added to each well to obtain the optimum ratio of effector: target cells - 40:1. The total volume of each well was made up to 200μl with RPMI media. Blank wells were prepared by the same procedure except only 1×10^6 effector cells or 25000 target cells were added. The plate was then spun at 325g (Heraeus Sepatech Megafuge 1.0R) for 1 minute and incubated at 37 °C for 3 hours. 3μl of propidium iodide was added to each well 15 minutes before the end of the incubation. At the end of the incubation, samples and blanks were transferred to flow cytometer tubes containing 350μl of PBS and then counted on the flow cytometer.

The principle underlying this technique is similar to that of phagocytosis assay of macrophages (personal communication with Dr. Kay Rutherfurd). Target cells (YAC–1) are labeled with a green fluorescent dye (D275). Propidium iodide is added and stains dead cells red. On the flow cytometer, the target cell population is gated to get a quadrant plot (i.e. a plot divided into 4 squares) with FL1 (green) on the X axis and FL2 (red) on the Y axis. Live target cells are labeled green and will appear in the lower right quadrant of the plot. However, when killed by effector cells (NK cells), they are doubly labeled red and green and will appear in the upper right quadrant of the plot. Thus, a shift in the cells from the lower right quadrant to the upper right quadrant, which means the percentage of the killed target cells, gives the % cytotoxic activity (NK cell activity).

2.23. Faecal Colonization Assay

At 8am on day 0 (just before *Salmonella* challenge) and on day 1, 5, 12, 21 post-*Salmonella* challenge, fresh individual faecal samples of six mice in each group were collected. A 0.5g (wet weight) sample of faeces from each mouse was placed in 9 ml of sterile PBS, pH=7.3, and thoroughly mixed by vortexing at the maximal setting. Then each faecal homogenate was made into 3 serial 1:10 dilutions in peptone water. Dilutions were then plated on MacConkey agar plates. Bacterial colonies were enumerated after incubation for 24 hours at 37°C. The plates with 10 to 60 colonies were selected for enumeration. *Salmonella typhimurium* was identified as described in Section 2.5.

2.24. Statistical Analysis of Results

The results were expressed as mean ± standard error of the mean (SE). Students't test and Duncan's new multiple range test in SAS (SAS® Analysis of Variance Produces) were used to determine the significant differences between groups (α=0.05). Chi-square was used to test the difference in survival rate between different groups. Correlation analysis was preformed by Correlation program in Analysis ToolPak in Microsoft Excel 97.

Chapter 3. Dose of *S. typhimurium* required for mice to show clinical / subclinical infection

3.1 Introduction

Salmonella infection is the one of the most common, food-borne, bacterial infections in the USA and Europe (Rampling, 1993). Most patients experience a self-limiting gastroenteritis, but 2-5% develop a systemic infection with bacteraemia and sepsis-associated complications (Blaser and Feldman, 1981). This is because *Salmonella* is an invasive pathogen, which may translocate to the Peyer's patches via the epithelium from the distal small intestine. Antibiotic treatment of salmonellosis has been discouraged by clinicians, since it does not reduce the duration or severity of illness and may prolong asymptotic carriage (Jewes, 1987). Considering this phenomenon, combined with the growing resistance of *Salmonella* species to clinically important antibiotics (Lee *et al.,* 1994), finding new methods to prevent and treat it becomes more and more important. LAB are likely to be our novel arms in fighting salmonellosis. Several strains of LAB have been found to be able to prevent *Salmonella* infection in some studies (Link-Amster *et al.*, 1994; Nader de Macias *et al.*, 1992; Perdigon *et al.*, 1990 and 1995). However, the efficacy of such effects varies greatly among different species and strains. Thus, to evaluate the efficacy of LAB, a suitable *Salmonella* infection model is needed.

A suitable animal bacterial infection model is one that can cause infection while allowing the animals to live long enough to measure both bacterial translocation and some of the immune response parameters. Carter *et al.,* (1974) found that it took 4 days for *Salmonella* to translocate into spleen and liver in the mice challenged with a mean lethal dose of *Salmonella enteritidis*. Perdigon, *et al.,* (1990) also observed that the viable bacteria in the liver and spleen appeared on day 4 and reached the highest level on day 7 post-challenge in mice fed with LAB and challenged with *Salmonella typhimurium*. They also found that these mice produced the highest level of anti-*Salmonella* antibody after 7 days post-challenge. It has also been reported

that it takes about one week for specific defenses such as antibodies to appear (Van der Meer *et al.*, 1998). Thus, if the challenge dose of bacteria is too high, animals will die in a very short time and the effect of LAB on preventing *Salmonella* translocation and stimulating immunity cannot be properly studied. On the other hand, if the dose is too low, the animals may not get sick or only have a slight infection.

Mean lethal dose (LD_{50}) is an important parameter in measurement of the toxicity of drugs and bacteria. It is determined by giving various doses of the drug or bacteria to groups of animals. Ordinarily only a single dose is given to each animal. The percentage of animals dying in each group within a selected period is plotted against the dose to get a dose-response curve. From this curve the dose that kills 50% of the animals is estimated and is referred to as the LD_{50}. Usually, this curve is S-shaped (Figure 3.1). The slopes of the two ends of this curve are flat, which means that changes in the high or low dose range do not cause much change in mortality. However, the slope in the middle of this curve is steep, which indicates that a small change of the dose in this range would lead to large change in mortality, especially in the part around LD_{50}. Therefore, LD_{50} is a very sensitive index in measuring the toxicity. Furthermore, it is also a very stable index. When repeating the dose-response experiments under the same condition, we may get several different curves but we always obtain almost all the same LD_{50} (Suqin, 1990). Based on the reasons above, LD_{50} has been chosen as a major reference to decide the dose of challenge in many studies (Perdigon *et al.*, 1990; Sato, 1984; Collins & Carter, 1978; Soila *et al.*, 1997).

The LD_{50} s (oral) of *Salmonella* infection in mice vary greatly in these studies, ranging from 2×10^3 to 1×10^7 cfu (Table 3.1). The LD_{50} of the strain of *Salmonella typhimurium* (ATCC 1772), has not been reported yet. In order to establish a bacteria-infected murine model, it is important to measure its LD_{50}. However, since the ethic approval of this trial only allows mice to be observed before they die (mice have to be euthanased when they reach General Health Appearance Score (GHAS)

of 5, which means the mice are dying), the Mean Dose to cause 50 % of the mice to reach GHAS of 5 will be measured.
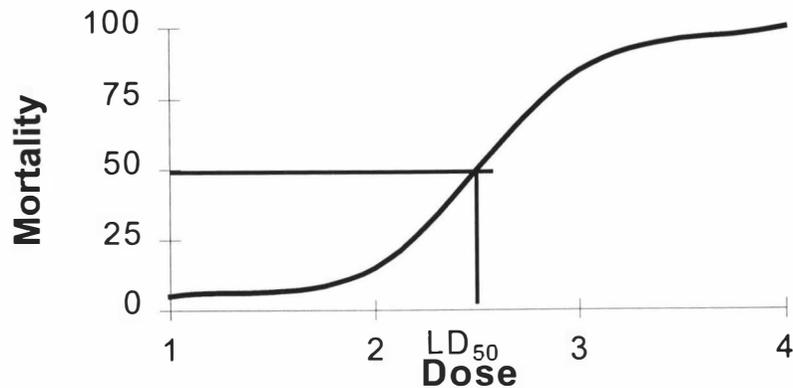
Figure 3.1: A typical Dose-Response curve of mean lethal dose experiment.

Table 3.1: The results of some studies about LD_{50} of *Salmonella* on mice

Bacteria	Mice	Duration	LD_{50}	Reference
S.typhimurium (Stm 754)	Female BALB/c mice	Not indicated	9×10^6 cfu	George, 1996.
S.dublin S11363	BALB/c mice	Not indicated	1×10^6 cfu	Kincy-Cain*et al.*, 1996.
S.choleraesuis (SL 2822)	BALB/c or Ct7B216 mice	Not indicated	1×10^6 cfu	Ndubisi *et al.*, 1989.
S.enteritidis 5694(wide-type)	CD-1 mice B6D2 mice	Not indicated	2×10^6 cfu 5×10^3 cfu	Carter *et al.*, 1974.
S.typhimurium Sr-11 Fli-8007 Mol-8008	BALB/c mice	Not indicated	2.4×10^4cfu 4.5×10^4 cfu 4.5×10^4 cfu	Hank *et al.*, 1990.
S.typhimurium X4252 fim⁺fla+ X 4252fim⁺-301 X4252fim⁺-401 X4308fim⁺-309	BALB/c mice	30 days	4.2×10^4 cfu 2.3×10^4 cfu 3.3×10^4 cfu $>>10^7$ cfu	Hank *et al.*, 1992
S.typhimurium 2000	BALB/c mice	14 days	3.8×10^5 cfu	Ramarathinam *et al.*, 1991.
S.typhimurium	Germfree mice Conventional mice	28 days	$> 5 \times 10^9$ cfu $> 5 \times 10^9$ cfu	Collins & Carter 1978
S.typhimurium X 3181 X 4666 X 4665	BALB/c mice	Not indicated	8×10^3 cfu 2×10^3 cfu 1×10^5 cfu	Soila *et al.*, 1997.
S. typhimurium	Swiss albino mice	Not indicated	1×10^6 cfu	Perdigon *et al.*, 1991.

3.2. Experimental Design

There were two steps in this trial.

1. To determine the standard curve in a turbidimetric assay (as described in Chapter 2).

2. To determine the dose of *S. typhimurium* required to induce subclinical / clinical symptoms in the animals.

One hundred (6-7 week old) male BALB/c mice were housed individually with a 12 hour light/dark cycle and a constant temperature ($22\pm2^{o}C$) in the Small Animal Production Unit (SAPU), Massey University. After 2 weeks acclimatization on a Skim Milk Powder (SMP) based diet (fed *ad libitum*), eighty mice were selected on the basis of liveweight and feed intake, and randomized into 4 treatment groups (n=20) (Table 3.2).

Table 3.2: Experimental design for Mean Dose Study

Group	Dose of *Salmonella typhimurium* (cfu)
1	1×10^8
2	1×10^7
3	1×10^6
4	1×10^5

Each experimental group was orally challenged with a different dose of *S. typhimurium* (Table 3.2). The experiment continued for another 3 weeks. During this period, the clinical signs were carefully monitored and recorded three times per day. Mice were withdrawn from the experiment if the General Health Appearance Score (GHAS) reached 5. The withdrawn animals were euthanased by isofluorance overdose. Based on this trial, a dose inducing subclinical / clinical symptoms in the animals would be chosen as a reference of dose for further study.

3.3 Results

3.3.1 Standard Curve

There was very good linear relationship between absorbance and bacterial count in the range between 1×10^7 to 1×10^{10} cfu/ml. All the data points were very close to the curve. The R (correlation coefficient) value between absorbance and bacteria count was 0.9952, indicating a strong linear relationship. Outside this range, the linear relationship was not very good (the curve isn't shown).
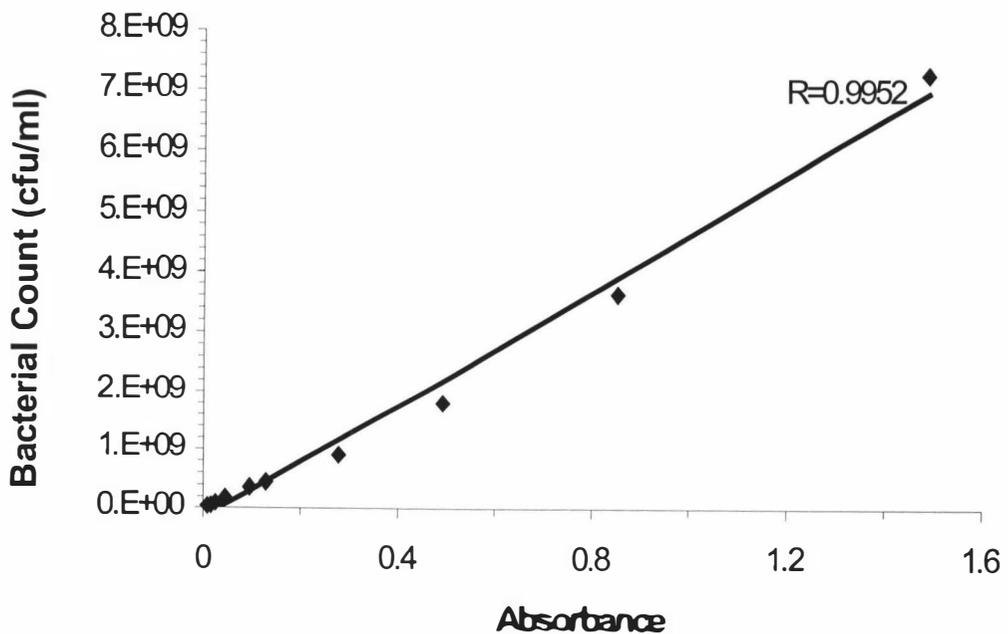
Figure 3.2: The standard curve using the turbidimetric assay.

3.3.2 Percentage of Survival in 3 Weeks

All mice in the highest dose group (1×10^8 cfu of *S. typhimurium*) reached GHAS of 5 within first 2 weeks. Most of this happened between day 6 to day 9 post-challenge. In the group challenged with 1×10^7 cfu of *S. typhimurium,* 14 mice achieved GHAS of 5 between day 7 and day 14. Only 2 mice reached such score (day 14 and day 18

post-challenge) in group 3 (1×10^6 cfu of *S. typhimurium*). All the mice except one survived in the group challenged with 1×10^5 cfu of *S. typhimurium*.
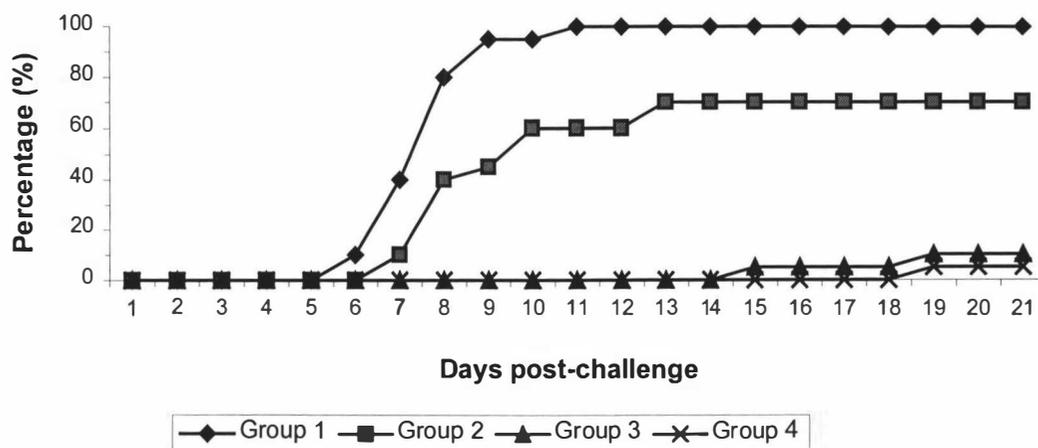
Figure 3.3. Rates of appearance of lethal clinical symptoms post-challenge in the mice (n=20).

3.3.3 Calculation of Mean Dose to cause 50 % of the mice to show lethal clinical symptoms:

Karber's method was used to calculate this dose (Suqin, 1990).

lg Mean Dose $= X_m - (d/2) \Sigma (p_i + p_{i+1})$

Here: X_m is the lg of maximum dose, d is the difference of lg of doses between the adjacent groups. P is the percentages of mice to reach GHAS of 5 in each group. Therefore, $X_m = lg 10^8 = 8$. d = 1,

Group	p	$p_i + p_{i+1}$
1×10^5	0.05	0.15
1×10^6	0.1	0.8
1×10^7	0.7	1.7
1×10^8	1	$\Sigma (p_i + p_{i+1}) = 2.65$

$\therefore$ lg Mean Dose $= 8 - (1/2) \times 2.65 = 6.675$

Mean Dose $= 10^{6.675} = 4466836 = 4.47 \times 10^6$.

3.3.4. The General Health Appearance Score (GHAS)
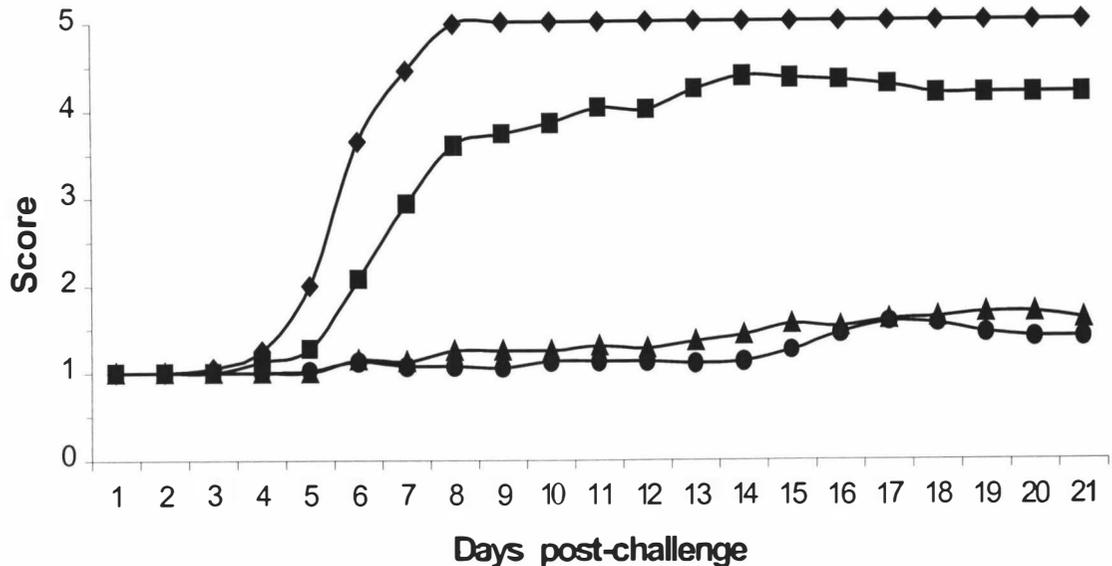
Figure 3.4: General Health Appearance Score of four groups of mice within 21 days post-challenge

- ($\blacklozenge$) group 1 (10^8); ($\blacksquare$) group 2 (10^7); ($\blacktriangle$) group 3 (10^6); ($\bullet$) group 4 (10^5).

