Immunomodulatory Properties Of Bovine Whey Proteins And Whey Protein Concentrates

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

In

Nutritional Science and Immunology

at Massey University, Palmerston North, New Zealand

Pauline Ping Lin Low

2004

Massey University
COLLEGE OF SCIENCES

INSTITUTE OF FOOD, NUTRITION
AND HUMAN HEALTH
Te Kura Hangarau o
Kai-oranga-ā-tāngata
Private Bag 11 222
Palmerston North
New Zealand
T 64 6 350 4336
F 64 6 350 5657
www.massey.ac.nz

SUPERVISOR'S DECLARATION

This is to certify that the research carried out for the Doctoral thesis entitled "**Immunomodulatory Properties of Bovine Whey Proteins and Whey Protein Concentrates**" was done by **Pauline Ping Lin Low** in the **Institute of Food, Nutrition and Human Health**, Massey University, Palmerston North, New Zealand. The thesis material has not been used in part or in whole for any other qualification, and I confirm that the candidate has pursued the course of study in accordance with the requirements of the Massey University regulations.

Supervisor's Name **Dr Kay Rutherfurd-Markwick**

Signature K J Rutherfurd-Markwick

Date 18/10/04

Massey University
COLLEGE OF SCIENCES

INSTITUTE OF FOOD, NUTRITION AND HUMAN HEALTH
Te Kura Hangarau o Kai-oranga-ā-tāngata
Private Bag 11 222
Palmerston North
New Zealand
T 64 6 350 4336
F 64 6 350 5657
www.massey.ac.nz

CANDIDATE'S DECLARATION

This is to certify that the research carried out for my Doctoral thesis entitled "**Immunomodulatory Properties of Bovine Whey Proteins and Whey Protein Concentrates**" in the **Institute of Food, Nutrition and Human Health**, Massey University, Palmerston North, New Zealand is my own work and that the thesis material has not been used in part or in whole for any other qualification.

Candidate's Name **Pauline Ping Lin Low**

Signature 

Date 15.10.04

Massey University
COLLEGE OF SCIENCES

INSTITUTE OF FOOD, NUTRITION
AND HUMAN HEALTH
Te Kura Hangarau o
Kai-oranga-ā-tāngata
Private Bag 11 222
Palmerston North
New Zealand
T 64 6 350 4336
F 64 6 350 5657
www.massey.ac.nz

CERTIFICATE OF REGULATORY COMPLIANCE

This is to certify that the research carried out in the Doctoral Thesis entitled:

Immunomodulatory Properties of Bovine Whey Proteins and Whey Protein Concentrates

in the **Institute of Food, Nutrition and Human Health**
at Massey University, Palmerston North, New Zealand:

(a) is the original work of the candidate, except as indicated by appropriate attribution in the text and/or in the acknowledgements;
(b) that the text, excluding appendices/annexes, does not exceed 100,000 words;
(c) all the ethical requirements applicable to this study have been complied with as required by Massey University, other organisations and/or committees which had a particular association with this study, and relevant legislation.

Please insert Ethical Authorisation code(s) here: 95/150, 96/103, 96/56

Candidate's Name:
Pauline Low

Signature:

Date: 15.10.04

Supervisor's Name:
Dr Kay Rutherfurd-Markwick

Signature:

Date: 18/10/04

ABSTRACT

In recent years, partly due to advances in protein separation technology, many studies have focused on the immunomodulatory activity of bovine milk and colostral protein components. Individual milk proteins have been purified for the purpose of studying their physical properties and physiological functions. Today there is substantial evidence to indicate that the major components of bovine milk, such as whey protein and several highly purified whey protein isolates, can regulate immune function in heterologous species. Intense research has focused on identifying biologically active components within bovine milk whey, as well as characterising the mode by which mammalian immune function is modulated by these components. However, information regarding the effect of bovine whey proteins on immune responses to orally and parentally-administered antigens is currently conflicting and far from exhaustive. Consequently, this thesis sought not only to investigate the immunomodulatory ability of previously untested bovine whey products on general immunoresponses but also to investigate the ability of bovine whey proteins to modulate murine immune responses to vaccines currently in routine medical use. Initially, individual whey proteins (α-lactalbumin, β-lactoglobulin and lactoferrin) were screened for their *in vitro* effects on lymphoid cell function and phagocytic function. These *in vitro* studies found that the individual whey protein components had a positive immunomodulatory effect, providing evidence that these components have the potential to enhance immune function, and investigation into their immunomodulatory capabilities in an *in vivo* murine model was consequently undertaken. The results of the *in vivo* studies demonstrated that the dietary whey protein isolates and whey protein concentrate tested in this study could not only enhance two important indices of *ex vivo* lymphoid and non-lymphoid cell function (lymphocyte proliferation and phagocytic function) but could enhance mucosal and systemic antibody responses to orally and systemically administered human vaccines. The demonstrated benefits to the immune system of dietary whey proteins in the murine model could result in the production of immune-boosting, nutritionally and physiologically advantageous food supplements suitable for human consumption. Of particular relevancy to modern human health is the use of whey proteins as dietary adjuvants or immunopotentiators to increase immune responses to commonly administered vaccines.

ACKNOWLEDGMENTS

I would like to thank, first and foremost, two people for whom without their efforts, incredible patience and kindness this thesis would not exist - my supervisors Dr Kay Rutherfurd-Markwick and Dr Frank Cross. I express my sincere thanks to Dr Kay Rutherfurd-Markwick for her superior expertise in all areas of this work, continued encouragement, optimism, understanding, delicious cakes and friendship throughout the entire course of my studies. I express my sincere thanks to Dr Frank Cross for his wonderful way with words, support, friendship, intellectual input and guidance especially during the write up of this thesis.

I would like to extend a special thank you to my dear friend Kim Kennedy for her invaluable helping hand (the photocopy of which I treasure), for putting up with me and for making me smile. I would like to thank Sarah Blackburn for her technical advice and assistance and for providing strong competition in our Hokowhitu pie-eating competitions. Thanks to Anne Broomfield for her excellent animal care and handling skills and providing me with a TV and desk. Special thanks to Jade Robertson for her helpful assistance in the laboratory and at wine and cheese evenings. I also wish to acknowledge the following people for their specific contributions to this study - the members of the Doctoral Research Committee, Massey University, Palmerston North for their generosity and understanding over the years, Bryden Zalom for continuously fixing my problem prone computer, Mark Pritchard for providing the whey proteins and information regarding these proteins and Dr Geoff Rickards from Victoria University, Wellington, New Zealand for inspiring me to embark on PhD study. I acknowledge the financial support of Massey University through a PhD scholarship award.