In group 1 (1×10^8 cfu) and group 2 (1×10^7 cfu), GHAS changed drastically from score 1 to score 5 from day 4 to day 9 post-challenge and remained score 5 afterwards (Figure 3.4). Similarly, GHAS rose sharply from score 1 to score 4 from day 4 to day 11 and the average score was about 4.2 in the remaining time of the trial. Thus, these results indicated that doses of 1×10^8 and 1×10^7 cfu of *S. typhimurium* caused very severe infection in mice. On the other hand, there were no significant changes in GHAS in group 3 (1×10^6) and group 4 (1×10^5). The GHAS only changed from score 1 to score 1.6 and 1.4 respectively from day 4 to the day 21 post-challenge. Therefore, these two doses only resulted in very slight infection in the mice.

45

3.4 Discussion:

The R (correlation coefficient, which is used to measure the strength of the linear relationship) between absorbance and bacteria count is 0.9952, which indicates a very good correlation between them. Thus, a reasonably accurate count of live bacteria in a suspension may be obtained by using this standard curve. However, this curve can only be used to measure the bacteria suspension with concentration between 1×10^7 to 1×10^{10} cfu/ml since there is no good linear relation outside of these concentration ranges. This is due to the sensitivity limits of the assay. There is only a good linear relation between the absorbance and a certain concentration of measured solution (Collins *et al.*, 1995). Anyway, since the *S. typhimurium* concentration used in the following trials will never exceed 10^8 cfu/ml (see discussion below), this standard curve can be used in further trials. If bacterial concentration below 1×10^7 cfu/ml are needed, this standard curve can also be used to measure the concentration between 1×10^7 to 1×10^{10} cfu/ml and then dilute it to get the desired concentration. Overall, the method established here provides a quick and accurate measurement of the concentration in a *S. typhimurium* suspension.

The GHAS in all four groups changed from day 4 post-challenge, which meant that mice were getting sick. The reason for this phenomenon might be that the *Salmonella* began to translocate into the spleen and liver of the mice. It has been found that *Salmonella* started to enter into spleen and liver after 4 days of oral challenge (Carter *et al.*, 1974). Perdigon *et al.*, (1990) also confirmed this finding in their study. They observed that *S. typhimurium* reached the highest level in the liver of mice on day 7 and maintained high levels until day 10 post-challenge. When *Salmonella* entered into the liver and spleen, it would result in a systemic infection and then the change of GHAS. It has been found that when the GHAS of a mouse exceeded 4, which indicated the mouse had a serious infection, it was not likely recover and would usually die within several days. If it scored below 4, it still had the chance to recover, especially for those who scored under 2.5.

The dose of 1×10^8 cfu is too high since all the mice in this group achieved score 4.5 at the end of first week and score 5 at the day 9 post-challenge, with some mice reaching GHAS of 5 and then being withdrawn from the experiment before the end of first week. Therefore, if this dose was used, mice might not survive long enough to produce an immune response and for bacteria translocation to be studied. On the other hand, the doses of 1×10^6 and 1×10^5 cfu seem too low. The average score in these two groups were 1.6 and 1.4 respectively within 3 weeks, which indicated there was only slight infection in these mice. In addition, there were no clinical symptoms of infection in many mice in these two groups. Those that had score 2 or 2.5 usually scored 1 after several days, indicating that they could recover by themselves.

The dose of 1×10^7 cfu seems to be the best choice. It is about two times of the value of the Mean Dose for 50% of the mice to reach GHAS of 5. The average score in this group in 3 weeks was 4, which indicated that mice suffered severe infection but were not dying. The mice in this group all survived after 7 days post-challenge, which gave sufficient time to study bacteria translocation and immune responses. Thus, this dose was chosen to be used in the following trials.

Chapter 4: The efficacy of DR10™ against *S. typhimurium* infection and its immune-stimulating properties in mice.

4.1. Introduction

Salmonella typhimurium establishes infection readily in mice, causing a severe typhoid-like illness (Heffernan *et al.*, 1987). It is considered to be a facultative intracellular organism and can survive and replicate within host blood cells and translocate after a simple oral inoculation (Van Leeuwen *et al.*, 1994). It can migrate readily across the mucosal barrier - a process called translocation, and enter the circulation to initiate the septic process and eventually lead to multiple organ failure. Thus, translocation is a vital step in the initiation of systemic infection. There are three mechanisms that are likely to promote bacterial translocation: (1) Changes in permeability of the intestinal mucosa as seen with haemorrhagic shock, sepsis, injury or administration of endotoxin; (2) Decreased host defence mechanisms; (3) Increased numbers of bacteria within the intestine under the conditions of bacterial overgrowth, intestinal stasis or exogenous bacteria applied experimentally (Van Leeuwen *et al.*, 1994). It has been proposed that translocation from the gut is probably controlled by the gut's own flora, a process called ' colony resistance' (Van Leeuwen *et al.*, 1994). Thus, LAB may play a very important role in prevention of bacterial translocation. In fact, the potential of LAB in preventing or even treating *Salmonella* infection has been studied in a few studies in recent years (Perdigon *et al.*, 1995b; Nader de Macias *et al.*, 1992).

Some strains of LAB, such as *Lactobacillus. casei* and *Lactobacillus. acidophilus*, have been found to be able to prevent *S. typhimurium* translocation and stimulate host immunity against *S. typhimurium*. It has been found that the number of viable of *S. typhimurium* in spleen and liver of the mice in LAB treatment group was significantly lower than that in the *Salmonella* control group (Perdigon *et al.*, 1990; 1991 & 1995a). In addition, some strains of LAB have been found to stimulate host immune responses against *S. typhimurium* in these studies. For example, it has been

found that LAB can increase the serum and mucosal anti-*Salmonella* antibody level and enhance the phagocytic activity of peritoneal macrophages. It was also demonstrated that the efficacy of this immunity-stimulation and anti-*Salmonella* translocation of LAB varied among different species and strains. For instance, *L. casei* has been found to have a greater efficacy in stimulating mucosal and serum antibody production than *L. acidophilus* (Perdigon *et al.*, 1990). *L. casei* has also been shown to be better at preventing *S. typhimurium* infection *than L. acidophilus* in another study by Perdigon *et al.*, (1995b). These studies show a promising direction in the research of health benefits of LAB. However, only a few parameters of immune responses, such as antibody production and phagocytic activity, have been studied while other aspects of the non-specific and specific responses have not been examined in these studies. In addition, the mechanisms of LAB against *Salmonella* infection have not been fully elucidated and the relative importance of these mechanisms is not clear.

The aim of present trial was to examine the ability and compare the efficacy of a newly identified strain of LAB - DR10™ (*Bifidobacterium lactis*) in preventing *S. typhimurium* infection and stimulating host immunity against it. In order to get a wider picture of the effects of DR10™, a broad range of parameters of immune responses was examined using the *Salmonella*-infected murine model established in Chapter 3.

(1) The general health status was measured by monitoring the GHAS, liveweight change, and feed intake daily.

(2) The severity of the infection was assessed by measuring the percentage of appearance of lethal clinical symptoms in 3 weeks post-challenge, the number of viable *Salmonella typhimurium* in the spleen and liver, and the spleen index (spleen weight divided by live weight).

(3) The non-specific immune response was examined by testing the phagocytosis of peripheral blood leukocytes and peritoneal macrophages.

(4) The specific immune response was studied by measuring the levels of serum lymphocytes to LPS and PHA, the numbers of $CD4^+$, $CD8^+$, $CD25^+$ and $CD40^+$ lymphocytes in the PP and MLN.

4.2. Experimental Design

One hundred and eight (6-7 week old) BALB/c mice were housed individually with a 12 hour light/dark cycle and a constant temperature ($22\pm^{o}$C) in the Small Animal Production Unit (SAPU), Massey University. After 7 days' acclimatisation on a Skim Milk Powder (SMP)-based diet (fed *ad libitum*), they were randomly allocated into 3 groups (n=36) on the basis of live weight, with a LAB treatment group (DR10™+Sal), a normal control group (SMP), and a non-probiotic *Salmonella* control group (*Salmonella* only). The LAB treatment group was fed *ad libitum* with SMP-based diet containing 2×10^{8} cfu/g of DR10™, until the end of the experiment (Table 4.1). After feeding LAB for 7 days, the DR10™+Sal group and the *Salmonella* only group were orally challenged with *S. typhimurium* ATCC 1772 (on day 0) with the dose of 1×10^{7} cfu. The trial lasted for another 21 days. During this period, the clinical signs were carefully monitored and recorded three times a day. Mice were withdrawn from the experiment when the GHAS reached 5 and then euthanased by isofluorance overdose. On day 7 post-challenge, six mice from each group were randomly chosen to be killed for immune function analysis. Blood, PP and MLN samples, gut fluid, peritoneal macrophages, spleen and liver were collected for assays. Various immune parameters were measured.

Table 4.1: Treatment schedule for feeding DR10™, challenging of *Salmonella*, and performing immune assays.

Day	DR10™+Sal	SMP	Sal. only
-14 ~ -7	Acclimatisation	Acclimatisation	Acclimatisation
-7	SMP+DR10™	SMP	SMP
0	SMP+DR10™+Sal	SMP	SMP+Sal
7	Killed 6 mice for immune assays	Killed 6 mice for immune assay	Killed 6 mice for immune assays
21	Killed all the remaining mice	Killed all the remaining mice	Killed all the remaining mice

4.3. Results

4.3.1. Appearance of lethal clinical symptoms

Table 4.2: The percentage of appearance of lethal clinical symptoms within 21 days post-challenge.

Group	Percentage of appearance of lethal clinical symptoms
DR10™+*Salmonella*	23.33%[a] (23 out of 30 rats survived)
SMP (control)	0%[b] (all rats survived)
Salmonella only	93.1%[c] (2 out of 20 rats survived)

- Chi-square test was used.
- Values with a different superscript are significantly different from each other (P < 0.01).
- n =29~30.
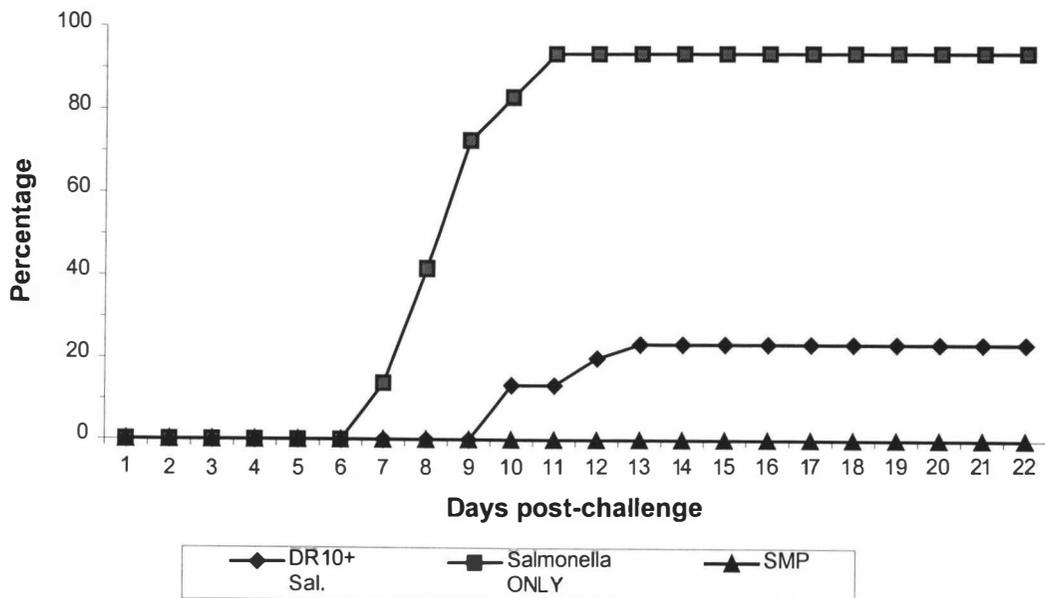
Figure 4.1: The percentage of appearance of lethal clinical symptoms in mice of the DR10™ treatment group, normal control group, and *Salmonella* control group in 3 weeks post-challenge. (n = 29-30).

After 7 days post-challenge, 41.38% mice in *Salmonella* control group had the appearance of lethal clinical symptoms (GHAS of 5) and were euthanased while no

mice in DR10™ treatment group and SMP control group had such symptoms and they all survived. After 14 days post-challenge, the percentages of such symptoms in mice in DR10™+Sal group was 23.33%, with SMP 0%, while with 93.1 % of the mice in *Salmonella* control group. At 21 days post-challenge, such percentages in each group remained the same. There was a significant difference between the percentages of lethal clinical symptoms of DR10™ treatment group, SMP control group, and *Salmonella* control group within 3 weeks post-challenge (P<0.01) (Table 4.2).

4.3.2. General Health Appearance Score

The mice in the *Salmonella* control group achieved a quite higher GHAS than that of the mice in the DR10™+Sal group and the SMP control group after 5 days and they scored over 4 at 7 days post-challenge then remained about at score 4.5 until 21 days (Figure 4.2). In comparison, the average GHAS in the DR10™+Sal group never exceeded 2.5 in the 21 days. There was a significant difference between the GHAS in the *Salmonella* control group and the DR10™+Sal group on 7, 14, and 21 days post-challenge (Table 4.3). In addition, a difference was also found between the DR10™+Sal group and the SMP group on day 14 and 21 post-challenge (Table 4.3).
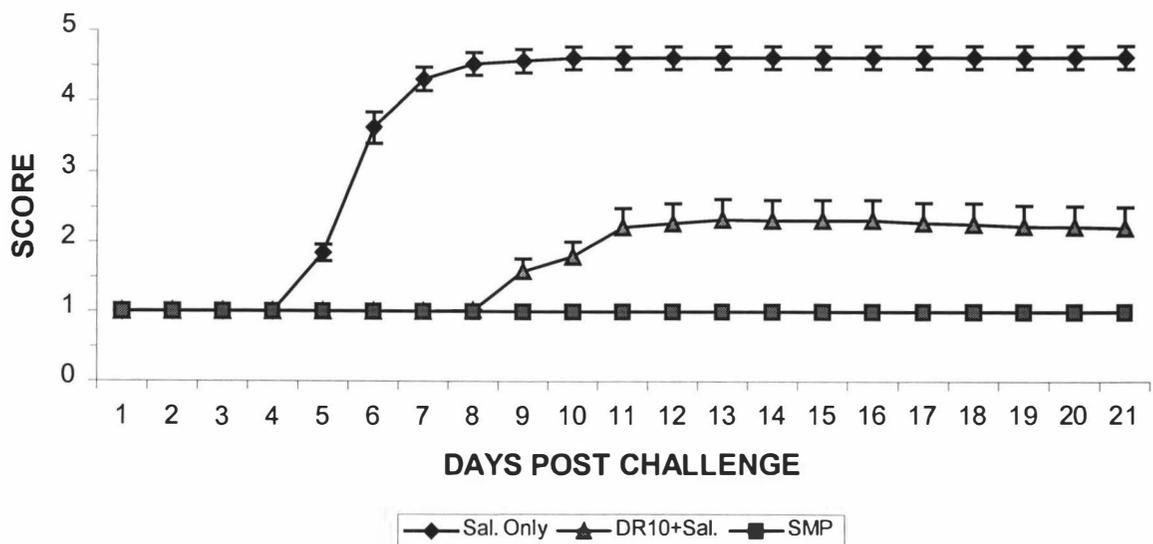
Figure 4.2: General Health Appearance Score in 21 days post-challenge.

Table 4.3: General Health Appearance Score in Day 7, 14, and 21 post-challenge.

Group	Day 7	Day 14	Day 21
Salmonella only	4.3 ± 0.17^a	4.6 ± 0.17^a	4.6 ± 0.17^a
SMP	1^b	1^b	1^b
DR10™+Sal.	1^b	2.3 ± 0.25^c	$2.2 \pm 0.3b^c$

- Values with a different superscript in a same day are significantly different from each other (P<0.05).
- All withdrawn mice were recorded score 5 until day 21 after they were euthanased.
- Mean ± SE (n=29-30)

4.3.3. Food and water intake

As shown in Table 4.4, there was a significant difference between the food intake of the *Salmonella* control group and the DR10™+Sal group in week 1 (P<0.05). The food intakes of the DR10™+Sal group and the SMP group were not significantly different during the 3 weeks of the trial. In addition, there was no significant difference in water intake between the groups during the 3 weeks post-challenge.

Table 4.4: Food and Water intake in 3 weeks post-challenge

Group	Week 1(g/week)		Week 2 (g/week)		Week 3 (g/week)	
	Food Intake	Water Intake	Food Intake	Water Intake	Food Intake	Water Intake
Salmonella	20.59 ± 1.03^a	26.95 ± 1.6	NA	NA	NA	NA
SMP	30.21 ± 0.93^b	27.36 ± 1.9	30.89 ± 1.1	35.73 ± 1.9	30.87 ± 1.3	38.76 ± 1.8
DR10™+Sal	29.44 ± 0.82^b	27.42 ± 1.8	30.34 ± 1.27	36.47 ± 1.63	29.07 ± 2.61	39.19 ± 1.11

- Mean ± SE (n = 22-36, for week 1, n=36 in each group; for week 2 & 3, n=22 in DR10+Sal group and n=30 in SMP control group)
- Values with a different superscript are significantly different from each other (P<0.05).

4.3.4. Live weight change

As shown Table 4.5, there were positive weight changes (weight gain) in the DR10™+Sal group and the SMP group while in the *Salmonella* control group there was a negative weight change (lost weight) in week 1. The difference of weight changes between the *Salmonella* control group and these in the DR10™+Sal group and the SMP group was significantly different (P<0.05). No significant difference has been found between the DR10™+Sal group and the SMP group during 3 weeks post- challenge.

Table 4.5: Live weight change in 3 weeks post-challenge.

Group	Week 1(g/week)	Week 2 (g/week)	Week 3 (g/week)
Salmonella Only	-2.33±0.36[a]	NA	NA
SMP	0.99±0.21[b]	2.67±0.81	0.98±0.23
DR10™+Sal	0.87±0.08[b]	2.25±0.42	0.59±0.08

- Mean ± SE (n = 22-36, for week1, n=36 in each group; for week2 & 3, n=22 in DR10™+Sal group and n=30 in SMP control group)
- Values with a different superscript are significantly different from each other (P<0.05).

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

As shown in Figure 4.3, the number of *S. typhimurium* in both the liver and spleen in the DR10™+Sal group was significantly lower than that of the *Salmonella* control group (P<0.05). No viable *S. typhimurium* has been found in the SMP group on day 7 post-challenge (That is not shown in Figure 4.3).
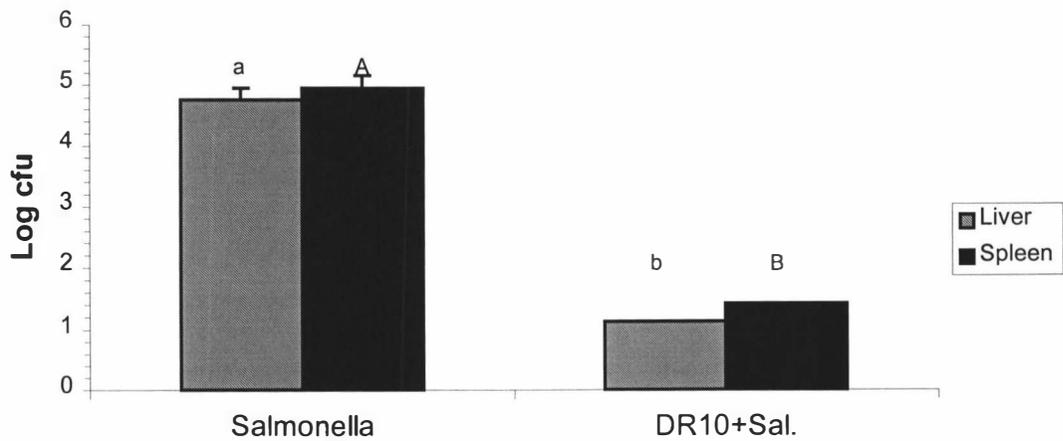
Figure 4.3: Viable *S. typhimurium* numbers in the livers and spleens on day 7 post-challenge.

- Mean ± SE (n=6)
- Values with a different letter in the same case are significantly different from each other (P<0.05).

4.3.6. Spleen Index (spleen weight/live weight) and Liver Index (liver weight/live weight)
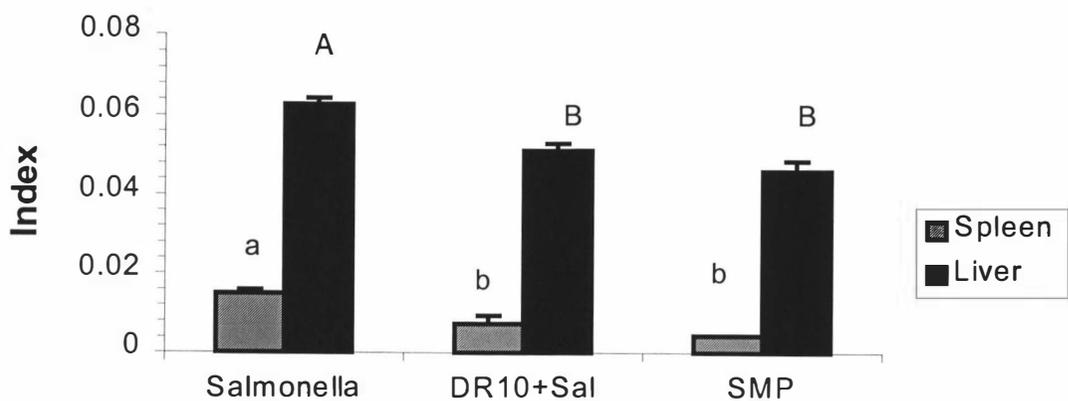
Figure 4.4: Spleen Index and Liver Index on day 7 post-challenge.

- Mean ± SE (n=6)
- Values with a different letter in the same case are significantly different from each other (P<0.05).

On day 7, the *Salmonella* control group had a significantly higher spleen index and liver index than those of the DR10™+Sal and the SMP group (P<0.05) (Figure 4.4). There was no significant difference between the DR10™+Sal group and the SMP group in spleen index and liver index.

4.3.7. Effect of Feeding DR10™ on the Phagocytic Activity of Peritoneal Macrophages and Peripheral Blood Leukocytes
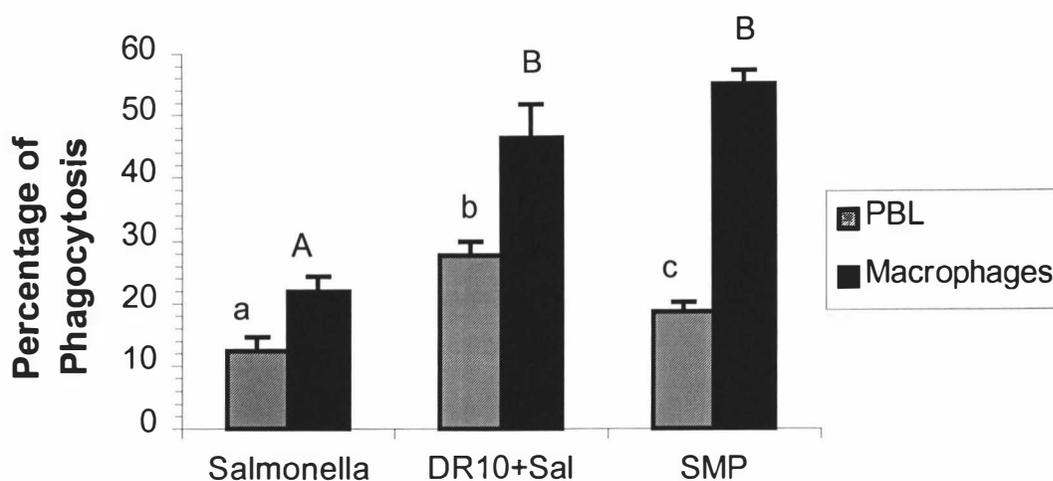
Figure 4.5: Phagocytic activity of peritoneal macrophages and peripheral blood leukocytes to FITC-*E. coli* on day 7 post-challenge.

- Mean ± SE (n=6)
- Values with a different letter in the same case are significantly different from each other (P<0.05).

As shown in Figure 4.5, on day 7, the phagocytic activities of peritoneal macrophages and PBL to FITC-*E. coli* from the DR10™+Sal group and the SMP group were significantly higher than that of the *Salmonella* control group. In addition, the DR10™+Sal group had a significantly higher phagocytic activity of PBL than the SMP group. However, such differences could not be found in phagocytic activity of peritoneal macrophages.

4.3.8. Effect of Feeding DR10™ on the Antibody Production against *S. typhimurium*

As shown in Figure 4.6, the mucosal antibody (mainly sIgA) production in response to LTA (lipoteichoic acid, derived from crude *S. typhimurium* LPS) in the *Salmonella* control group and the SMP group were significantly lower than the DR10™+Sal group on day 7 post-challenge. There was no significant difference between the *Salmonella* control group and the SMP group in mucosal antibody production. In addition, the DR10™+Sal group and the *Salmonella* control group produced a higher level of serum antibody in response to LTA than the SMP group did. There was no significant difference between the DR10™+Sal group and the *Salmonella* control group in such antibody production.
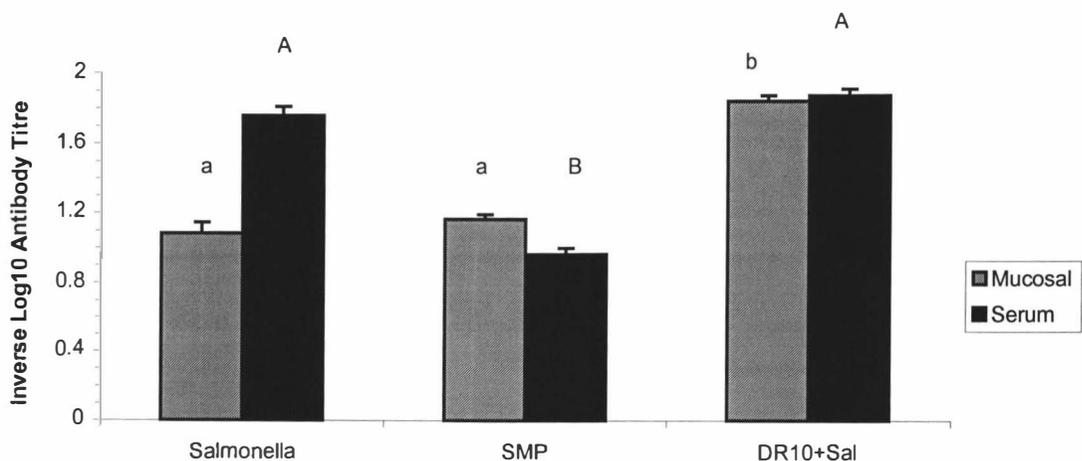
Figure 4.6: Mucosal & serum antibody in response to LTA on 7 days post-challenge.

- Mean ± SE (n = 6)
- Values with a different letter in the same case are significantly different from each other (P<0.05).

4.3.9. Effect of Feeding DR10™ on the Expression of CD4/CD8 ratio, CD25, CD40 on PP (Peyer's Patch) Lymphocytes

CD4, CD8, CD25, and CD40 are cell surface markers of Helper T cells; Suppressor or Cytotoxic T cells; B cells; and activated T cells, B cells or macrophages respectively (Roitt, 1997). As shown in Table 4.6, on day 7 post- challenge, there was no significant difference in expression of CD40 and the ratio of CD4 / CD8 on PP lymphocytes among three groups. However, the DR10™+Sal group expressed significantly higher percentage of CD25 on PP lymphocytes than other two groups (P<0.05).

Table 4.6: Percentage of CD4 / CD8 ratio, CD25, CD40 expression on PP lymphocytes on day 7 post-challenge

Group	CD4/CD8	CD25 (%)	CD40(%)
Salmonella Only	2.9±0.23	5.2±0.5[b]	39.8±3
SMP	2.9±0.14	4.4±0.16 [b]	33±2.3
DR10™+Sal	3.1±0.29	6.2±0.1[a]	38±4.4

- Mean ± SE (n=3).
- Values with a different superscript in the same column are significantly different from each other (P<0.05).

4.3.10. Effect of Feeding DR10™ on the Expression of CD4/CD8 ratio, CD25, CD40 on MLN (Mesenteric lymph nodes) Lymphocytes

Table 4.7: Percentage of CD4/CD8 ratio, CD25, CD40 expression on MLN lymphocytes on day 7 post-challenge.

Group	CD4/CD8	CD25 (%)	CD40(%)
Salmonella Only	2.5±0.2	6.4±0.76[b]	23.3±5.3
SMP	2.8±0.43	6.8±0.15[b]	29±3.7
DR10™+Sal	2.6±0.2	7.4±1[a]	26.4±2.3

- Mean ± SE (n=3).
- Values with a different superscript in the same column are significantly different from each other (P<0.05).

As shown in Table 4.7, the DR10™+Sal group had a significantly higher percentage of CD25 than the *Salmonella* control group and the SMP group did (P<0.05). No

other significant difference could be found on the expression of CD4/CD8, CD25, and CD40 on MLN lymphocytes.

4.3.11. Effect of Feeding DR10™ on the Cell Proliferative Responses of Spleen Lymphocytes
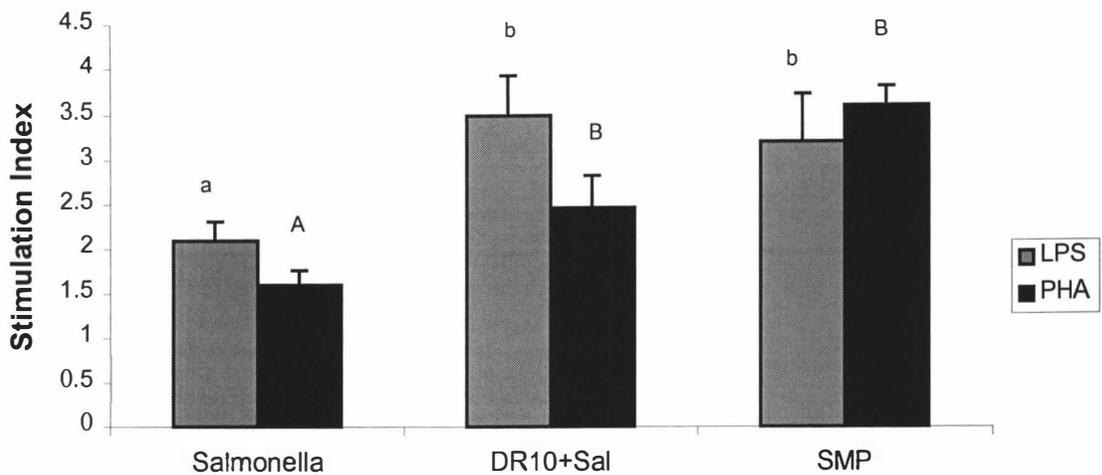
Figure 4.7: Proliferative responses of spleen lymphocytes to LPS & PHA on day 7 post-challenge.

- Mean ± SE (n=3).
- Significantly different from values with different letter in the same case (P<0.05).

As shown in Figure 4.7, the DR10™+Sal group and the SMP group showed a significantly higher stimulation indexes (SI) of spleen lymphocytes to both LPS and PHA than those of the *Salmonella* control group (P<0.05). There was no significantly difference in SIs of LPS and PHA between the DR10™+Sal group and the SMP group.

4.3.12. Effect of Feeding DR10™ on the Cell Proliferative Responses of PP (Peyer's Patch) Lymphocytes

As shown in Figure 4.8, the DR10™+Sal group and the SMP group showed a significantly higher proliferative response of PP lymphocytes to LPS than that of the *Salmonella* control group on day 7 post-challenge. Such difference could not be found between the DR10™+Sal group and the SMP group. There was no significant difference in SI to PHA among these three groups.
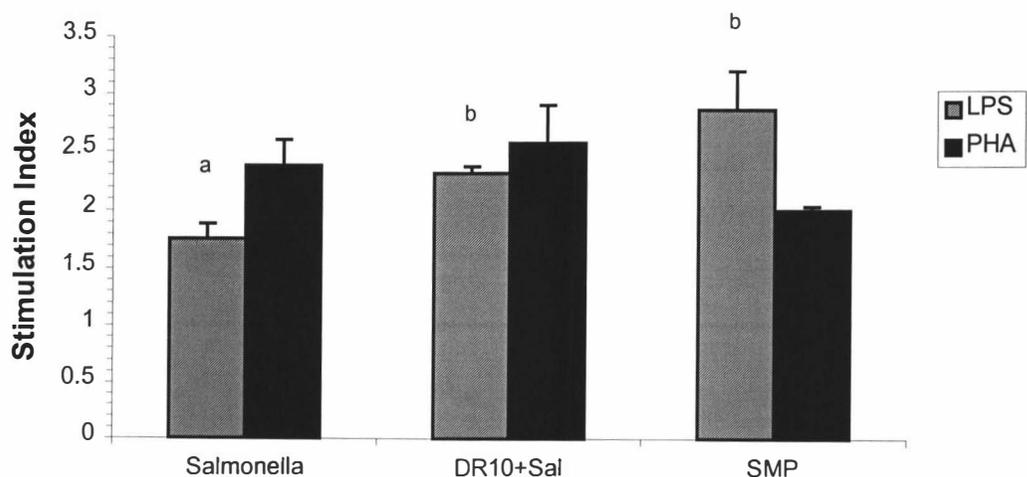
Figure 4.8: Proliferative responses of PP lymphocytes to LPS & PHA on day 7 post-challenge.

- Mean ± SE (n=3).
- Significantly different from values with different letter in the same case (significant difference in SI to LPS between *salmonella* control group and DR10™+Sal group & SMP group; no significant difference in SI to PHA).

4.3.13. Effect of Feeding LAB on the Cell Proliferative Responses of MLN (Mesenteric lymph nodes) Lymphocytes

As shown in Figure 4.9, on day 7 post-challenge, the DR10™+Sal groups had significantly higher SI in response to PHA than that of the SMP group. No other significant difference could be found among three groups.
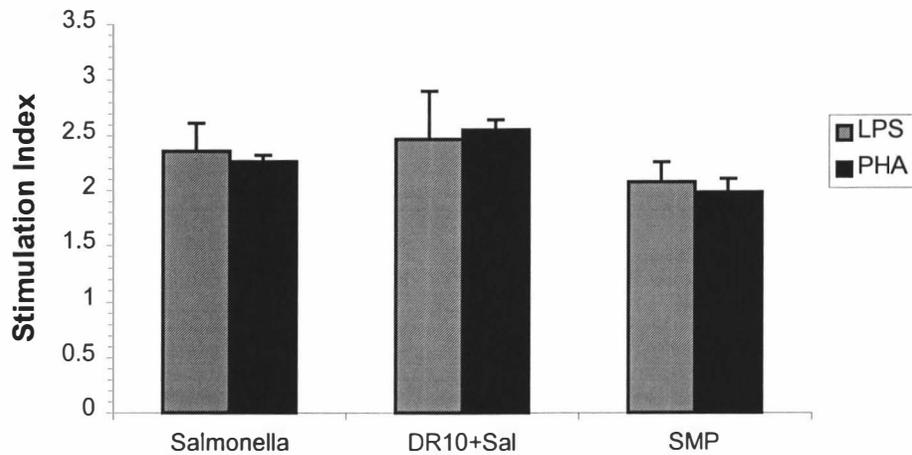

Figure 4.9: Proliferative responses of MLN lymphocytes to LPS & PHA on day 7 post-challenge

- Mean ± SE (n=3).
- Significant difference in SI to PHA between DR10™+Sal group and SMP group. No other significant difference could be found.