Heartfelt thanks to my family for their unconditional love and support. Thank you to Mum, Dad, Angeline and all my special furry friends for always 'being there' for me in so many ways. Special thanks to Joe Kelly for his undying confidence and faith in me and for demonstrating remarkable patience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

α	alpha
ACE	angiotensin converting enzyme
ANOVA	analysis of variance
β	beta
B cell	a lymphocyte that matures in bone marrow and when activated differentiate into plasma cells that secrete antibodies
BCG	*Mycobacterium bovis,* strain BCG
BCM-7	β-casomorphin-7
BrdU	5-bromo-2'deoxyuridine
C	Celsius
CLA	conjugated linoleic acid
CO_2	carbon dioxide
Con A	concanavalin A
CGP	caseinoglycopeptide
CT	cholera toxin (B subunit) vaccine
DHA	docosahexanoic acid
Dip	diphtheria toxoid vaccine
DMH	dimethylhydrazine
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
E. coli	*Escherichia coli*
EDTA	ethylenediaminotetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPA	eicosapentaenoic acid
FACS	fluorescence-activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Flu	influenza virus vaccine
γ	gamma
g	grams

GALT	gut-associated lymphoid tissue
GF	growth factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSH	glutathione (L-gamma-glutamyl-L-cysteinyl-glycine)
hrs	hours
Ig	immunoglobulin
IU	international units
kDa	kilo Daltons
L	litre
LF	lactoferrin
LPS	lipopolysaccharide
μ	micro
m	milli
M	molar
max	maximum
MHC	major histocompatibility complex
min	minute
mM	milli molar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	nano
NK	natural killer
NZDB	New Zealand Dairy Board (now known as Fonterra)
NZDRI	New Zealand Dairy Research Institute (now known as Fonterra Marketing and Innovation)
ω	omega
OD	optical density
OV	ovalbumin
PBL	peripheral blood leucocytes
PBS	phosphate buffered saline
pers. comm.	personal communication
PHA	phytohaemagglutinin
PWM	pokeweed mitogen
RNA	ribonucleic acid

RPMI 1640	Roswell Park Memorial Institute 1640
S. aureus	*Staphylococcus aureus*
SEM	standard error of the mean
SRBC	sheep red blood cells
S. typhi	*Salmonella typhi*
S. typhimurium	*Salmonella typhimurium*
T cell	a thymus-derived lymphocyte that differentiates after activation into a cytotoxic T cell or a helper T cell
$TCID_{50}$	log_{10} 50% tissue culture infective dose
T. cruzi	*Trypanosoma cruzi*
TT	tetanus toxoid vaccine
U	units
UHT	ultra high temperature
WPC	whey protein concentrate
WPI	whey protein isolate
w/w	weight/weight
v/v	volume/volume

PUBLICATIONS

Publications arising from this work:

Enhancement Of Mucosal Antibody Responses By Dietary Whey Protein Concentrate. Low PPL, Rutherfurd KJ, Cross ML & Gill HS (2001) *Food and Agricultural Immunology* **13**, 255-264.

Effect Of Dietary Whey Protein Concentrate On Primary And Secondary Antibody Responses In Immunized BALB/c Mice. Low PPL, Rutherfurd KJ, Gill HS & Cross ML (2003) *International Immunopharmacology* **3**, 393-401.

CHAPTER 1

Literature Review and General Introduction

1.1 Immunonutrition

"If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health"

Hippocrates (c. 460-377 B.C.)

Since ancient times health and immunity has been thought to depend, to some extent, on nutrition. Beyond supplying a source of calories and nutrients to prevent nutritional diseases such as malnutrition, scurvy and rickets, the food we eat can contribute to the maintenance of health and the prevention of a broad range of diseases (reviewed by Sanders, 1998). This ancillary effect, exhibited by functional foods ("a modified food or food ingredient that provides a health benefit beyond satisfying traditional nutritional requirements" (Regester *et al.*, 1996)), occurs through the modulation of our basic physiology, including immune, endocrine, nerve, circulatory, and digestive systems (Sanders, 1998). In the 1970s the interdependency between the science of nutrition and immunology was recognised when immunological measures were introduced as a component of assessing nutritional status (Bistrian *et al.*, 1975). In the last 30 years systematic studies have confirmed that nutrient deficiencies impair immune responses and lead to frequent severe infections resulting in increased mortality, especially in children (Chandra, 1996).

It is now well established that nutritional deficiency is commonly associated with impaired immune responses, particularly cell-mediated immunity, phagocyte function, cytokine production, secretory antibody response, antibody affinity and the complement system (Cunningham-Rundles & Ho Lin, 1998; Chandra, 1999). Thus the optimisation of the immune system, through dietary intervention, provides the opportunity to improve disease resistance and health.

1.2 Immunomodulatory Dietary Components

Several dietary components have been shown to influence physiological systems, including the immune system. These dietary components include the trace elements and vitamins, components that modify the gut microflora (such as pro-/pre-biotics) or components that modulate fatty acid metabolism. Dietary proteins are also important, both quantitatively (e.g. protein energy malnutrition can lead to immune suppression) and qualitatively (e.g. different protein types have been shown to effect the immune system in different ways). The research described in this section illustrates how the two disciplines of nutrition and immunology have been combined to study the modulation of the immune system.

1.2.1 Trace elements and vitamins

The association between micronutrient deficiencies of trace elements/vitamins and immune response was first recognised by Good *et al.,* (1977) who proposed that the deficiency of certain key trace elements may be the cause of immune deficiency in the malnourished host. Subsequent research has now established that dietary deficiencies of trace elements (such as zinc and iron) and vitamins (such as A, C, and E) impair immunity (Cunningham-Rundles & Ho Lin, 1998; Chandra, 1999; Field *et al.*, 2002). For example, zinc deficiency has been shown to impede host-defence systems, leading to susceptibility to a variety of pathogens (Prasad, 1988; Fraker *et al.*, 2000) and iron deficiency is estimated to affect 20 - 50% of the world's population, making it the most widely spread nutritional deficiency in industrialised and developing countries (Field *et al.*, 2002). Iron has also been found to regulate the function of T lymphocytes and in most animal studies (*in vitro* and *in vivo*) iron deficiency results in impaired cell mediated immunity and bactericidal activity and may also delay the development of cell-mediated immunity (reviewed by Beard, 2001; Oppenheimer, 2001). Dietary vitamin A (retinol) deficiency can affect host defences directly through its essential functions in metabolism in various immune cells (Ross, 1992) or indirectly through its role in epithelial cell differentiation and host barrier function (McDowell *et al.*, 1984). Additionally, vitamin C (ascorbic acid) is highly concentrated in leucocytes and is used rapidly during infection (preventing oxidative damage) (Field *et al.*, 2002). Reduced concentrations of vitamin C in leucocytes is associated with reduced immune function (Schwager & Schulze, 1998) and in humans the clinical deficiency of vitamin C results

in scurvy, a disease where infections occur and there is anergy (poor or immeasurable immune response) of components of the immune system (Friedman *et al.*, 1994).

Micronutrients have been found to be most effective at boosting immunity in those predisposed to immunosuppression; consequently many studies have focused on the immunomodulatory role of micronutrients and vitamins in infants and the elderly (reviewed by Gill *et al.*, 2001a). In children, low levels of iron or zinc have been shown to be associated with a decrease in the *in vitro* production of interleukin-2 (Thibault *et al.*, 1993) and proliferation of lymphocytes (Licastro *et al.*, 1994), respectively. In the elderly, dietary multivitamin and mineral supplementation resulted in increased numbers and/or activity of T cells ($CD3^+$, $CD4^+$, $CD25^+$) and natural killer (NK) cells, an increase in mitogen stimulated T-lymphocyte proliferation and cytokine production, and higher antibody titers to an influenza vaccine compared to placebo-supplemented subjects (Chandra, 1992). The capacity of dietary supplementation to enhance cell-mediated immune functions was confirmed by a study demonstrating that mixed micronutrient supplementation can enhance delayed-type hypersensitivity (DTH) responses to skin test antigens in elderly subjects (Bogden *et al.*, 1994). Elderly subjects have also been shown to benefit from defined vitamin supplementation, for example a mixture of vitamins A, and C and E enhanced the proliferation of circulating T cells ($CD4^+$, $CD8^+$) (Penn *et al.*, 1991) and a mixture of vitamins C and E increased both lymphocyte proliferative responses and the phagocytic capacity of polymorphonuclear granulocytes *in vitro* (de la Fuente *et al.*, 1998).

In general, it is recognised that antioxidants play an important role in all aspects of the immune response: phagocyte function, cytokine production, cell mediated immune responses and immunoglobulin production (Tengerdy, 1990). Vitamin E and selenium are also known to have anti-carcinogenic effects by means of their antioxidant properties, and are supplemented routinely in the diets of domestic animals for their immune benefits (such as reduced immunosuppression, anti-viral and anti-inflammatory activity) (Tengerdy, 1989). Vitamin E (α-tocopherol) is the main membrane-bound antioxidant and plays a role in human nutrition as a free radical scavenger, preventing lipid peroxidation damage (Levy, 1998). However, it has been suggested that antioxidant vitamins may also reduce the action of immunosuppressive eicosanoids, such as prostaglandins. In aged mice, dietary antioxidant vitamins have been shown to

not only enhance parameters of cell mediated immunity (DTH and lymphocyte proliferation) but also reduce the production of prostaglandin $E1_2$ (Meydani *et al.*, 1986). In addition to the vitamins E and C, carotenoids (present in many plants providing photo-protection and acting as accessory pigments in photosynthesis) have been shown to affect pro-oxidant/antioxidant balance and reduce the consequent risk of degenerative damage (reviewed by Morrissey & Brien, 1998). Epidemiologic studies have also shown strong associations between diets rich in carotenoids and a reduced incidence of many forms of cancer, leading to the suggestion that the antioxidant properties of these compounds might help protect immune cells from oxidative damage (reviewed by Hughes, 2001). Vitamin A has also received a great deal of research attention and has been shown to play a role in cellular differentiation, antitumourgenicity and immunomodulation (Bates, 1995). In children, vitamin A supplementation has been shown to enhance specific IgG production and total lymphocyte numbers (Coutsoudis *et al.*, 1992) and confer a protective effect against viral infections, specifically measles (Rumore, 1993). The excessive intake of vitamin A has however been shown to be toxic, especially in the elderly (Ward, 1996). Additional studies on the effect of dietary vitamin supplementation in the elderly have found that vitamin C reduces autoimmune responses (via a glucocorticoid-induced mechanism) (Kodama *et al.*, 1994) and vitamin B6 enhances several aspects of cell-mediated immunity (lymphocyte proliferative responses to mitogens and increased proportion of circulating $CD3^+$ and $CD4^+$ T cells) (Talbott *et al.*, 1987).

1.2.2 Prebiotics, probiotics and fibre

In recent years the concept of functional foods has been directed towards food additives that may exert a positive effect on gut microbial composition such as probiotics ("a live microbial feed supplement that exerts beneficial effects for the host via improvement of the microbial balance in the intestine" (Fuller & Gibson, 1997)) and prebiotics ("a non-viable food component that selectively stimulates the growth and or activity of one or a limited number of beneficial colonic bacteria" (Gibson & Roberfroid, 1995; Ziemer & Gibson, 1998)). The most commonly used probiotics are lactic acid excretors such as lactobacilli and bifidobacteria that are usually added to fermented milk products or given in lyophilised forms (Ziemer & Gibson, 1998) and non-pathogenic, antibiotic-resistant ascospore yeasts, principally *Saccharomyces boulardii* (Levy, 1998). Several studies have shown that dietary supplementation with a probiotic such as lactic acid

bacteria can exert physiological and therapeutic effects to the consumer, including stimulation of the immune system, increased resistance to infectious diseases and suppression of cancer development (reviewed by Gill, 1998). Reports from clinical studies have also indicated that humans who consume fermented foods or products containing lactic acid bacteria can experience some improvements in atopic symptoms, associated with an increase in interferon-γ production (Majamma & Isolauri, 1997; reviewed by Cross & Gill, 2001).