4.4. Discussion

In the present study, the effect of feeding DR10™ on the resistance of mice to *S. typhimurium* infection and the role of immune enhancement in this process was investigated.

Results indicated that DR10™ provided significant protection against *S. typhimurium* infection. This was shown by lower percentage of and slower appearance of clinical manifestations of disease (Figure 4.1), better general health appearance score (Figure 4.2), maintenance of feed intake and live weight (Table 4.4 & 4.5), lower spleen and liver index (Figure 4.4), and lower numbers of *S. typhimurium* in the liver and spleen (Figure 4.3) in the DR10™ treatment group. The enhanced resistance to *S. typhimurium* was accompanied by an increase in both non-specific and specific immune responses.

The finding that there were significantly lower numbers of *S. typhimurium* in the liver and spleen and lower spleen index and liver index in the DR10™ treatment group indicates that feeding of DR10™ prevented *S. typhimurium* translocation to the liver and spleen. This is consistent with the observation of other investigators in similar experiments (Perdigon *et al.*, 1990 & 1991, Nader de Macias *et al.*, 1992). The pathogenic sequence of *Salmonella* infection starts with the survival and growth of the pathogen in the lumen of the gastro-intestinal tract. Subsequently, the pathogen adheres to and colonises the intestinal mucosa. Eventually, it may translocate to the mucosa via transepithelial transport by M cells of PP (Bradley *et al.*, 1994) to extra-intestinal tissues such as liver and spleen and/or blood, resulting in a systemic infection (Carter *et al.*, 1974). In addition, absorptive enterocytes can also be invaded and damaged, providing an additional portal of entry (Takeuchi, 1967). *S. typhimurium* is a gram-negative bacterium with endotoxin. Translocation of *S. typhimurium* results in a systemic endotoxaemia, which may cause organ dysfunction, impairs the mucosal barrier, the clotting system, the immune system and may depress Kupffer cell function (Van Leeuwen *et al.*, 1994). Host defences against bacterial pathogens can be divided in two categories: constitutive,

nonspecific defences directed against most bacteria, and specific, induced defence directed against a specific pathogen. The former defences are of particular importance in the first encounter between host and pathogen because it takes about one week for the specific defences (such as antibodies) to appear (Van der Meer *et al.*, 1998). Therefore, strengthening of these nonspecific defences could be a successful strategy with regard to the prevention of intestinal bacterial infection. The relevant nonspecific defences in the intestinal tract can be summarized as follows: (a) acid environment in the gut, bile acids, and pancreatic enzymes which can kill many pathogens; (b) the motility of the intestine, epithelial mucin secretion, exfoliation of epithelial cells, and the presence of an autochthonous microflora, which act together to kill pathogens and /or to prevent their colonisation and subsequent translocation across the intestinal mucosa. Consumption of LAB exposes *S. typhimurium* to bactericidal lactic acid, which may inhibit the survival of acid-sensitive pathogens such as *Salmonella* (Mishra & Lambert, 1996). In addition to acidification of the environment of gut, there may be other mechanisms involved in the resistance of *S. typhimurium*. Bovee-Oudenhoven *et al.,* (1996) performed a strictly controlled infection experiment, in which they compared the resistance of rats to a *Salmonella* infection when fed milk fermented with LAB or milk acidified with hydrochloric acid. Within an hour, more than 95% of the inoculum of *Salmonella* was killed. In contrast, milk acidified with hydrochloric acid to pH 4.2 (the pH of the milk fermented with LAB) did not affect the viability of this *Salmonella.* They suggested that fermentation metabolites (like lactic acid and other substances) in milk fermented with LAB might improve the resistance to *Salmonella* infection. In a study by Bernet-Camard *et al.,* (1997), it was demonstrated that *L. acidophilus* strain LA1 developed an antibacterial activity in conventional or germ-free mouse models orally infected by *S. typhimurium*. They found that the spent culture supernatant of strain LA1 (LA1-SCS) contained antibacterial components active against *S. typhimurium* . The LA1-SCS antibacterial activity was observed *in vitro* against a wide range of gram-negative and gram-positive pathogens. This antibacterial activity was insensitive to proteases and independent of lactic acid production. Mishra & Lambert (1996) suggested that the production of antimicrobial substances such as lactic and acetic acids, H_2O_2, diacetyl, CO_2, and bacteriocins such

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as nisin, lactocidin, acidolin and reuterin might participate in this process. Other suggested mechanisms include the ability of LAB to adhere to the intestinal wall thus competing with pathogenic bacteria for adhesion receptors (Bernet-Camard *et al.,* 1994), the 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 by LAB (Mitchell & Kenworthy, 1976). Although these antibacterial substances were not directly examined in this study, it is very likely that above mechanisms may be involved in the improved resistance to *Salmonella* infection by DR10™. The number of *S. typhimurium* in the liver and spleen was inversely related to the phagocytic activity of peritoneal macrophages & peripheral blood leukocytes, and the antibody levels in the gut on day 7 post-challenge (Figure 4.3; 4.5; 4.6). This result indicated that both the non-specific and specific immune systems were involved in the host defence response to *Salmonella* infection. The significantly enhanced phagocytic activity of the peripheral blood leukocytes and peritoneal macrophages observed in the DR10™ treatment group was consistent with the observations of other investigators (Perdigon *et al.,* 1986a, 1986b, 1988, 1990, Nader de Macias *et al.,* 1993, Moineau *et al.,* 1989, Paubert-Braquet *et al.,* 1995) though the different species or strains of LAB were used. This enhanced phagocytic activity might play an important role in the early stage of elimination of *S. typhimurium* in the gut and blood. This hypothesis was supported by a study by Sato (1984). He found that the enhanced resistance against *Listeria monocytogenes* in the early phase (e.g. 2-3 days post-challenge) induced by *L. casei* was impaired by CAR (carrageenan), a chemical which abrogated the phagocytic cell functions, but it was not affected by cyclophosphamide and anti-mouse thymocyte serum, which abrogated lymphocytes. He also found that the enhanced host resistance by *L. casei* to *L. monocytogenes* infection may be mediated by macrophages migrating from the blood stream to the reticuloendothelial system in response to *L. casei* feeding before or after *L. monocytogenes* infection. Perdigon *et al.* (1990) also demonstrated that the effective killing of *S. typhimurium* on day 3 and 4 could be due to the large increase in macrophage activity or to activation of non-specific defence systems produced by feeding with *L. acidophilus.*

The DR10™ treatment group showed a significantly higher mucosal anti-LTA (a cell surface component of *S. typhimurium)* antibody level than that of the *Salmonella* control group on day 7. The mucosal antibodies against *S. typhimurium* are mainly secretory immunoglobulins (sIgA) (Perdigon *et al.*, 1991). These antibodies can inhibit bacterial colonization by agglutinating microorganisms in the gut lumen, or by interfering with motility and blocking the bacterial surface structures responsible for adherence to the epithelium (Cantey, 1978). This hypothesis has been supported by a study by Michetti *et al.* (1992). They used a monoclonal anti-*S. typhimurium* sIgA alone to treat the mice infected with *S. typhimurium.* It was found that monoclonal sIgA could directly attack a surface epitope of *S. typhimurium* so that it could intercept *S. typhimurium* in the intestinal lumen, and prevent its epithelial contact, M-cell transport and invasion of the Peyer's patch mucosa. No *S. typhimurium* was found in the spleens of these mice after oral or intraperitoneal challenge with *S. typhimurium.* Although whether sIgA could affect the viability of organisms in the intestinal lumen was not determined, they proposed that clearance of *S. typhimurium* could be facilitated by enhanced phagocytosis of sIgA-coated *Salmonella* by lumenal macrophages or even by IgA-enhanced killing by lumenal T lymphocytes. Thus, the enhanced production of mucosal antibody in mice fed with DR10™ may play an important role in the resistance against *Salmonella* infection in the gut in this trial.

There was no significant difference in serum antibody against *Salmonella* between the DR10™+Sal and *Salmonella* control groups (Figure 4.6). This might indicate that DR10™ didn't translocate into the blood to stimulate the production of serum antibody against *Salmonella*. Serum antibody could have opsonized or in some way helped clear the organisms during systemic spread (Michetti *et al.*, 1992). However, the relative importance of serum antibody in preventing *S. typhimurium* is not clear. It was found that serum antibody alone was not sufficient to prevent systemic infection (Michetti *et al.*, 1992). This result was consistent with other studies in which most serum antibodies were shown to lack bactericidal, opsonizing, or complement-fixing activities (Kerr, 1990; Mestecky, 1988). Thus, there are most likely other mechanisms participating in the clearance of *S. typhimurium* from the

blood. They could include increased phagocytic activity of peripheral blood leukocytes or the activation of spleen lymphocytes. This hypothesis was supported by the results of this study. The DR10™ treatment group had significantly higher phagocytic activity of PBL than that of the *Salmonella* control group (Figure 4.5). In addition, it has been found that the DR10™ treatment group had significantly higher proliferative responses of spleen lymphocytes to LPS (a B lymphocyte mitogen) and PHA (a T lymphocyte mitogen) on day 7 (Figure 4.7).

The mechanism(s) by which DR10™ stimulates the production of mucosal antibody against *Salmonella* is not very clear. There are a number of possible mechanisms that could be participating this process. One is that DR10™ might increase the number of B lymphocytes and thus raise the mucosal antibody level. This is supported by the finding that the increased level of mucosal antibody in the DR10™ treatment group was accompanied by an increased in CD25 marker (the cell marker for B lymphocytes) on PP and MLN (Table 4.6 & 4.7). Another possible mechanism is the activation of B or T lymphocytes in PP and MLN. It was observed that the DR10™ treatment group had higher proliferative responses of PP and MLN lymphocytes to LPS and PHA than the *Salmonella* group on day 7, though most of the differences were not significant (Figure 4.8 & 4.9). In addition, $CD4^+$ and $CD8^+$ lymphocytes might play a role in the mucosal antibody production. Sawamura *et al.,* (1994) found feeding LAB increased the percentage of $CD4^+$ (Helper T cells) and decreased $CD8^+$ (Suppressor or Cytotoxic T cell) in colorectal cancer patients. The increase in sIgA in mice after feeding with LAB, resulted from the normalization of the $CD4^+$ T lymphocytes helper cells, with a decrease in the number of $CD8^+$ T suppressor cells, which would improve the synthesis of sIgA (Perdigon *et al.*, 1991). However, the results of this trial did not support this mechanism since there was no significant difference in $CD4^+/CD8^+$ ratio in PP and MLN lymphocytes between the DR10™ treatment group and the *Salmonella* control group.

Shu *et al.,* (1999) found no abnormal clinical signs, histological and haematological parameters were observed in the mice fed with DR10™. In addition, no DR10™

translocation to spleen was found in these mice. Zhou *et al.*, (2000) also proved that no acute oral toxicity or bacterial translocation was found in mice fed DR10™. The current study also demonstrates the safety of DR10™ from another aspect. The spleen and liver indices in the DR10™ treatment group were significantly lower than that of the *Salmonella* control group on day 7, but were not significantly different from that of the SMP control group (Figure 4.4), indicating that prolonged feeding of DR10™ had not caused hepatomegaly or splenomegaly. This finding was consistent with that of Perdigon *et al.*, (1988). Thus, together with other findings mentioned above, this finding might permit the use of DR10™ as an immunostimulating agent to resist bacterial infection without any harmful effects on the host. On the other hand, some *lactobacilli*, such as *L. plantarum*, cause an increase in the liver and spleen weight irrespective of whether dead or viable cells are used for inoculation (Bloskma *et al.*, 1979).

It is also of interest that there was a very good correlation between GHAS, and the number of viable *S. typhimurium* in the spleen and liver. The correlation coefficient between GHAS and the Log10 number of viable *S. typhimurium* in the spleen and liver was 0.84 and 0.79 respectively. This indicates that GHAS could reasonably accurately reflect the number of viable *S. typhimurium* in the spleen and liver and thus the severity of infection.

There were some significant differences in immune parameters between the DR10™ treatment group and the SMP control group. It was found that the DR10™ treatment group had a significantly higher level of mucosal and serum antibody (Figure 4.6), higher phagocytic activity of PBLs (Figure 4.7), higher percentage of CD25 on PP and MLN lymphocytes (Table 4.6 & 4.7), and higher proliferative response of MLN lymphocytes to PHA (Figure 4.9) This suggests that, compared to the normal control, DR10™ enhances the immunity even when mice were infected by *Salmonella*.

Previous research work in our laboratory has demonstrated that DR10™ enhances the natural and acquired immunity under normal condition in mice (Gill *et al.*, 2000). It was found that supplementation with DR10™ resulted in a significant increase in the phagocytic activity of peripheral blood leukocytes and peritoneal macrophages, proliferative responses of spleen cells to ConA (a T-cell mitogen) and LPS compared with control mice. Arunachalam *et al* (2000) also found that dietary consumption of DR10™ enhanced the natural immune function in healthy elderly subjects. Consumption of DR10™ produced significantly enhanced levels of interferon-α, and phagocytic capacity of polymorphonuclear cells. The current study was carried out based on these findings and it was the first time to study the immune-enhancing capacity of DR10™ under the condition of pathogenic infection. The significance of the positive findings in this study is that it proves the wider potential use of DR10™ - to stimulate immunity and produce beneficial effects on the host under both normal and bacterial infection conditions.

In summary, DR10™ provides highly significant protection against *S. typhimurium* infection. This increased resistance against infection was accompanied by an increase in several aspects of non-specific and specific immune responses, indicating that DR10™ is able to enhance the murine immune system against *S. typhimurium* infection.

Chapter 5. The synergism between DR10™ and milk calcium in the resistance against *S. typhimurium* infection

5.1 Introduction

Milk products have a high nutrient density and thus contribute significantly to the daily intake of several nutrients, including calcium. A high percentage of habitual dietary calcium comes from milk products. For example, in Netherlands, 70% of habitual dietary intake of calcium derives from milk products (Dutch National Food Consumption Survey, 1993). Since absorption of calcium is relatively low, it is hypothesized that unabsorbed calcium might significantly affect the composition of the luminal contents throughout the intestine (Van der Meer *et al.*, 1998). Milk is supersaturated with equimolar amounts of calcium and phosphate, stabilized as colloidal calcium phosphate by casein. Thus, most of this calcium phosphate precipitates in the intestinal lumen and this sediment binds and precipitates cytotoxic surfactants like bile acids. This hypothesis has been supported by some studies. For example, in a metabolic study on human subjects (Govers *et al.*, 1996), the effect of dairy calcium on intestinal fatty acids and secondary bile acids was examined. They found that milk calcium significantly increased faecal pH and faecal excretion of phosphate, total fat, free fatty acids, and bile acids, indicating intestinal complexation. They also observed that the concentrations of long-chain fatty acids, secondary bile acids, neutral sterols, and phospholipids were approximately halved in faecal water. Thus, they concluded that calcium in milk products precipitated luminal cytotoxic surfactants and thus inhibited cytotoxicity. The effect of calcium on duodenal bile acid composition was studied by Van der Meer *et al.,* (1990) since intestinal precipitation can affect the enterohepatic cycling of bile acids. Supplemented calcium was found to decrease the hydrophobic and cytotoxic dihydroxy bile acids (chenodeoxycholate and deoxycholate) and increase the hydrophilic, less cytotoxic trihydroxy bile acid cholate in duodenal bile. These results indicated that dietary calcium lowered the cytotoxicity of the bile acids secreted into the proximal small intestine (duodenum), implying that the epithelial

cells in the small intestine were also exposed to a less cytotoxic environment. Therefore, it was hypothesized that these cytoprotective effects of calcium phosphate throughout the intestine might increase host defence against bacterial infections as calcium can inhibit damage to the epithelium so thus strengthen mucosal barrier to resist bacterial infection.

Bovee-Oudenhoven *et al.,* (1996) studied the effects of milk calcium on the resistance of rats to a *Salmonella* infection, by feeding them either low calcium milk or high calcium milk. Specific pathogen-free rats were infected with *S. enteritidis*. The course of the infection was followed for 12 days. Infection-induced diarrhea was only noticed in the low-calcium milk group, indicating that milk calcium inhibited the intestinal infection. On day 5, population levels of *Salmonella* in faeces were decreased 100 to 1000-fold in the high-calcium groups. In contrast, rats fed the low-calcium milk continued to excrete high levels of *Salmonella* in their faeces. This was still the case on day 12 post-infection. The population level of *Salmonella* in faeces several days after oral infection was inversely related to the intestinal colonisation resistance to this pathogen. Therefore, rats fed the milk products containing the high amount of calcium had a much higher colonisation resistance to *Salmonella*e than rats fed the low-calcium milk. Bovee-Oudenhoven *et al.,* (1997) also examined the effects of feeding different amounts of milk calcium on the bacterial translocation. They found that dietary calcium increased the daily faecal output and specifically increased the faecal excretion of organic phosphate, phospholipids, and nitrogen indicating an increased faecal excretion of bacteria. In addition, calcium decreased the concentration of bile acids in the faecal water, reflecting precipitation of bile acids by amorphous calcium phosphate in the intestinal lumen. Therefore, the cytotoxicity of faecal water was drastically decreased. This improved colonisation resistance of rats fed the calcium-supplemented diet coincided with a significantly lower level of bacterial translocation as measured by urinary NO derived nitrate excretion. The inhibiting effect of calcium on *Salmonella* translocation was further supported by the results of tissue cultures in an additional infection experiment (Bovee-Oudenhoven *et al.,* 1997). Supplemented calcium reduced viable *Salmonella* counts in ileal PP and in

the spleen. Therefore, taken together, these results indicated that milk calcium improved the colonisation and translocation resistance to *Salmonella*.