Although the precise mechanisms by which probiotics act on the immune system are not fully understood, studies suggest that they may exert their immune enhancing effects by augmenting both non-specific (e.g. phagocytic function, NK-cell activity) and specific (e.g. antibody production, cytokine production, lymphocyte proliferation, delayed type hypersensitivity) host immune responses (Gill, 1998). It has been proposed that certain strains of lactic acid bacteria may affect immunity, such as anti-allergy immunity, by inducing the pro-Th1 responses (primarily through a receptor-mediated cytokine pathway) and inducing the ability of individual cell wall components to produce pro-interferon monokines (especially interleukin-12) at a sub inflammatory level (Cross *et al.*, 2001). Indeed, animal models of allergy have demonstrated that the dietary inclusion of potent interleukin-12-inducing probiotics, such as *Lactobacillus casei* (Shirota strain), can lead to the down regulation of T helper 2-type effector molecules (Matsuzaki *et al.*, 1998; Matsuzaki & Chin, 2000). A T helper 2 lymphocyte-dominated response can cause the cytokine-mediated recruitment of granular effector cells to sites of allergic inflammation (in the presence or absence of IgE class antibodies) and promote the clinical manifestations of allergy and atopy (Wills-Karp, 1999).

There is also strong evidence indicating that the consumption of prebiotic fibres (inulin and oligofructose) increases the proportion of beneficial lactic acid bacteria in the human colon (Gibson, 1999; Menne *et al.*, 2000). Combining prebiotics and probiotics in what is called a symbiotic may also benefit the host by improving the survival of selected microbial supplements (such as bifidobacteria and/or lactobacilli) (Gibson & Roberfroid, 1995) thereby enhancing their effects in the large bowel, however the creation of a symbiotic has not been extensively investigated (Roberfroid, 2000).

Animal studies have also demonstrated that dietary fibre content can modulate the immune system (reviewed by Schley & Field, 2002). Changing or adding fibre to the diet has yielded various effects on immune function such as increasing systemic and mucosal immunoglobulin production, altering cytokine production in the mesenteric lymph nodes (Lim *et al.*, 1997; Yun *et al.*, 1998), altering leucocyte and lymphocyte numbers in the spleen and intestinal mucosa (Kudoh *et al.*, 1998) and significantly increasing the proportion of T cells ($CD4^+$ and $CD8^+$) and their *in vitro* responses to mitogens (Field *et al.*, 1999). The mechanism by which fermentable dietary fibres affect immune function in the gut has not yet been established, however several mechanisms have been proposed, such as direct contact of lactic acid bacteria or bacterial products (cell wall or cytoplasmic components) with immune cells in the intestine or the modulation of mucin production (reviewed by Schley & Field, 2002). The gut microflora may modulate immune cells through the fermentation of dietary fibres to short chain fatty acids such as acetate, butyrate and propionate. Short chain fatty acid production, particularly butyrate, in the colon may reduce the requirement of glutamine by epithelial cells, sparing it for the cells of the immune system (Schley & Field, 2002). The immuno-nutritive properties of carbohydrates (whose major role is the provision of energy) have also been related to short chain fatty acid production or other effects of bacterial fermentation in the colon (reviewed by Vanderhoof, 1998).

1.2.3 Dietary fatty acids

While some studies have suggested that over-nutrition, particularly the excess intake of fats has a suppressive effect on immune response (Lorenz & Good, 1993), the presence of some fatty acids in the diet have been shown to be able to modulate the immune system through several mechanisms such as reducing cytokine synthesis and increasing phagocytic activity (Stark & Madar, 2002). In Western countries adults obtain 30 - 40% of their caloric intake from dietary fat (Field *et al.*, 2002), a small proportion of which consists of long chain polyunsaturated fatty acids that have been shown to influence immune and other cellular responses (Table 1.1).

Table 1.1

Some long-chain fatty acids that influence immune and other cellular responses

α-Linolenic acid	C18:3ω-3[1]
Eicosapentaenoic acid	C20:5ω-3[1]
Docosahexanoic acid	C22:6ω-3[1]
Linolenic acid	C18:2ω-6[1]
γ-Linolenic acid	C18:3ω-6[1]
Arachidonic acid	C20:4ω-6[1]
Oleic acid	C18:1ω-9[1]

Adapted from: Alexander, 1998

[1] C(number of carbon atoms):(number of double bonds)ω-(site of first double bond from the distal or methyl end)

The major unsaturated long chain fatty acids which are incorporated into the cell membrane of humans are eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) (ω-3), arachidonic acid (ω-6) and oleic acid (ω-9) (Alexander, 1998). The essential fatty acids, linoleic (ω-6) (found in most plant oils) and α-linolenic (ω-3) (found in flaxseed, soybean and canola oils), cannot be synthesised by mammalian cells so must be obtained in the diet (Field *et al.*, 2002). Long chain (ω-3) polyunsaturated EPA, and DHA can be synthesised from α-linolenic acid by humans but can also be obtained from marine fish oils (Field *et al.*, 2002). The dietary fatty acids of the ω-3 series are rapidly incorporated into cell membranes and profoundly influence biological responses (Alexander, 1998). These lipids are important in brain development, cardiovascular disease (Field *et al.*, 2002) and cancer (Rose & Connolly, 1999) and there is now convincing evidence that dietary ω-3 polyunsaturated fatty acids (particularly EPA and DHA) have a major impact on the immune system. Feeding EPA and DHA has been shown to modulate specific functions of innate and acquired immunity. In general, feeding high levels (> 10% of total fat) of ω-3 polyunsaturated fatty acids (compared with diets high in ω-6 polyunsaturated fatty acids) to healthy animals or human subjects results in suppression of the ability of lymphocytes to respond to mitogen stimulation (Calder, 1998a), a decrease in NK activity and impaired DTH reactions (Calder, 1998b).

Dietary ω-3 polyunsaturated fatty acids have been shown to exert anti-inflammatory activities in several animal studies (reviewed by Calder, 1998b). Clinical studies of the

effects of dietary fatty acids in inflammatory and autoimmune disease (reviewed by Calder, 1996a) have found that dietary ω-3 polyunsaturated fatty acids could have a protective role towards diseases such as psoriasis, asthma, Type 1 diabetes and multiple sclerosis. Fish oil administered in clinical trials and in animal models of rheumatoid arthritis, ulcerative colitis, and organ transplantation has resulted in measurable beneficial effects on the immune system (Meydani, 1996; Calder, 1998b). Similarly feeding fish oil to animals reduces the severity and prolongs survival in animal models of lupus (Blok *et al.*, 1996). Feeding ω-3 polyunsaturated fatty acids has also been shown to decrease tumour growth, incidence and/or metastasis in a large number of animal studies (Rose & Connolly, 1999) and prolong the survival of cancer patients in a human clinical trial (Gogos *et al.*, 1998).

Dietary lipids or free fatty acids not only affect the immune system directly, but also interfere with the production of other substances. Fatty acids (released from plasma membranes or incorporated from the extracellular medium) may suffer biochemical degradation through the eicosanoid pathways and influence the production of eicosanoids (enzymes that participate as secondary messengers within the cytoplasm) (Stark & Madar, 2002). Overall, these events may be responsible for the modulation of the immune system. For example arachidonic acid-derived eicosanoids such as prostaglandin E_2 are pro-inflammatory and regulate cell function. The consumption of fish oils leads to the replacement of arachidonic acid in cell membranes with eicosapentaenoic acid, altering the balance of eicosanoids produced (Pablo & Cienfuegos, 2000). Several other potential mechanisms have been proposed to explain the immunomodulatory effects of dietary ω-3 polyunsaturated fatty acids, including effects on eicosanoid formation, signal transduction, gene expression and lipid peroxidation (reviewed by Miles & Calder, 1988; Calder, 1996a; Calder, 1996b; Calder, 1998a; Calder, 1998b; Calder, 1998c; Calder *et al.*, 2002). The long chain ω-3 fatty acids, especially eicosapentaenoic acid (fish oil) appear to be the most potent when included in the human diet and can down regulate the T-helper 1 type response which is associated with chronic inflammatory disease (Calder *et al.*, 2002). Hence, the immunomodulation induced by dietary fatty acids may be applied to the amelioration of inflammatory disorders, such as autoimmune diseases (Stark & Madar, 2002).

Olive oil (monounsaturated fat) also appears to be an example of a functional food. Accumulating evidence suggests that dietary olive oil may have health benefits including the reduction of risk factors of coronary heart disease, the prevention of cancer, and the modification of immune and inflammatory responses (reviewed by Stark & Madar, 2002). Animal studies have suggested that dietary olive oil is capable of modulating functions of cells of the immune system in a manner similar to, although weaker than, fish oils (Yaqoob, 1998).

The fatty acid composition of phospholipids (a major part of cellular membranes and membrane fluidity and permeability) changes with age and disease and is largely influenced by nutrient supply. The administration of dietary phospholipids has been effective in protecting cellular membranes such as those of the gastrointestinal tract against inflammation and ulceration in rats (reviewed by Bengmark, 1998). Polar lipids have also been shown to be effective in preventing microbial translocation and this effect has been found to be further potentiated by an external supply of probiotic fibres such as pectin, guar gum and oat gum (reviewed by Bengmark, 1998).

1.2.4 Dietary nucleotides

Nucleotides are obtained from nucleoprotein-rich foods such as organ meats, fish and poultry and are especially high in human breast milk (Barness, 1994). Animals fed nucleotide-free diets suffer impaired cellular and humoral immune function, including decreased antibody production (Jyonouchi, 1994), increased susceptibility to infection (Kulkarni *et al.*, 1994), lower DTH responses, lower cytokine production and decreased NK cell and macrophage activity (Carver, 1994). The addition of nucleotides to nucleotide-free diets has been shown to reverse or restore many of the changes observed with nucleotide deficiency, such as increasing antibody production (IgG2a) (Jyonouchi *et al.*, 2001), increasing spleen cell proliferation (Adjei *et al.*, 1999) and increasing Th1-type cytokines (Nagafuchi *et al.*, 1997). In addition, human infants fed breast milk supplemented with nucleotides had higher NK cell activity and interleukin-2 production compared with infants fed formula without nucleotides (Carver, 1994).

1.2.5 Dietary protein

Research examining the effect of protein nutrition on immunity has led to the observation that the quantity of dietary protein supplied to an individual can affect the immune system and consequently increase susceptibly to environmental pathogens, viral reactivation and development of opportunistic infection, as well as influencing the course of any established infectious disease (Chandra, 1972; Malave & Layrisse, 1976; Gross & Newberne, 1980; reviewed by Gershwin, 1985). In fact, protein energy malnutrition has been cited as the major cause of immunodeficiency worldwide (Delafuente, 1991; Chandra, 1993; Chandra, 1999). Immune cells have a high requirement for energy and amino acids for cell division and protein synthesis (Field *et al.*, 2002) therefore nutritional state and specific nutrients such as amino acids may affect the immune system directly (e.g. by triggering immune cell activation or altering immune cell interactions) or indirectly (e.g. by changing substrates for DNA synthesis, altering energy metabolism, changing the physiological integrity of cells or altering signals or hormones) (Field, 2000).