LAB have been proven to be able to prevent bacterial infection in many studies. The previous trial (discussed in Chapter 4) has shown that a new LAB strain, DR10™, has a great efficacy in preventing *S. typhimurium* infection and enhancing immunity. Here, an interesting question arises: is there any synergism between DR10™ and milk calcium in protecting against enteric infection and stimulating immunity? It appears that no such research work has been done so far. To test this hypothesis, DR10™ was combined with milk powders with different contents of calcium, SMP (1220mg/100g) or a high calcium milk-ANLENE (2000mg/100g). The results of such a study might help to develop novel, value-added immunity-enhancing dairy products to fight bacterial infection and maintain good health for humans.

5.2 Experimental Design

One hundred and twenty (6-7 week old) male BALB/c mice were housed individually with a 12 hour light/dark cycle and a constant temperature ($22\pm^{\circ}$C) in the SAPU, Massey University. After 7 days acclimatisation on a SMP-based on diet (fed *ad libitum*), they were randomly allocated into 6 groups based on liveweight, with each group having 20 mice. The 6 groups were Group 1 (ANLENE + DR10™ + *Salmonella)*, Group 2 (SMP + DR10™ + *Salmonella*), Group 3 (ANLENE + *Salmonella*), Group 4 (SMP + *Salmonella*), Group 5 (ANLENE only), and Group 6 (SMP only) (Table 5.1). The three ANLENE feeding groups and SMP feeding groups were fed with ANLENE and SMP respectively for one week. After which, the two DR10™ treatment groups were fed with SMP or ANLENE containing 2×10^{8} cfu/g of DR10™ until the end of the trial. After feeding DR10™ for 7 days, four groups were orally challenged with *S. typhimurium* ATCC (1772) (on day 0) with the dose of 1×10^{7} cfu/mouse. The trial lasted for another 21 days. During this period, the clinical signs were carefully monitored and recorded three times a day. Mice were withdrawn from the experiment when the GHAS reached 5 and euthanased by

isofluorance overdose. On the day 7 post-challenge, six mice from each group were randomly chosen for immune function analysis. Blood, PP and MLN samples, gut fluid, peritoneal macrophages, and spleen were collected for assays. Various immune parameters such as serum and gut antibody, cell proliferation, phagocytic activity of peripheral blood leukocytes and peritoneal macrophages, NK cell activity, and immunophenotyping were measured. In addition, faecal samples were collected at 8 am on day 0 (just before challenge), day 1, day 5, day 12, day 21 for faecal colonisation assay. The remaining mice were observed until day 21 post-challenge.

Table 5.1: Treatment Schedule for feeding milk powders and probiotics, challenging *Salmonella typhimurium*, and performing immune assays.

Group	Day-21~	Day-14~	Day-7~	Day0~	Day7	Day 21
Group 1 (Anl+DR10™ +Sal)	SMP	ANLENE	Anl+DR10™	Anl+DR10™ +Sal	Killed 6 mice for immune assays	Killed all the remaining mice
Group 2 (SMP+DR10™ +Sal)	SMP	SMP	SMP+DR10™	SMP+DR10™ +Sal	Killed 6 mice for immune assays	Killed all the remaining mice
Group 3 (Anl+Sal)	SMP	ANLENE	ANLENE	Anl+Sal	Killed 6 mice for immune assays	Killed all the remaining mice
Group 4 (SMP+Sal)	SMP	SMP	SMP	SMP+Sal	Killed 6 mice for immune assays	Killed all the remaining mice
Group 5 (Anl only)	SMP	ANLENE	ANLENE	ANLENE	Killed 6 mice for immune assays	Killed all the remaining mice
Group 6 (SMP only)	SMP	SMP	SMP	SMP	Killed 6 mice for immune assays	Killed all the remaining mice

5.3. Results

5.3.1. Appearance of lethal clinical symptoms
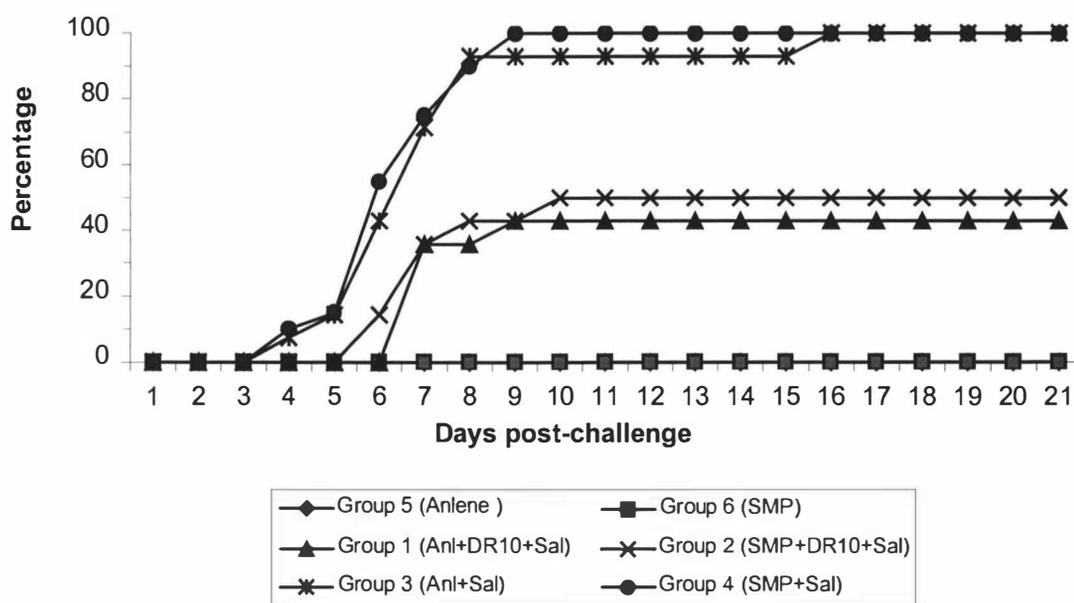
Figure 5.1: Percentage of appearance of lethal clinical symptoms in 21 days post-challenge (n=14).

No mice died in the Group 5 and Group 6 during the 3 weeks of the trial. After 7 days the percentages of appearance of lethal clinical symptoms (GHAS of 5) of mice in Group 1 and Group 2 were both 35.71% while 71.43% of mice in Group 3, and 75% in Group 4 had such symptoms (Figure 5.1). After 14 days post-challenge, such percentage in Group 1 was 42.86%, Group 2 50%, Group 3 92.86%, and Group 4 100%. After 21days, the percentages remained the same except that Group 5 went up to 100%.

5.3.2. General Health Appearance Score

The mice in Group 5 and Group 6 all stayed at score 1 during the 3 weeks. On day 7, the GHAS of Group 4 was significantly higher than that of Group 3 but they all scored 5 on day 14 and 21(Table 5.2). There was no significant difference of GHAS

between Group 1 and Group 2 on day 7 but Group 1 had a significantly lower GHAS than Group 2 on day 14, and 21. Group 3 and 4 achieved significantly higher GHAS than these of Group 1 and 2 on day 7, 14, and 21.
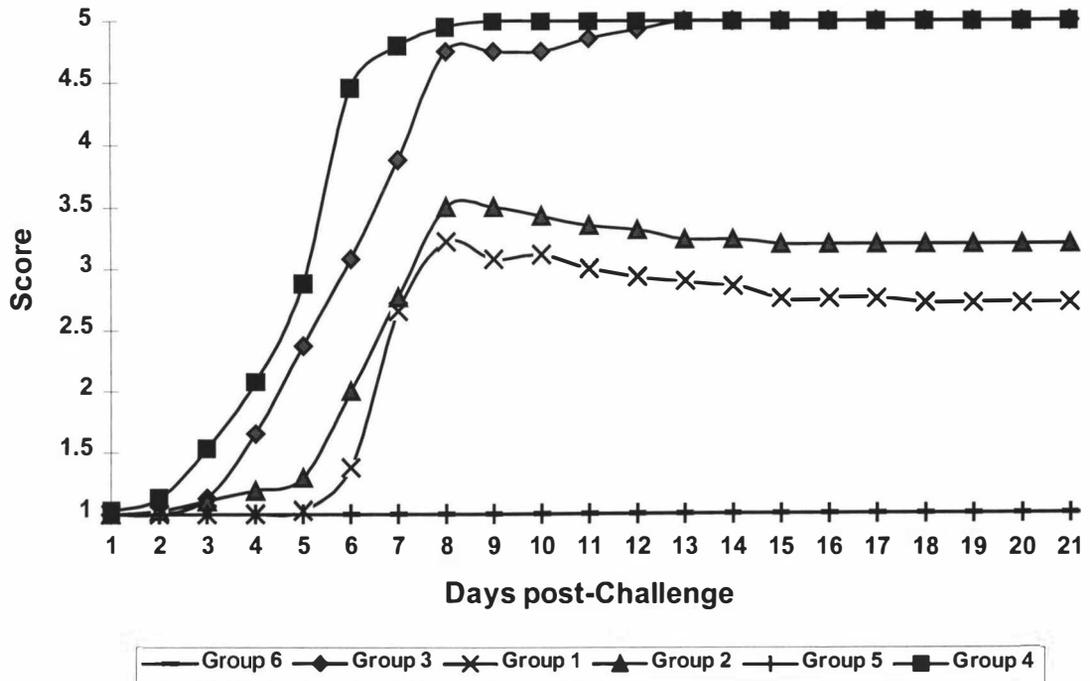
Figure 5.2: General Health Appearance Score in 21 days post-challenge (n=14).

Table 5.2: General Health Appearance Score at Day 7, 14, and 21 post-challenge.

Group	Day 7	Day 14	Day 21
Group 1 (Anl+DR10™+Sal)	2.67 ± 0.11^{c}	2.8 ± 0.21^{c}	2.7 ± 0.22^{c}
Group 2 (SMP+DR10™+Sal)	2.76 ± 0.11^{c}	3.2 ± 0.18^{b}	3.2 ± 0.21^{b}
Group 3 (Anl+Sal)	3.88 ± 0.17^{b}	5^{a}	5^{a}
Group 4 (SMP+Sal)	4.8 ± 0.13^{a}	5^{a}	5^{a}
Group 5 (ANLENE)	1^{d}	1^{d}	1^{d}
Group 6 (SMP)	1^{d}	1^{d}	1^{d}

- Mean±SE (n=20 in week 1, n=14 in week2 and week3)

- Values with a different superscript on the same day were significantly different from each other (P<0.05).

- All withdrawn mice were recorded score 5 until day 21 after they were euthanased.

5.3.3. Food intake

As shown in Table 5.3, in week 1, there was a significant difference in food intake between Group 5 & 6, Group 1 & 2, and Group 3 & 4 with Group 5 & 6 consuming more than Group 1 & 2, who were consuming more than Group 3 & 4. In addition, Group 3 had a significant higher food intake than that of Group 4.

In week 2 and 3, Group 5 & 6 had a significantly higher food intake than that of the Group 1 & 2. No significant difference in food intake could be found between Group 5 and Group 6 or between Group 1 and Group 2 during 3 weeks.

Table 5.3: Food intake within 3 week post-challenge.

Group	Week 1 (g/week)	Week 2 (g/week)	Week 3 (g/week)
Group 1 (Anl+DR10™+Sal)	25.4 ± 0.69^b	23.75 ± 1.1^b	25.45 ± 0.98^b
Group 2 (SMP+DR10™+Sal)	25.1 ± 0.76^b	23.97 ± 1.1^b	25.3 ± 1.4^b
Group 3 (Anl+Sal)	20.45 ± 1.1^c	NA	NA
Group 4 (SMP+Sal)	17.2 ± 0.8^d	NA	NA
Group 5 (Anl Only)	31.59 ± 0.79^a	32 ± 0.66^a	31.25 ± 0.66^a
Group 6 (SMP Only)	31.32 ± 0.91^a	30.63 ± 0.54^a	31.15 ± 0.93^a

- Mean $\pm$SE (n=20 in week 1, n=7 in group 1 & 2 in week 2 & 3, n=14 in group 5 & 6 in week 2 & 3)
- Values with a different superscript in the same week were significantly different from each other (P<0.05).

5.3.4. Water Intake

As shown in Table 5.4, in week 1, there was a significant difference in water intake between Group 5 & 6, Group 1 & 2, and Group 3 & 4 with again Group 5 & 6 intaking more than Group 1 & 2 which were drinking more than Group 3 & 4. In addition, Group 3 had a significant higher water intake than that of Group 4.

In week 2, the only significant difference in water intake that could be found was between Group 2 and the other 3 groups. In week 3, we could only find a significant difference between Group 2 and Group 5 & 6.

Table 5.4: Water intake within 3 weeks post-challenge.

Group	Week 1 (g/week)	Week 2 (g/week)	Week 3 (g/week)
Group 1 (Anl+DR10™+Sal)	30.68 ± 0.79^{b}	32 ± 0.9^{a}	31.6 ± 1.1^{ab}
Group 2 (SMP+DR10™+Sal)	29.33 ± 0.75^{b}	28.1 ± 1.1^{b}	29.6 ± 1.3^{b}
Group 3 (Anl+Sal)	22.2 ± 0.85^{c}	NA	NA
Group 4 (SMP+Sal)	19.5 ± 0.7^{d}	NA	NA
Group 5 (Anl Only)	33.4 ± 0.76^{a}	32.48 ± 0.84^{a}	33.2 ± 0.9^{a}
Group 6 (SMP Only)	32.39 ± 0.8^{a}	32.2 ± 0.76^{a}	32.9 ± 0.74^{a}

- Mean $\pm$SE (n=20 in week 1, n=7 in group 1 & 2 in week 2 & 3, n=14 in group 5 & 6 in week 2 & 3)

- Values with a different superscript in the same week were significantly different from each other ($P<0.05$).

5.3.5 Live Weight Change

In week 1, the live weight gain was significantly different between Group 5 & 6, the Group 1 & 2, and Group 3 & 4 with the order: Group 5 & 6 > Group 1 & 2 > Group 3 & 4 (Figure 5.3).

In week 2 and 3, no significant difference was found in live weight change between Group 5 and Group 6 or between Group 1 and Group 2 (data not shown).
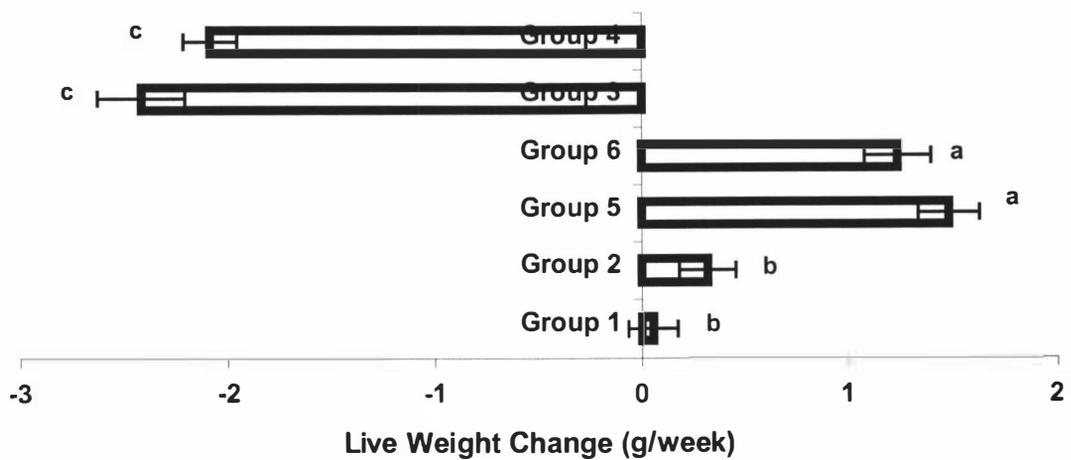
Figure 5.3: Live Weight Change in Week 1 post-challenge.

- Mean ±SE (n=20 in week 1, n=7 in group 1 & 2 in week 2 & 3, n=14 in group 5 & 6 in week 2 & 3)

- Values with a different letter were significantly different from each other (P<0.05).

5.3.6. Number of Viable *S. typhimurium* in the Spleen of Mice

As shown in Figure 5.4, each ANLENE feeding group (Group 1 and 3) always had a significantly lower number of viable *S. typhimurium* in the spleen than that of its corresponding SMP feeding group (Group 2 and 4). That is: Group 3 < Group 4 and Group 1 < Group 2. No viable *S. typhimurium* was found in Group 5 & 6 (data not shown). In addition, Group 1 & 2 had significant lower number of viable *S. typhimurium* in the spleen than Group 3 & 4.

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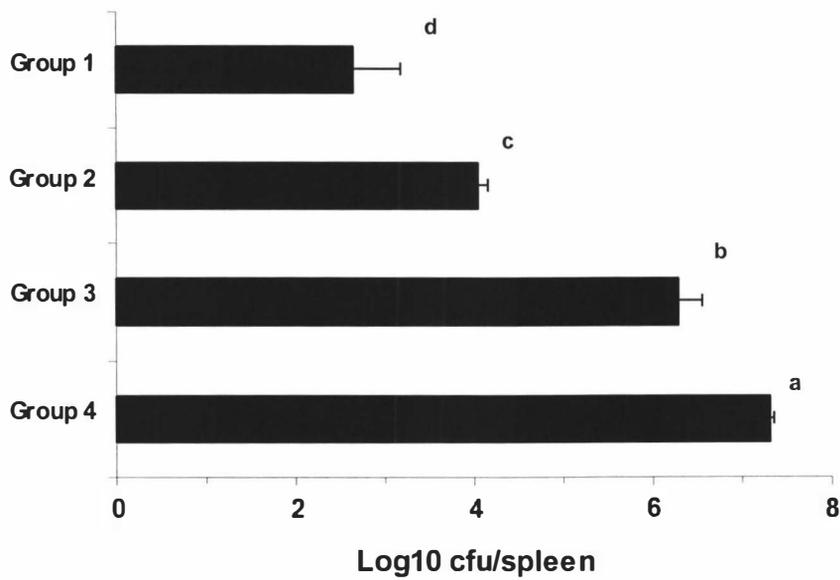
Figure 5.4: Viable *S. typhimurium* number in the spleens

- Mean ± SE (n = 6)
- Values with a different letter were significantly different from each other (P<0.05).