Several experimental animal models have been used to determine the effect of protein deficiency on immunity (reviewed by Gershwin, 1985) for example, in a life-long dietary protein restriction study, *in vivo* primary antibody responses (assessed by plaque-forming cell assay) were found to be significantly lower in mice fed (*ad libitum*) 4% protein (casein), compared to mice fed 24% protein (casein). Primary antibody responses fell with age in both the experimental and control groups (Stoltzner & Dorsey, 1980). Mice fed diets with 8% (low) or 27% (control) protein content showed a significant decrease in splenic cell numbers and primary and secondary humoral immune responses against allogeneic cells (assessed by the titration of serum hemagglutinins and plaque-forming cell assay), within 1 week of protein depletion (Malave and Layrisse, 1976). Low protein diets have, however, been found to have a differential effect on lymphocyte proliferation. Diets containing 4% protein (casein) have been found to either have no effect (Stoltzner & Dorsey, 1980) or a suppressive effect (Mann, 1978) on mitogen-stimulated splenic proliferation (measured by ^{3}H-thymidine uptake) *in vitro*. The interaction of protein calorie malnutrition and various immune parameters has lead to some understanding of the aetiology of the increased susceptibility to infections associated with a protein depleted diet. The dysfunction of one or several components of immunity caused by the lack of dietary protein can render

a host more susceptible to infection by obligate intracellular organisms such as *Mycobacterium tuberculosis,* herpes simplex virus, *Pneumocystis carinii,* measles, malaria and gram negative infection and sepsis (Gross & Newberne, 1980). An examination of macrophage function demonstrated that animals deficient in protein and essential amino acids had a reduced ability to clear intracellular particles and exhibited poorer chemotaxis responses *in vitro* (Petro & Watson, 1982). Macrophages from protein deficient animals also showed an impaired ability to trigger T lymphocytes to respond to antigen (Rose *et al.*, 1982). T lymphocytes also performed poorly in *in vitro* studies when derived from protein deficient donors; exhibiting a decreased response to mitogens and a reduction in lymphokine production (Mann, 1978; Gross & Newberne, 1980; Floyd *et al.*, 1983). However, the dendritic cell antigen-presenting capacity (assessed by mixed lymphocyte reaction, host-versus-graft responses and $CD11c^{+}F4/80^{-/low}$ phenotype) was found to be retained in mice fed an isocaloric low protein diet (combined deficiencies of protein and energy), despite the daily loss of 1.5 - 2% of initial body weight (Zhang *et al.*, 2002). Protein nutrition has also been found to influence the immunity of groups of people who are already considered to be immunocompromised. An example of such a group is the elderly who have been found to have decreased lymphocyte proliferation, reduced cytokine release (interleukin-2) and lower antibody responses to vaccines due to protein-energy malnutrition (Lesourd, 1997; Rosenthal, 1999).

Both the type of dietary protein, as well as overall protein levels (amount of protein energy) in the diet have been shown to influence immunity. Bounous & Kongshavn (1982) investigated the effect of feeding graded amounts (20, 30, or 40 g/100 g) of four naturally occurring purified proteins (lactalbumin hydrolysate, casein, soy or wheat) on humoral immune responsiveness and non-specific splenic cell responsiveness in mice. This study found that, nutritionally, the various 20 g/100 g protein formula diets (providing approximately 17% total amino acid content in the diet) and control diet (mouse chow) (providing approximately 23% total amino acid content in the diet) sustained the normal growth of mice, the 30 g/100 g diets (with higher amino acid content) did not enhance body growth beyond that of the 20 g /100 g diets and the 40 g/100 g protein diets resulted in comparatively lower body and spleen weights. Immunologically, the humoral immune response to sheep red blood cells (SRBC) and the splenic cell responsiveness to mitogen after stimulation with *Mycobacterium bovis*

(strain BCG) were up to 5 times greater in mice fed lactalbumin than mice fed casein, soy or wheat diets at all protein levels (20, 30, 40 g/100 g) investigated (Bounous *et al.*, 1983a). This finding demonstrates that changes in the protein type of nutritionally adequate diets can also influence the immune response of the host. The minimum dietary requirement of protein with optimal digestibility and biological value, such as milk protein, for a mouse to sustain normal growth is estimated at approximately 12% (John & Bell, 1976) and experiments have demonstrated that the spleen cell responsiveness to SRBC (measured by plaque-forming cell assay) of mice is significantly increased when the content of lactalbumin hydrolysate in the diet is raised from 12 to 28 g /100 g with maximal immune enhancement occurring at approximately 20 g lactalbumin/100 g diet (Bounous *et al.*, 1981; Bounous & Kongshavn, 1982). However, increasing the dietary concentration of casein from 12 to 28% failed to produce any change in the splenic plaque-forming cell response to SRBC (Bounous & Kongshavn, 1982).

Studies have also been conducted on the immunomodulatory effects of single amino acids. Amino acids and polyamines such as glutamine may also influence aspects of the immune response which depend on DNA and protein synthesis during lymphocyte proliferation and antibody synthesis (reviewed by Grimble & Grimble, 1998). Laboratory and clinical data have suggested that glutamine (once thought of as a non-essential amino acid) is essential during certain inflammatory conditions, such as infection and injury (reviewed by Wilmore & Shabert, 1998). Furthermore, glutamine has been found to be an important ammonium donor group needed for the biosynthesis of purines and pyrimidines, and glutamine depleted diets result in significant atrophy of the gut mucosa in experimental animals (Levy, 1998). Mice fed diets limited in the single amino acids tryptophan, leucine, lysine, phenylalanine, histidine, methionine or threonine demonstrated increased susceptibly to *Salmonella typhimurium* (*S. typhimurium*) versus *Listeria monocytogenes* infection (Bitar & Bhattacharjee, 1982). Mice fed leucine-limited diets had an impaired ability to clear *S. typhimurium* challenges, while mice on lysine-limited diets exhibited a slightly enhanced clearance of the pathogen. A diet limited in histidine or threonine rendered mice more susceptible to *Salmonella* infection and diets limited in methionine or threonine rendered mice more susceptible to *Listeria* infection. Mice fed diets limited in lysine or leucine were not susceptible to infection by *Listeria*. It was also noted that a leucine-excess diet in

conjunction with moderate protein deficiency resulted in an 11 fold decrease in the number of antibody plaque-forming cells in the spleen (Aschkenasy, 1973). These findings suggest that the balance of nutrients, not simply their general availability, may have an influence on cellular functions in the immune system, consequently there has been considerable interest in establishing a minimum requirement for essential amino acids and protein intake that would adequately support the entire immune system (Bounous & Kongshavn, 1978).

1.2.6 Milk protein

Ensuing research has established that not only nutritional factors *per se* have an effect on immune function, but specific types of protein particularly those derived from milk, have a major effect on immunity (Minehira *et al.*, 2000). For young mammals, including humans, milk is the first and for most, the only food ingested for a considerable period of time (Swaisgood, 1996). It is well documented that, in addition to providing early nutrition, breast feeding provides a large variety of protective factors and cells that have a direct effect on neonatal resistance to bacterial and viral infections (Heine *et al.*, 1991). Consequently, the lower incidence of infectious illness in breast-fed infants compared with formulae fed infants is also well documented (Rassin *et al.*, 1977; Balmer & Wharton, 1989; Heine *et al.*, 1991). Immunoglobulins, lysozyme, interferon, lactoferrin, Bifidus factor, cortisol and fatty acids are some of the components of breast milk that have been identified to have immuno-nutritive properties (Levy, 1998). The protective effects of breast milk have, in part, been ascribed to its antioxidant properties, bactericidal and phagocytic characteristics (Levy, 1998) and the transfer of passive immunity through secretory IgA (Goldman *et al.*, 1994). Breast milk is therefore the most obvious example of a food with recognised immuno-nutritive properties and is the model that human infant formula manufacturers have tried to emulate primarily using bovine milk (Levy, 1998).

1.3 Immunomodulatory Components And Properties Of Bovine Milk

Bovine milk and its by-products have long been recognised as important foodstuffs as part of a balanced nutritional diet and have become an integral part of the human diet (Bounous & Kongshavn, 1989). The nutritional benefits of milk-derived proteins, vitamins and minerals have been promoted extensively by commercial dairy enterprises in recent times. The vast range of milk-based products now available has come into being as a direct result of the consumer image of milk as a healthy product. Evidence also suggests that the protective effect of bovine milk derived factors is not completely nutritional or passive in nature. Some milk components contribute to the immunological development of the neonate, whereas others enhance the efficacy of host defence factors produced by the neonate (Goldblum & Goldman, 1994). The means by which particular nutrients or immunomodulatory components affect the balance of the immune response is therefore a critical issue. Research has identified and characterised bovine milk-based products in terms beyond their nutritional value, and the scientific community is giving credibility to the opinion that bovine milk, as a functional food, has a direct and measurable influence on the health of its recipient (Clydesdale, 1997; Korhonen *et al.*, 1998; Gill *et al.*, 2000a). This concept is not new (Newby *et al.*, 1982) but the identification of factors that may be relevant to improving human health, and the potential development of bovine milk-containing preparations into products with proven health-promoting properties certainly is (Cross & Gill, 2000). It is now widely accepted that the protein components within bovine milk can influence and direct the physiological and immunological development of infants as environmental exposure increases (reviewed by Korhonen *et al.*, 1998).

In recent years, partly due to advances in protein separation technology, many studies have focused on the immunomodulatory activity of bovine milk and colostral protein components. Individual milk proteins have been purified for the purpose of studying their physical properties and physiological functions (Fiat *et al.*, 1993). Today there is evidence indicating that the major components of bovine milk, such as casein and whey, as well as several highly purified individual constituents of sub-fractions, can regulate immune function in non-ruminant as well as ruminant species. Research has focused on identifying biologically active components within bovine milk, as well as characterising the mode by which mammalian physiological function is modulated by these

components. As commercial interest in the production of functional foodstuffs that have health-promoting properties increases, such identification has become commercially as well as medically important.

1.3.1 Bovine milk-derived bioactive peptides

Dietary milk not only contains hormones and growth factors which have physiological effects but also provides amino acids for the synthesis of proteins and other nitrogen-containing compounds essential for growth and development and in the last two decades a number of studies have investigated the bioactive properties of the peptides present in the amino acid sequence of milk proteins. The biological properties of milk proteins or milk-derived proteins with antibacterial activity (lactoferrin), peptides which facilitate nutrient assimilation and peptides with a regulatory activity on physiological function have been studied (Tome & Debabbi, 1998). Milk-protein derived bioactive peptides are obtained by the enzymatic proteolysis of the parent protein during *in vitro* enzymatic food processing or *in vivo* gastrointestinal digestion. *In vivo,* milk proteins are subjected to digestion and absorption processes in the gastrointestinal tract hence the putative mechanisms of milk-derived peptides are largely influenced by different intestinal processes including gastric and intestinal motility, lumenal digestion, mucosal absorption and other transport and degradative mechanisms in the gut (Tome & Debabbi, 1998). Once liberated in the body, bioactive peptides may act as regulatory compounds with specific functions such as enzyme inhibitor activities, specific or non-specific binding properties, protective properties, mediator, hormone and growth factor activities, blood coagulation properties and immunomodulatory activities (reviewed by Meisel, 1998; Schanbacher *et al.*, 1998; Tome & Debabbi, 1998; Gill *et al.*, 2000b; Rutherfurd & Gill, 2000; Gobbetti *et al.*, 2002; Kilara & Panyam, 2003; Aimutis, 2004).

The immunomodulatory effects of bovine milk-derived bioactive peptides have been characterised by several *in vitro and in vivo* test systems (reviewed by Gill *et al.,* 2000b; Kilara & Panyam, 2003). For example, intravenous administration of fragments of α_{s1}-casein (residues 194 - 199) and β-casein (residues 63 - 68) stimulated the phagocytosis of SRBC by murine peritoneal macrophages and exerted a protective effect against *Klebsiella pneumoniae* infection in mice (Migliore-Samour *et al.,* 1989). The fragments of bovine α-lactalbumin (N-terminal end of α-lactorphin) and κ-casein (residues 38 and 39) significantly enhanced the proliferation of peripheral blood lymphocytes *in vitro* at