5.3.7. Spleen Index (spleen weight/live weight)

As shown in Figure 5.5, Group 3 had a significantly higher spleen index than other 4 groups. No significant difference in spleen index could be found between the other groups.
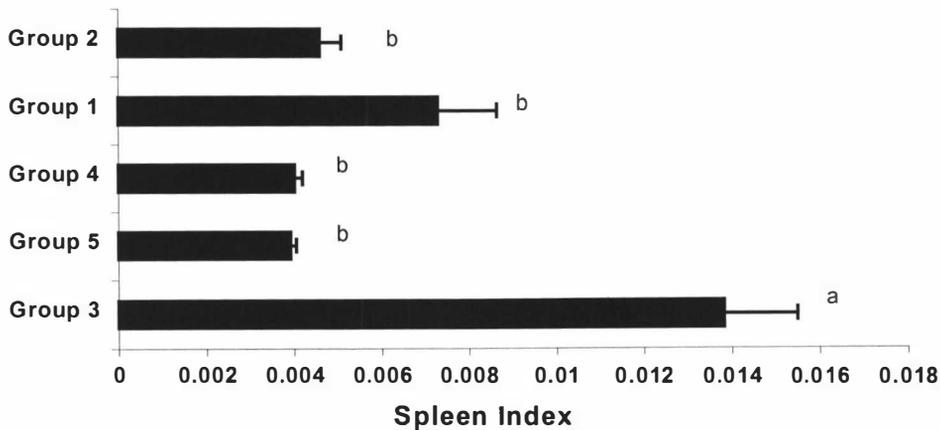
Figure 5.5: Spleen Weight/Live Weight (Spleen Index).

- Mean ± SE (n=6)
- Values with a different letter were significantly different from each other (P<0.05).

5.3.8 Effect of Feeding DR10™ with ANLENE or SMP on the Faecal *S. typhimurium* Excretion.

As shown in Table 5.5, on day 0 just before the *Salmonella* challenge and on day 21, no significant difference in the faecal *S. typhimurium* excretion between any group could be found. On day 1 and day 5, Group 1 and Group 3 were found to have a significantly lower number of faecal *S. typhimurium* than Group 2 and Group 4 respectively, but no such significant difference could be found on day 12. In addition, Group 1, Group 2, Group3, and Group 4 (*Salmonella* challenged groups) always had a significantly higher number of faecal *Salmonella* than Group 5 and Group 6 (non *Salmonella* challenged groups).

It has been observed that the number of faecal *S. typhimurium* in Group 1, Group 2, Group 3, and Group 4 all reached their highest level on day 1 and then dropped gradually. By day 21, the number of faecal *S. typhimurium* in Group 1 and Group 2

went down to the same level as that in Group 5 and Group 6, which received milk powders but no DR10™ (Figure 5.6).
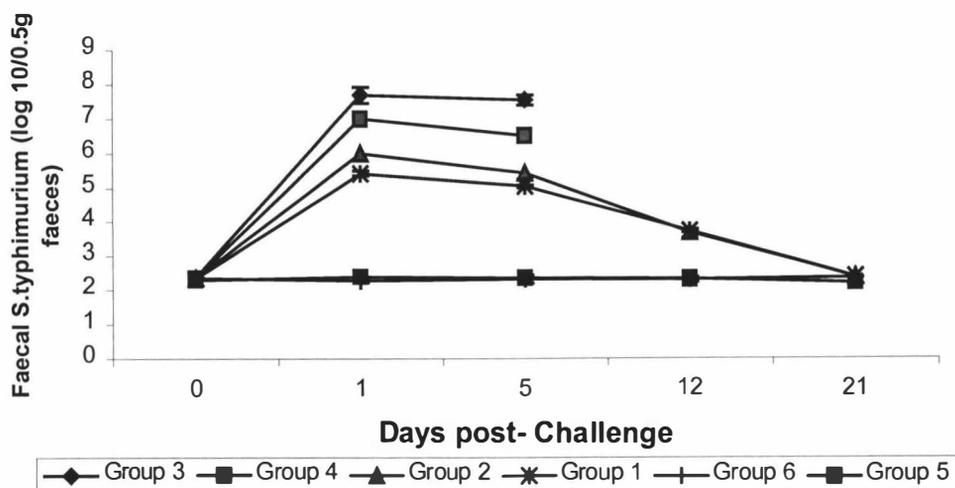
Figure 5.6: Effect of the different diets and LAB on the faecal *S. typhimurium* excretion within 3 weeks post-challenge.

- Mean ± SE (n=6)

Table 5.5: Comparison of effect of different diets and LAB on the faecal *S. typhimurium* excretion (log 10 cfu/0.5g faeces).

Group	Day 0	Day 1	Day 5	Day 12	Day 21
Group 4	2.37±0.23	7.69±0.15^a	7.55±0.19^a	NA	NA
Group 3	2.35±0.06	6.99±0.27^b	6.5±0.28^b	NA	NA
Group 2	2.35±0.07	6±0.3^c	5.4±0.09^c	3.68±0.02^a	2.38±0.03
Group 1	2.34±0.06	5.4±0.05^d	5.03±0.03^d	3.72±0.03^a	2.37±0.02
Group 6	2.35±0.06	2.28±0.12^e	2.3±0.07^e	2.3±0.05^b	2.35±0.7
Group 5	2.3±0.03	2.38±0.04^e	2.3±0.06^e	2.3±0.07^b	2.2±0.09

- Values with a different superscript on the same day were significant different from each other (P<0.05).
- Mean ± SE (n=6).

5.3.9. Effect of Feeding DR10™ with ANLENE, or SMP on the Phagocytic Activity of Peritoneal Macrophages
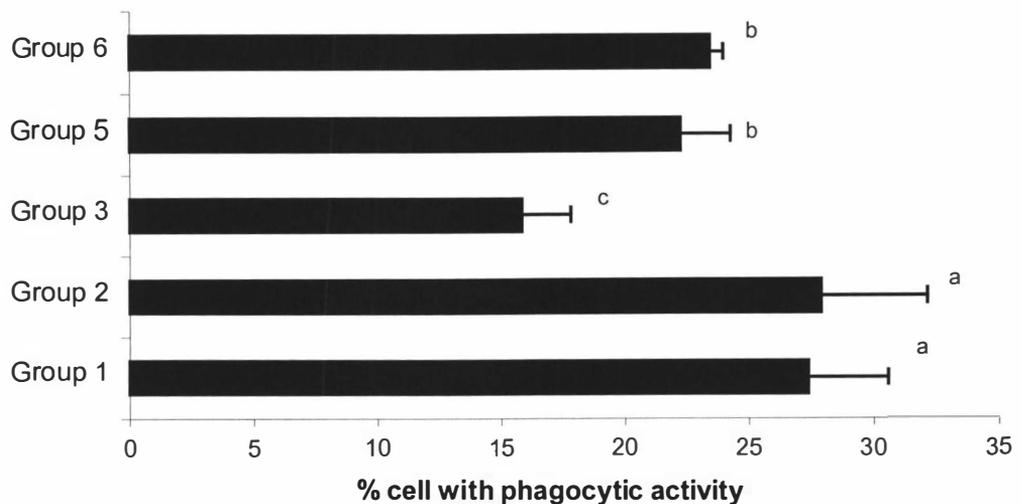
Figure 5.7: Phagocytic Activity of Peritoneal Macrophages.
- Mean±SE (n=3)
- Values with a different letter were significantly from each other (P<0.05).

As shown in Figure 5.7, the phagocytic activity in Group 3 was significantly lower than that in other 4 groups. Group 1 and Group 2 had a significantly higher phagocytic activity than Group 5 and Group 6. There was no significant difference between Group 1 and Group2 or between Group 5 and Group 6.

5.3.10. Effect of Feeding DR10™ with ANLENE or SMP on the Phagocytic Activity of Peripheral Blood Leukocytes

As shown in Figure 5.8, the phagocytic activity in Group 3 was significantly lower than that in other 4 groups. Group 1 and Group 2 had a significantly higher phagocytic activity than Group 5 and Group 6. However, there was no significant difference between the ANLENE feeding and SMP feeding.
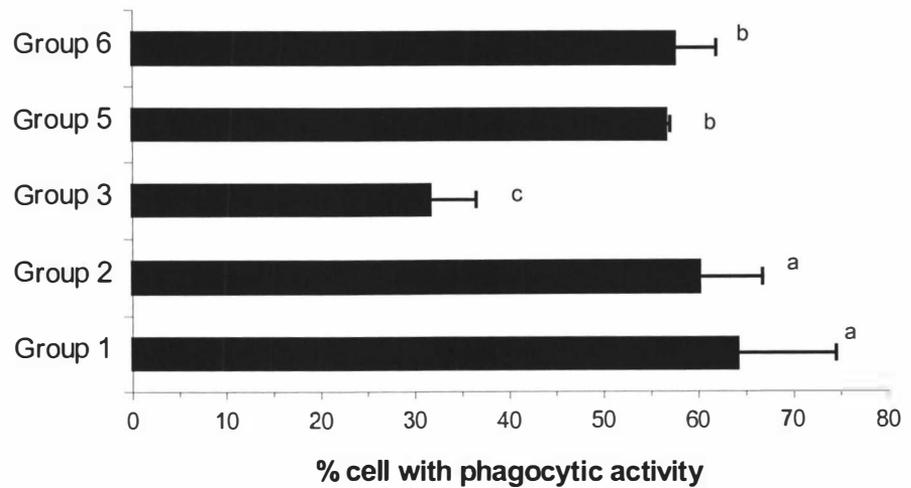
Figure 5.8: Phagocytic Activity of Peripheral Blood Leukocytes.

- Mean±SE (n=3)
- Values with a different letter were significantly different from each other (P<0.05).

5.3.11. Effect of Feeding DR10™ with ANLENE or SMP on the NK Cell Activity

As shown in Figure 5.9, the NK cell activity in Group 3 was significantly lower than that in other 4 groups. Group 1 and Group 2 showed a significantly higher NK cell activity than Group 5 and Group 6. There was no significant difference between ANLENE feeding and SMP feeding.
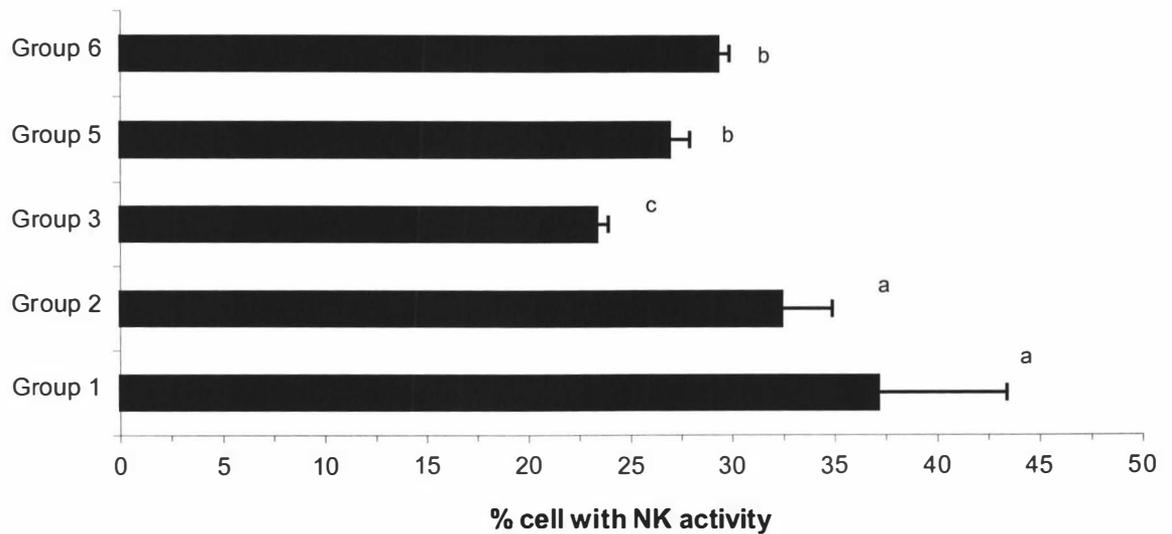
Figure 5.9: Effect of Feeding DR10™ with ANLENE or SMP on NK cell activity.

- Mean±SE (n=6)
- Values with a different letter are significantly different from each other (P<0.05).

5.3.12 Effect of Feeding DR10™ with ANLENE or SMP on the Antibody Production
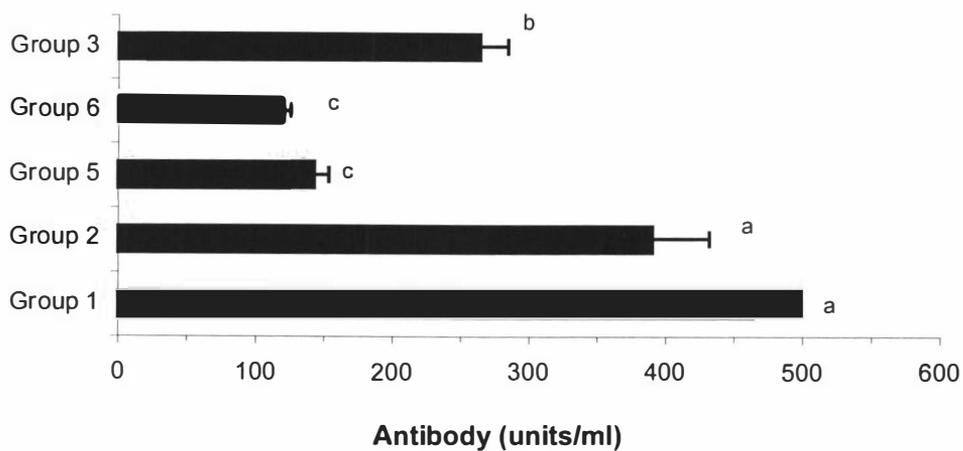
Figure 5.10: Effect of Feeding DR10™ with ANLENE or SMP on the Mucosal Anti-*Salmonella* Antibody Production.

- Mean±SE (n=6)
- Values with a different letter are significantly different from each other (P<0.05).
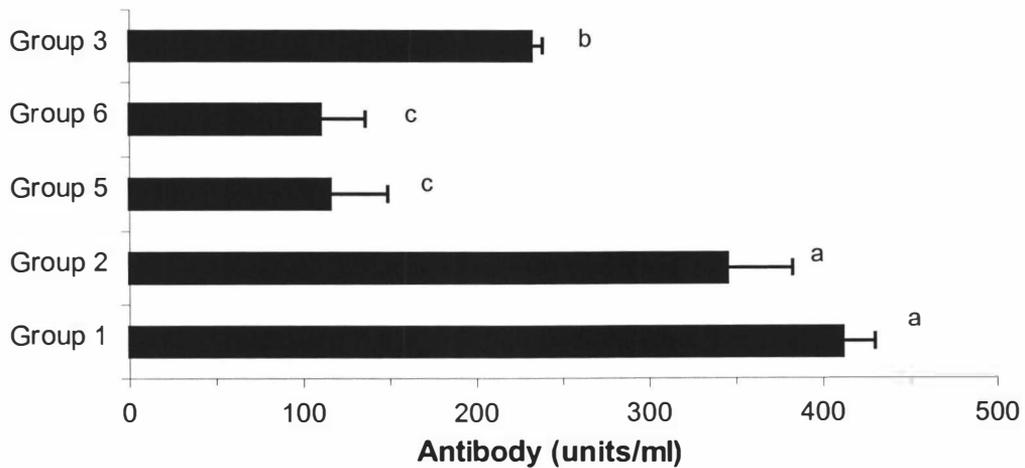
Figure 5.11: Effect of Feeding DR10™ with ANLENE or SMP on the Serum Anti-*Salmonella* Antibody Production.

- Mean±SE (n=6)
- Values with a different letter were significantly different from each other (P<0.05).

As shown in Figure 5.10, Group 1 and Group 2 produced significantly higher levels of mucosal anti-*Salmonella* antibody than Group 5, Group 6 and Group 3 did. In addition, Group 3 had a significantly higher level of mucosal anti-*Salmonella* antibody than Group 5 and Group 6 had. No other significant differences could be found.

This was also the case with the production of serum anti-*Salmonella* antibody. The significant difference among these groups was exactly the same as that in the production of mucosal anti-*Salmonella* antibody (Figure 5.11).

5.3.13. Effect of Feeding DR10™ with ANLENE or SMP on the Expression of CD4/CD8 ratio, CD25, CD40 on PP Lymphocytes

As shown in Table 5.6, Group 1 and Group 2 showed a significantly higher ratio of CD4 to CD8 and CD25 than those in Group 5, Group 6 and Group 3. No significant difference between Group1 and Group 2, between Group 5 and Group 6, and between Group 5 & 6 and Group 3 was found. The significant difference in CD25 among these groups was exactly the same as that in CD4/CD8 ratio.

Group 1 and Group 2 showed a significantly higher percentage of CD40 expression than that in Group 3. There was no significant difference in CD40 expression between Group 1 and Group 2, between Group 5 and Group 6, and between Group 5 & 6 and Group 3.

Table 5.6: CD4/CD8 ratio, CD25, and CD40 in PP Lymphocytes.

Group	CD4/CD8	CD25 (%)	CD40 (%)
Group 1	4.33 ± 0.68^a	6.06 ± 0.01^a	61.59 ± 3.53^a
Group 2	4.87 ± 0.39^a	6.17 ± 0.63^a	60.57 ± 2.66^a
Group 5	3.58 ± 0.24^b	4.85 ± 0.14^b	54.71 ± 1.64^{ab}
Group 6	3.43 ± 0.32^b	5.45 ± 0.21^b	53.86 ± 2.27^{ab}
Group 3	2.99 ± 0.06^b	5.29 ± 0.40^b	50.54 ± 3.07^b

- Mean±SE (n=3)
- Values with a different letter were significantly different from each other (P<0.05).

## 5.3.14. Effect of Feeding DR10™ with ANLENE or SMP on the Expression of CD4/CD8 ratio, CD25, CD40 on MLN Lymphocytes.

As shown in Table 5.7, Group 1 and Group 2 showed a significantly higher percentage of CD25 expression on MLN lymphocytes than Group 3. No other significant difference in CD25, CD4 /CD8 ratio, and CD40 expression among all the groups could be found.

Table 5.7: CD4/CD8 ratio, CD25, and CD40 on MLN Lymphocytes.

Group	CD4/CD8	CD25 (%)	CD40 (%)
Group 1	5.35±0.3	9.33±0.95[a]	18.77±3.24
Group 2	5.37±0.42	7.99±0.24[a]	16.25±2.48
Group 5	4.72±0.19	7.25±1.4[ab]	18.13±3.34
Group 6	4.63±0.26	7.24±0.54[ab]	20.74±4.28
Group 3	4.16±0.25	5.71±0.49[b]	16.38±1.06

- Mean±SE (n=3)

- Values with a different letter were significantly different from each other (P<0.05).

5.3.15. Effect of Feeding DR10™ with ANLENE or SMP on the Proliferative Responses of Spleen Lymphocytes to PHA and LPS.

As shown in Figure 5.12, there were significant differences in proliferative responses of spleen lymphocytes to LPS between Group 1 & Group 2, Group 5 & Group 6, and Group 3 with the order Group 1 & Group 2> Group 5 & Group 6> Group 3. No other significant difference could be found.

The significant differences in proliferative responses of spleen lymphocytes to PHA among all the groups were exactly the same as those to LPS (Figure 5.13).
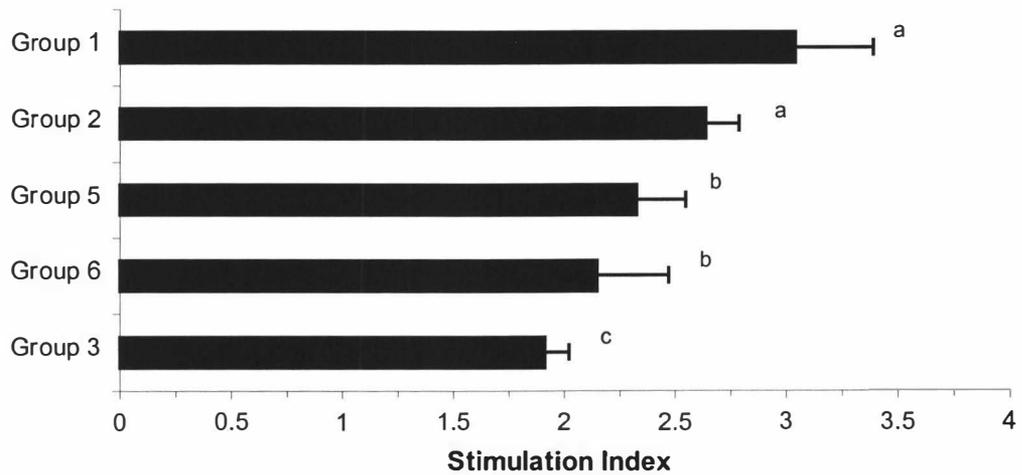
Figure 5.12: Proliferative Responses of Spleen Lymphocytes to PHA.

- Mean±SE (n=3)
- Values with a different letter were significantly different from each other (P<0.05).
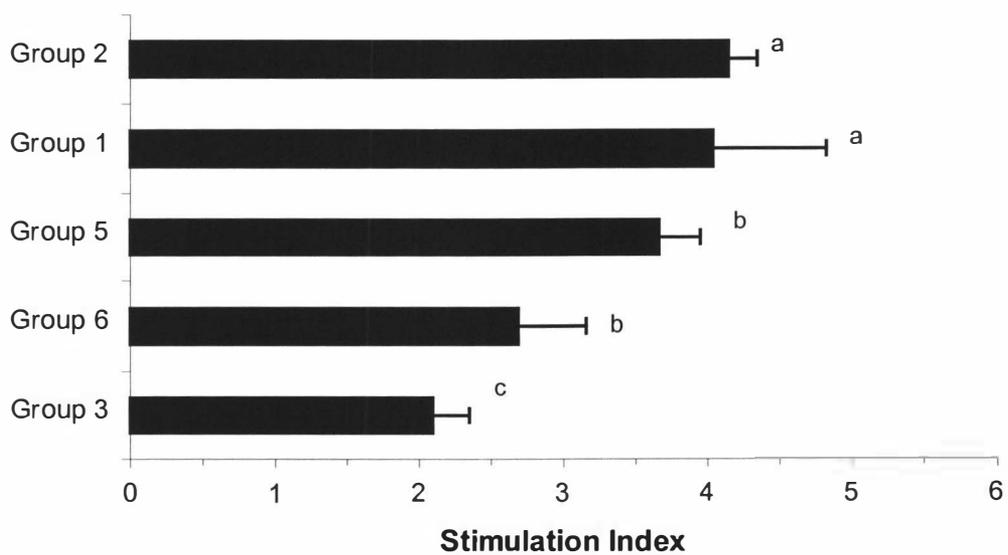
Figure 5.13: Proliferative Responses of Spleen Lymphocytes to LPS.

- Mean±SE (n=3)
- Values with a different letter were significantly different from each other (P<0.05).

5.3.16. Effect of Feeding DR10™ with ANLENE or SMP on the Proliferative Responses of PP Lymphocytes to PHA and LPS
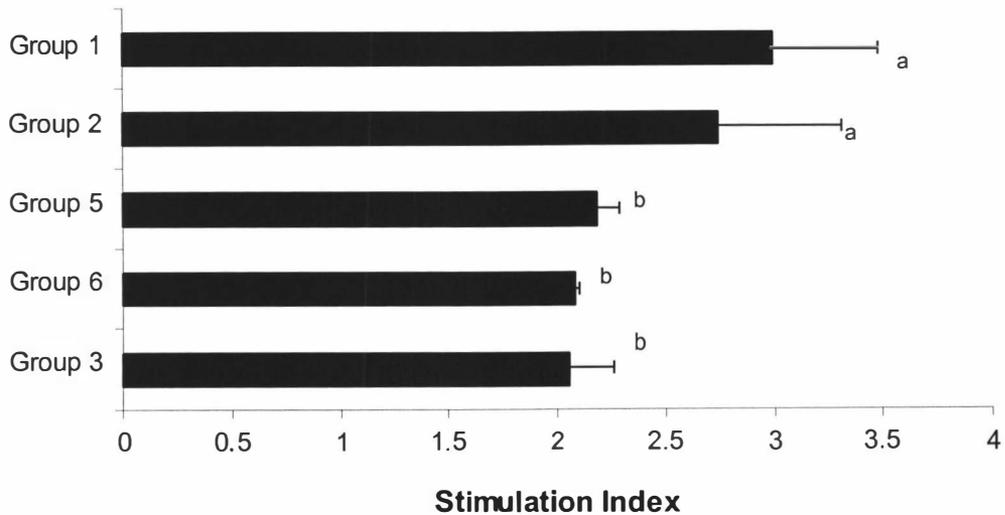
Figure 5.14: Proliferative Responses of PP Lymphocytes to PHA.

- Mean±SE (n=3)
- Values with a different letter were significantly different from each other (P<0.05).

As shown in Figure 5.14, Group 1 and Group 2 had a significantly higher SI to PHA than that in Group 5, Group 6, and Group 3. No other significant difference could be found.

There was no significant difference in the SI of PP lymphocytes to LPS among all the groups.

5.3.17. Effect of Feeding DR10™ with ANLENE with SMP on the Proliferative Responses of MLN Lymphocytes to PHA and LPS.

As shown in Figure 5.15, Group 3 showed to have significantly lower SI to LPS than other 4 groups. No other significant difference in the SI of MLN to LPS could be found.

The significant differences in SI to PHA among all groups were exactly the same as those in LPS (Figure 5.16).
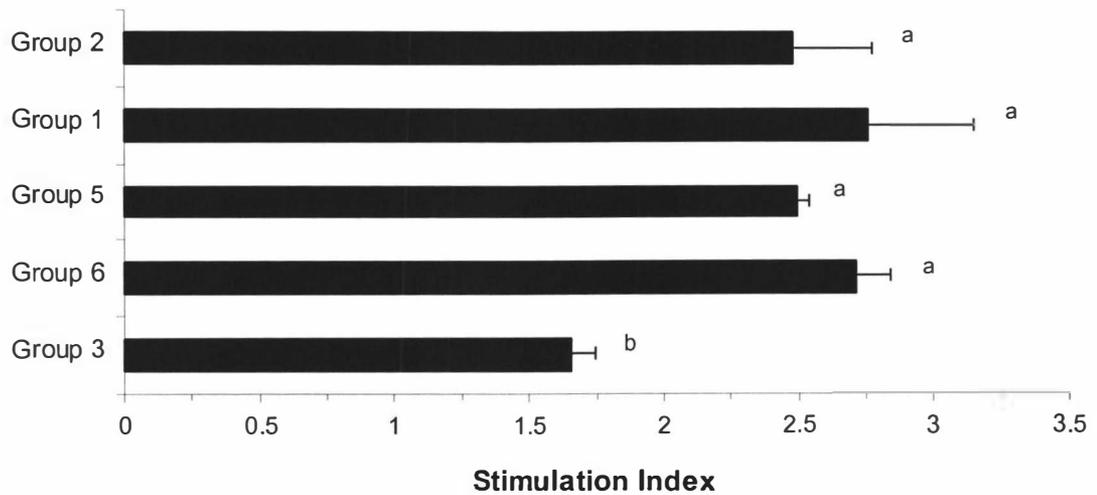
Figure 5.15: Proliferative Responses of MLN Lymphocytes to LPS.

- Mean±SE (n=3)
- Values with a different letter are significantly different from each other (P<0.05).
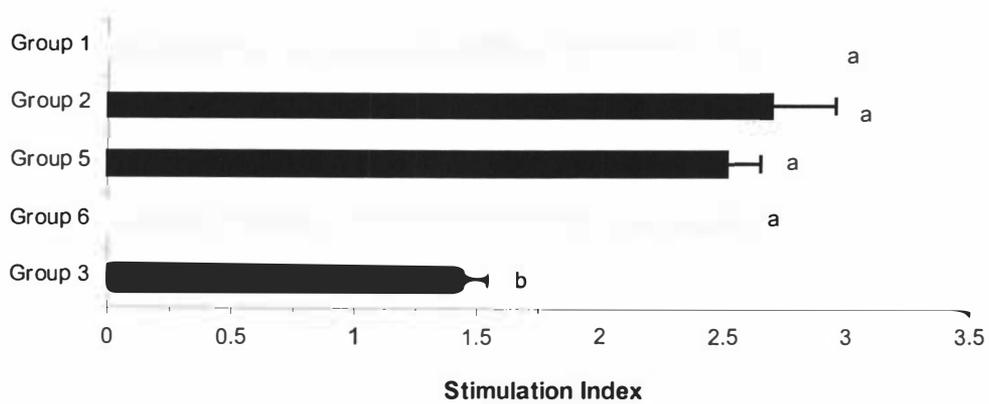
Figure 5.16: Proliferative Responses of MLN Lymphocytes to PHA.

- Mean±SE (n=3)
- Values with a different letter are significantly different from each other (P<0.05)

5.4. Discussion

Previous studies regarding DR10™ have demonstrated that DR10™ stimulates the host immunity under the normal and *Salmonella* infection condition (Gill *et al.*, 2000, Arunachalam *et al.*, and the previous study discussed in Chapter 4). In the present study, the immunomodulatory properties of milk powders containing different calcium concentrations with added DR10™ was assessed in *S. typhimurium* infection in mice. The resistance of the mice to this pathogen was also investigated.

The results of this study showed that calcium is very effective in reducing the severity of *S. typhimurium* infection. An important parameter in studies of the resistance to bacterial gastrointestinal infections is colonisation resistance. This is defined as the limiting action of the normal flora on colonization of the bowel by exogenous as well as indigenous potentially pathogenic microorganisms (Vollaard *et al.*, 1994). The results of the faecal excretion of *S. typhimurium* showed that feeding high calcium milk powder markedly improved the colonization resistance to this pathogen, as indicated by a rapid decline in faecal *S. typhimurium* excretion. As shown in Figure 5.6 and Table 5.5, each ANLENE-feeding group had a significantly lower number of faecal *S. typhimurium* than its corresponding SMP-feeding group. The faecal *S. typhimurium* excretion in Group 4 (SMP + Sal group) remained quite high while that in Group 3 (Anl + Sal group) dropped markedly after 5 days post-challenge. From these results, it can be stated that milk calcium inhibited colonization and thus shortened faecal shedding of the pathogen. The results from other studies also support this argument (Bovee-Oudenhoven *et al.*, 1996 & 1997). For example, in a study by Bovee-Oudenhoven *et al.*, (1997), it was found that colonization resistance to *Salmonella* was markedly impaired when the rats were fed the low-calcium diet while it was strengthened by feeding with medium and high-calcium diets.

Probably even more important than reducing the duration of carriage of *Salmonella* are the significant inhibitory effects of calcium supplementation on the translocation of *Salmonella* across the gut wall to the spleen and liver. Each ANLENE group had

a significantly lower number of viable *S. typhimurium* in the spleen than their corresponding SMP group (Figure 5.4). This becomes clearer when only Group 1 (Anl + Sal group) was compared with Group 2 (SMP + Sal group) since the compound factor, LAB, was excluded. This finding is consistent with that of Bovee-Oudenhoven *et al.,* (1997). They measured the translocation of *Salmonella* by urinary NO-derived nitrate excretion and by the viable *Salmonella* counts in the PP and spleen. Urinary excretion of NO derived nitrate has been demonstrated to be a sensitive and quantitative biomarker for the severity of bacterial translocation or systemic infections (Oudenhoven *et al.*, 1994). They found that there was significantly reduced urinary excretion of NO derived nitrate and a significant reduction in viable *Salmonella* counts in PP and spleen as well as the infection-induced increase in MLN weight in the calcium-supplemented groups compared to the low-calcium group.

So far, the mechanism(s) by which calcium exerts its protective effect against gastrointestinal infections are unclear. Dietary calcium induces gastric acid secretion by stimulating gastrin release (Floor *et al.*, 1991), which might explain the significantly reduced faecal *Salmonella* excretion of mice in Group 3 (Anl+Sal group) on the first day after oral infection. In the intestine, dietary calcium exerts cytoprotective effects on epithelial cells. Some studies showed that in the upper small intestine, calcium forms an insoluble complex with phosphate (Govers *et al.*, 1993, Van der Meer *et al.*, 1990). Cytotoxic components in the intestinal lumen, such as bile acids and fatty acids, are strongly precipitated by amorphous calcium phosphate, thereby decreasing the cell-damaging potential and reducing epitheliolysis (Govers *et al.*, 1994, Lapre *et al.*, 1993). Results of a study by Bovee-Oudenhoven *et al.,* (1997) supported this hypothesis. They found that calcium decreased the cytotoxicity of faecal water, probably by reducing the bile acid concentration. By lowering the cytotoxicity of intestinal contents and reducing epithelial cell damage, the mucosal integrity and resistance to infection might have been enhanced by supplemented calcium.

It is known that divalent cations such as calcium and magnesium play a regulatory role in the expression of virulence genes of *Salmonella* from *in vitro* studies (Vescovi *et al.*, 1996). For example, low concentrations of extracellular calcium induce *Salmonella* to express the pag genes, which may increase its survival in the phagosomes of macrophages. Vescovi *et al.*, (1996) also found that these virulence genes were repressed when *Salmonella* was present in extracellular fluids (i.e., at calcium levels of > 1mmol/L). Thus, calcium in the gastrointestinal lumen may play a role in the inhibition of expression of virulence genes of *Salmonella* in the gut. It was observed that Group 3 had a better GHAS from day 2 to day 13 and lower percentage of appearance of lethal clinical symptoms (ALCS) from day 5 to day 16 than these in Group 4, though they reached the same GHAS and percentage of ALCS afterwards. This indicates that calcium might reduce the virulence of *Salmonella* and thus decrease the severity of infection. However, since the severity of infection in these mice was quite high, calcium alone could not provide complete protection against this infection. At this stage, we can not reach a definite conclusion that calcium represses the expression of virulent genes of *Salmonella* and thus reduces the severity of infection since we do not know the concentration of calcium in the gastrointestinal lumen of the mice.

Soluble iron is an essential nutrient for growth of most bacteria, except some *lactobacilli* (Payne, 1988). Systemic iron overload increases the susceptibility for infections, including *Salmonella* (Jones *et al.*, 1977; Ampel *et al.*, 1989). Furthermore, iron is a catalyst in the generation of reactive oxygen species and plays a critical role in inflammatory processes, induced by bacterial infections (Kent *et al.*, 1994). Iron can be precipitated by calcium phosphate in the intestinal lumen (Bovee-Oudenhoven *et al.*, 1997), and thus, coprecipitation of iron can be helpful to minimize epithelial cell damage and to maximize the resistance against *Salmonella*.

It has been observed that Group 1 (ANLENE + DR10™ group) had better resistance against *Salmonella* infection than Group 2 (SMP + DR10™ group). This was shown by the lower percentage of ALCS (Figure 5.1), better GHAS (Figure 5.2), lower number of viable *Salmonella* in the spleen (Figure 5.4) and faeces (Figure 5.6).

These results support our hypothesis that LAB combined with milk calcium increases resistance to *Salmonella* infection.

The mechanism(s) by which LAB combine with calcium to increase resistance to *Salmonella* infection is not yet clear. It is likely that calcium may not only protect the epithelial cells, and thus improve the mucosal barrier via the mechanisms mentioned above, but also protect indigenous bacteria, like *lactobacilli*, thereby improving their antagonistic action towards pathogens. This hypothesis was supported by a study by Van der Meer *et al.,* (1998). As discussed above, the cytoprotective effects of dietary calcium are mediated by precipitation of hydrophobic, cytotoxic bile acids in the intestinal lumen. Thus, they first carried out an *in vitro* study to evaluate the possibly differential effects of bile acids on growth of *Salmonella* and *Lactobacillus acidophilus*, as a representative of the indigenous bacteria. Taurine-conjugated bile acids were studied to simulate the effects of bile acids secreted into the small intestine, while unconjugated bile acids were used to simulate the effects of deconjugation of bile acids by the intestinal microflora. Bile acids were found to hardly affect growth of *Salmonella*. In contrast, *L.acidophilus* was much more sensitive to bile acids. Conjugated bile acids dose-dependently inhibited growth, with 50% growth inhibition at about 5 mmol/L. Moreover, the lowest concentration of unconjugated bile acids tested (3 mmol/L) was enough to inhibit growth of *L. acidophilus* completely. This observed differential effect of bile acids on bacteria growth suggests that decreasing the concentration of soluble bile acids in the intestinal lumen, for instance by precipitating bile acids with calcium phosphate, might improve the competitive, antagonistic activity of LAB against *Salmonella*.

There was no significant difference found between Group 1(Anl +DR10™ group) and Group 2 (SMP+DR10™ group) in the immune parameters, such as: phagocytic activity of peritoneal macrophages & PBLs (Figure 5.7 & 5.8), CD4/CD8 ratio in PP and MLN lymphocytes, percentage of CD25 and CD40 expression on PP and MLN lymphocytes (Table 5.6 & 5.7), mucosal and serum anti-*Salmonella* antibody levels (Figure 5.10 & 5.11), proliferative responses of spleen, PP, and MLN lymphocytes

to LPS and PHA (Figure 5.12, 5.13, 5.14, 5.15, 5.16). This indicates that, compared to feeding of SMP (1200mg/100g milk powder), higher content of calcium (2000mg/100g milk powder) did not increase DR10™ 's ability to stimulate many aspects of immunity against *Salmonella* in the mice.

Based on the discussion above, the mechanisms of the effect of dietary calcium on resistance to intestinal bacterial pathogens were proposed as shown in Fig 5.17.
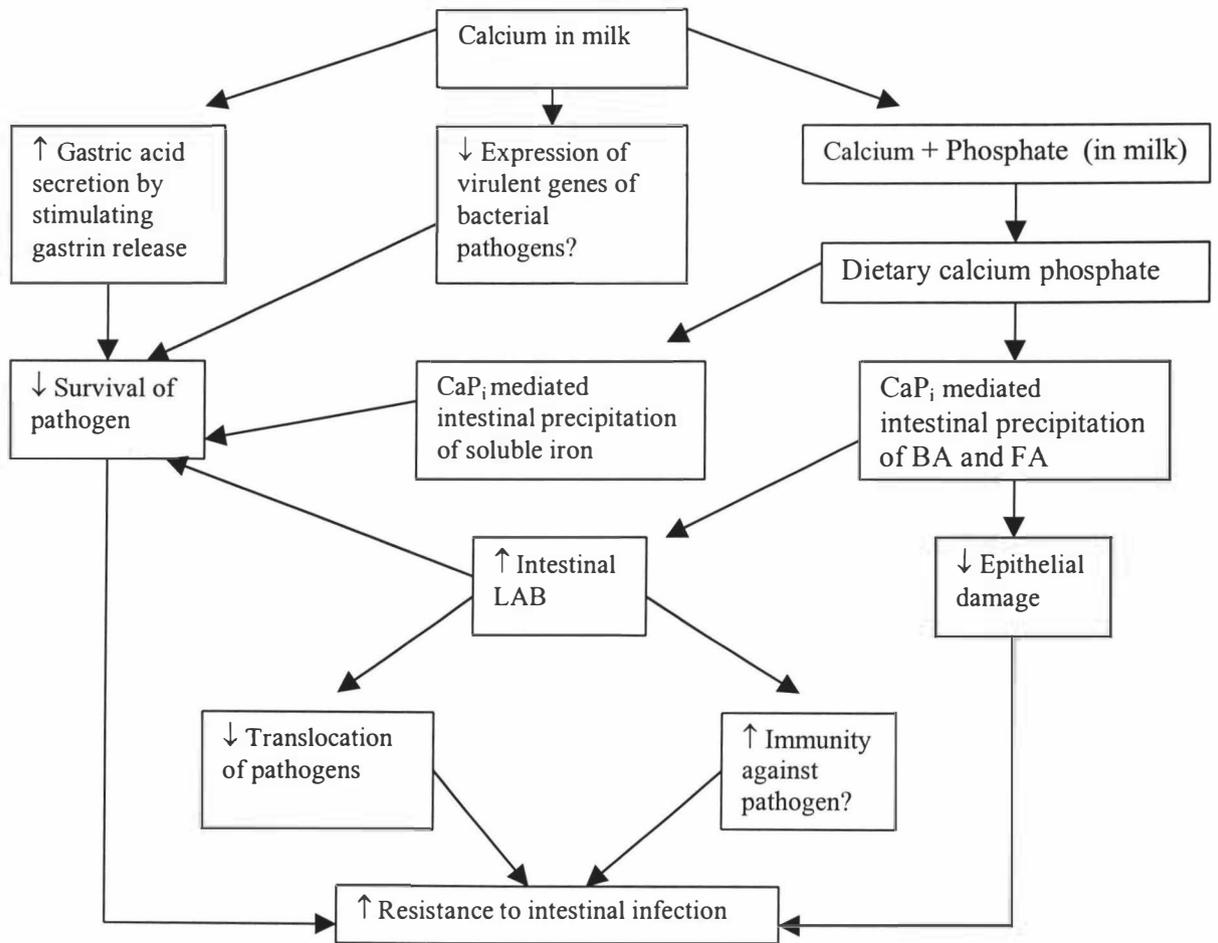

Figure 5.17: Proposed mechanisms of the effects of milk calcium on the resistance to intestinal bacterial pathogens (CaP$_i$: insoluble, amorphous calcium phosphate; BA: bile acids; FA: fatty acids).

The two DR10™ treatment groups (Group 1 & 2) showed a better resistance to *Salmonella* than that of the two *Salmonella* control groups (Group 3 & 4) in this

trial. This is shown by a lower percentage of ALCS (Figure 5.1), better GHAS (Figure 5.2), higher food intake (Table 5.3) and water intake (Table 5.4), positive live weight change (Figure 5.3), lower number of viable *Salmonella* in the spleen (Figure 5.4) and faeces (Figure 5.6), and lower spleen index (Figure 5.5). These results totally agree with those in the second trial. In addition, it was found that there was no significant difference in spleen index between DR10™ treatment groups (Group 1 & 2) and Group 5 & 6. That result also confirms the finding in the second trial that feeding DR10™ does not cause hepatomegaly or splenomegaly.

The increased resistance in the DR10™ treatment groups was accompanied by enhanced non-specific and specific immune responses to *S. typhimurium*. It was observed that there was a significant increase in phagocytic activity of peritoneal macrophages and PBLs (Figure 5.7 & 5.8), NK cell activity (Figure 5.9), production of both mucosal and serum anti-*Salmonella* antibody (Figure 5.10 & 5.11), CD4/CD8 ratio, CD25 and CD40 expression in MLN and PP lymphocytes (Table 5.6 & 5.7), proliferative responses of spleen & MLN lymphocytes to LPS & PHA, and PP lymphocytes to PHA (Figure 5.12, 5.13, 5.14, 5.15, 5.16) in the two DR10™ treatment groups (Group 1 & 2) compared to the *Salmonella* control group (Group 3 & 4). Most of these results are consistent with those in the second trial. However, the increased CD4/CD8 ratio in PP and MLN lymphocytes, CD40 in PP and MLN lymphocytes (Table 5.6 & 5.7), proliferative responses of PP lymphocytes to PHA (Figure 5.14) and MLN lymphocytes to LPS and PHA in the DR10™ groups (Group 1 & 2) (Figure 5.15 & 5.16) were not found in the second trial. Calcium seems not to be a factor that could be used to explain this difference in the results between two trials since no significant differences in these immune parameters between Group 1 and Group 2 were found in this trial. This difference in the results between two trials might only be explained as the variation between the experiments. It was observed that the DR10™ treatment groups tended to have higher levels of these immune parameters than the *Salmonella* control group in the previous trial (discussed in Chapter 4). In addition, the performance of these immune assays in the current trial was better than that in the previous one (discussed in Chapter 4) because of the

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improvement of skills. Thus, the results from the current trial seem more reliable. However, one should be very cautious in interpreting these results since they were based on a very small samples (n=3). A larger number of samples are needed to confirm these results in future trials.

Another important finding in this trial was that LAB feeding enhanced NK cell activity. The two DR10™ treatment groups (Group 1 & Group 2) were shown to have a significantly higher level of NK cell activity than the two *Salmonella* control groups (Group 3 & Group 4) (Figure 5.9). This result is consistent with other studies (De Simone *et al.,* 1989; 1986; and Gill, 1998). Since comparatively few studies have examined the effect of LAB consumption on NK cell activity, this finding of current trial makes some contribution to the research in this area. NK cells are the principal effectors of anti-tumour immunity and also participate in the destruction of virus-infected cells. Thus, it can be concluded from this result, together with the finding that DR10™ can enhance phagocyte function, that DR10™ could stimulate aspects of non-specific immunity and thus may impact positively on human health.

In summary, the results from current trial confirm most findings in the previous trial (discussed in Chapter 4). The main significance of this trial is that it has demonstrated the calcium in milk products and the combination of high content of milk calcium combined with DR10™ increase resistance to *Salmonella* infection.

Chapter 6: General Discussion and Conclusions

Over the last decade, the effects of LAB on the immune system and their anti-infection properties have been subjected to the extensive research (IDF, 1991; De Simone *et al.*, 1991; Perdigon and Alvaraz; 1992, Marteau and Rambaud, 1993). In many of these studies, LAB have been found to be able to stimulate a wide range of immune parameters including both non-specific and specific immune systems in human and animals (discussed in chapter 1). In addition, LAB have been found to be able to protect against enteric infection in some studies (Majamaa *et al.*, 1995; Perdigon *et al.*, 1990 & 1995a; Popova *et al.*, 1993; Nader de Macias *et al.*, 1992; De Pertrino *et al.*, 1995; Kaila *et al.*, 1992). However, the results of these studies have been varied, due mainly to differences in species and strains of LAB, dose, delivery medium and experimental protocols (Gill, 1998). These variables make it difficult to compare the results of the different studies, and thus, many questions and gaps still remain in our knowledge of LAB's effect on the immune system and their anti-infection properties.

Not all LAB have immunoenhancing abilities and different strains and species of LAB have different abilities to stimulate the immune system (De Simone *et al.*, 1987; Perdigon *et al.*, 1986 &1990; Francisco *et al.*, 1995; Majamaa *et al.*, 1995; Paubert-Braquet *et al.*, 1995; Schiffrin *et al.*, 1995). Consequently, it is necessary to determine what strains or species of LAB have immunomodulatory and anti-infection 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. This research project was carried out to evaluate the efficacy of a newly identified LAB strain, DR10™, on stimulating immunity and protecting against bacterial infection. This was one of the aims of this project.

There is some evidence that milk calcium is able to increase host defense against bacterial infection (Van der Meer *et al.*, 1998; Bovee-Oudenhoven *et al.*, 1997 & 1996). The probable mechanisms for this effect could be that milk calcium

stimulates protective bacteria such as LAB, and strengthens the mucosal barrier in the small intestine (Van der Meer, *et al.*, 1998). Since both LAB and calcium in the milk products have been found to be able to protect host against bacterial infection, it could be suggested that LAB combined with calcium in the milk can increase resistance to bacterial infection. This was another aim of the project.

A suitable bacteria-infected model was first needed to evaluate the LAB's efficacy in protecting against bacterial infection and enhancing immunity. This was the initial aim of the project.

A suitable animal model of bacterial infection should be the one that can cause clinical signs of infection whilst allowing the animals to live long enough to let us measure both bacterial translocation and some parameters of the immune response, since bacterial translocation and immune responses take some time to happen, especially antibody production.

First, the dose of *S. typhimurium* to cause 50 % BALB/c mice to reach GHAS of 5 (appearance of lethal clinical symptoms) was determined since it is very sensitive and stable index in measuring the toxicity. Based on this dose, the virulence of *S. typhimurium* could be found out and then the dose concentration in further trial could be decided. The correct enumeration of bacteria was essential in determining the dose. There are three kinds of methods for enumerating the bacteria number: plate count, direct microscopic count, and turbidimetric assay. Each has some advantages and disadvantages. The turbidmetric assay was chosen to enumerate the bacterial number in the end as it provides rapid, convenient, and reasonably accurate enumeration of the bacterial number. In this method, a standard curve is required. The correlation coefficient (R) between absorbance and the number of cells in our standard curve was 0.9952 (Figure 3.2), indicating a very good linear relationship between them. Hence, an accurate enumeration of live bacteria in a suspension could be obtained by using this standard curve.

The dose of 1×10^7 cfu/mouse was chosen for the future trials since it met the criteria outlined at the beginning of the trial. Under this dose, mice appeared clinical signs of

severe infection without dying too soon. The mice challenged with this dose reached GHAS of 5 all after 7 days post-challenge, which left enough time to examine bacterial translocation and immune responses.

In the second trial, mice were fed with DR10™ then challenged with *Salmonella typhimurium*. It was found that the DR10™ group had significantly increased protection against *S. typhimurium* infection. The enhanced resistance to *S. typhimurium* in the DR10™ fed group was accompanied by increased both non-specific and specific immune responses to *S. typhimurium*. It was found that the number of *S. typhimurium* in the liver and spleen was inversely related to the antibody levels in the gut and serum, and the phagocytic activity of peritoneal macrophages and PBLs on day 7 post-challenge. The DR10™ treatment group had a significantly higher mucosal antibody level and enhanced phagocytic activity of peritoneal macrophages and PBLs than those in the *salmonella* control group. The increased antibody level in the DR10™ treatment group was accompanied by an increase in the proliferative responses of spleen lymphocytes to PHA and LPS, and an increase in CD25 expression on PP and MLN lymphocytes.

Not many significant differences in immune parameters were found between the DR10™ treatment group and the SMP control group. However, since *S. typhimurium* infection led to immunosuppression in mice (Van Leeuwen *et al.*, 1994), and there was no significant difference in immune parameters between the DR10™ feeding group and the SMP control group, this means that DR10™ might increase the immunity under non-*Salmonella* infection conditions. This has been proven by the previous research work done in MHRC, Massey University (Gill *et al.*, 2000). In addition, long term feeding of DR10™ was found not to cause either hepatomegaly or splenomegaly, indicating that it may not have harmful effects on the host.

In the third trial, milk calcium was found to be very effective in reducing the severity of *S. typhimurium* infection. The results of the faecal excretion of *S. typhimurium* showed that feeding high calcium milk powder markedly proved the colonization resistance to *S. typhimurium,* as indicated by a rapid decline in faecal *S.*

typhimurium excretion. Another important finding is the significant inhibitory effects of calcium supplementation on the translocation of *Salmonella*. Each high calcium-feeding group had a significantly lower number of *S. typhimurium* in the spleen than that in its corresponding SMP feeding group.

Group 1(ANLENE+DR10™ fed group) was observed to have better resistance against *Salmonella* infection than Group 2 (SMP+DR10™ fed group). This was shown by the lower rate of appearance of lethal clinical symptoms, better GHAS, higher food intake, lower number of viable *Salmonella* in the spleen and faeces. These results indicate that DR10™ combined with milk calcium increases resistance to *Salmonella* infection. However, combination of higher content of milk calcium with DR10™ was found not to increase its ability to stimulate many aspects of immune responses.

The mechanism(s) for the effect of milk calcium on the resistance to intestinal bacterial pathogens, again, are not very clear. Milk calcium combined with DR10™ may act via the mechanism shown in Figure 5.17 to promote resistance to pathogenic bacterial infection.

All DR10™ treatment groups were shown to have a better resistance against *S. typhimurium* and higher immune parameters in many aspects of immune responses than the *Salmonella* control groups. These results agreed with the finding in the second trial. In addition, feeding of DR10™ was observed to enhance the NK cell activity in the last trial.

In summary, in these three trials, a suitable *Salmonella* -infected mouse model was first established and successfully used in the second and third trial. In general, this model is reliable, and easy to produce, thus it can be useful in evaluating the efficacy of other strains of LAB in preventing *Salmonella* infection and stimulating immunity in the future studies. The other two trials were then successfully carried out based on the animal model established in the first trial. The results of the second trial

presented here suggest that the resistance against *Salmonella* infection and a range of immune parameters including both non-specific and specific immune responses were enhanced by feeding with DR10™. The enhanced immune responses in the DR10™ treatment group provided the protection against *Salmonella* infection. Thus, this study provides additional evidence of the role played by the enhanced immune system in protecting enteric infection. The third trial demonstrated the effect of milk on enhancing resistance to *Salmonella* infection. In addition, it proved that DR10™ combined with milk calcium might increase its ability to prevent *Salmonella* infection. In addition, it confirmed many findings in the second trial. In these studies, the exact time that DR10™ will survive in the diet exposed to normal conditions is unknown since DR10™ (*Bifidobacterium lactis*) is anaerobic and may not survive under such conditions. Thus, the exact viable cells being taken by each mouse daily are unclear, and might be lower than 2×10^8 cfu/gram. The interaction between DR10™ and calcium was also not examined in chapter 5, it would be interesting to study such interaction and to find out whether the different amounts of calcium can enhance DR10™'s immune-stimulating ability.

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