concentrations ranging from 10^{-11} to 10^{-4} mol/L (Kayser & Meisel, 1996) and the C-terminal sequence 193 - 207 of β-casein (released by the pepsin-chymosin digestion of bovine casein) induced a significant increase in the proliferation of rat lymphocytes *in vitro* (Coste *et al.,* 1992). In contrast, bovine κ-caseinoglycopeptide (residues 106 - 169 of κ-casein) inhibited both mitogen induced proliferation of murine lymphocytes and suppressed antibody production in murine splenocyte cultures (Otani *et al.,* 1995). Similarly, the peptide residues derived from a pepsin/trypsin hydrolysis of β-casein, such as β-casomorphin-7 (residues 60 - 70) have been found to suppress the mitogen induced proliferation of human lymphocytes *in vitro* (Kayser & Meisel, 1996) and inhibit the proliferation of human lamina propria lymphocytes *in vitro* (Elitsur & Luk, 1991). The anti-proliferative effect of β-casomorphin-7 was reversed by an opiate receptor antagonist suggesting that opioid peptides may affect the immunoreactivity of lymphocytes via opiate receptors for endorphins present on T lymphocytes and human leucocytes (Faith *et al.*, 1984). The precise mechanisms by which milk protein derived peptides exert their immunomodulatory effects are, however, not yet fully defined. It has been suggested that specific receptors expressed by lymphocytes and macrophages may recognize arginine residues present at the N- or C-terminal and may act on the immune system by preventing the break down of bioactive bradykinin (Maruyama *et al,* 1987). Antihypertensive angiotensin converting enzyme (ACE)-inhibitory peptides such as β-casokinin-10 (residues 193 - 202 of β-casein) contain arginine as the C-terminal residue, and *in vivo* studies have demonstrated that the intravenous administration of ACE-inhibitory fragments derived from β-casein can stimulate phagocytosis and exert a protective effect against *Klebsiella pneumoniae* infection in mice (Jolles *et al.,* 1993). The immunomodulatory properties of bovine whey-protein-derived bioactive peptides have also been studied and will be reviewed in Section 1.4.1.

1.3.2 Conjugated linoleic acid

The role of dietary fat in regulating immune function has received considerable research attention, and reports have indicated that both the form and intake level of fats and oils are important determinants of immunocompetence (Section 1.2.3; reviewed by Calder, 1998b). In particular, essential polyunsaturated fatty acids are thought to exert a strong influence on the immune system due, in part, to their ability to be metabolised into long-chain ω-3 or ω-6 derivatives, which in turn can give rise to competitive inhibitors against or substances for the biosynthesis of immunoregulatory eicosanoids (Section 1.2.3; Calder, 1996a; 1998b). The polyunsaturated fatty acids of the ω-6 series comprise linoleic acid and its derivatives, which are present in certain seed/grain oils such as sunflower and corn. In ruminants, biohydrogenation of dietary linoleic acid by the rumen microflora can yield a series of biologically active isomers, collectively termed conjugated linoleic acid (CLA). CLA can be incorporated into cell membrane phospholipids and is bioaccumulative in tissues (Ip *et al.*, 1991). Consequently, CLA is the predominant form of linoleic acid present in ruminant derived foods, including meat (Ha *et al.*, 1987), milk (Parodi, 1997) and other fat containing dairy foods (Shantha *et al.*, 1995). Bovine milk represents one of the richest sources of naturally-occurring dietary CLA (Chin *et al.*, 1994). The lipid fraction of bovine milk contains up to 30 mg CLA/g fat, consisting almost entirely of the *cis*-9, *trans*-11 isomer (Parodi, 1996) which is believed to be the biologically active form of the molecule (Ip *et al.*, 1991). Additionally, milk fat contains other lipid molecules that may have immunomodulatory activity, including sphingomyelin, whose metabolites are thought to play an active role in cell membrane signal transduction pathways leading to cytokine production (Zhang & Kolesnick, 1995).

Numerous studies have confirmed the anticarcinogenic activity of CLA, in particular, its ability to retard tumourgenesis and tumour development at a number of tissue sites, in both *in vitro* and *in vivo* models (Ip *et al.*, 1991; Cunningham *et al.*, 1997; Wong *et al.*, 1997a). Several reports have demonstrated the ability of CLA to enhance the function of immune cells that play a role in preventing tumour growth. For example, Miller *et al.* (1994) found that 0.5% CLA fed to mice for two weeks increased phytohaemagglutinin-induced lymphocyte proliferation and the phagocytosis of the fluorescein isothiocyanate-labelled yeast cells by peritoneal macrophages. Mice fed synthetic CLA-supplemented diets have also been shown to enhance macrophage-mediated responses,

lymphocyte proliferation and cytokine production (Wong *et al.*, 1997a). Chew *et al.* (1997) also reported that CLA stimulates mitogen-induced lymphocyte proliferation, lymphocyte cytotoxic activity and macrophage bactericidal activity *in vitro* but inhibits interleukin-2 production by lymphocytes and suppressed the phagocytic activity of macrophages when included in cell culture. While the primary mechanisms by which CLA modulates cell-mediated immune activity remain uncertain, the molecule may exert its influence by modifying expression of cell surface receptors or by altering intracellular events via one or more signal induction pathways (Cook & Pariza, 1998).

1.3.3 Casein

Extensive research on the immunomodulatory properties of casein and its derivatives (Section 1.3.1) has revealed a wide range of *in vitro* effects on immune function. Caseins are phosphoproteins precipitated from raw milk at pH 4.6, 20°C and comprise approximately 80% of the total protein content in milk (Eigel *et al.*, 1984). The immunomodulatory effects of casein and its components have been reviewed at length by Jolles *et al.* (1981-1982), Berthou *et al.* (1987), Migliore-Samour *et al.* (1989), Carr *et al.* (1990) and Cross & Gill (2000). Briefly, caseins (whole casein, α-, β-, and κ-casein) have been shown to modulate lymphocyte proliferation both *in vitro* and *in vivo.* For example when included in *in vitro* cell culture, β-casein enhanced mitogen-induced proliferation of ovine T and B lymphocytes (Wong *et al.*, 1996a), α-S_1casein enhanced mitogen-stimulated proliferation of murine splenic T lymphocytes (Carr *et al.*, 1990) and an isolated sub-fraction of caseinoglycopeptide (CGP) derived from κ-casein promoted proliferation of non-mitogen-induced murine spleen cells (Yun *et al.*, 1996). In contrast, Otani *et al.* (1995) found that CGP suppressed T and B mitogen induced murine and rabbit lymphocyte proliferation when included in *in vitro* cell culture. However, this suppressive effect was not observed after treatment with pepsin, chymotrypsin or neuraminidase (Otani & Monnai, 1993). *In vivo* studies have also revealed diverse effects of casein components on immune function. For example, the intramuscular co-injection of β-casein suppressed specific antibody responses to ovalbumin in mice even in the presence of an immunopotentiating adjuvant (Wong *et al.*, 1996a), and mice fed a CGP-containing diet showed enhanced T (but not B) lymphocyte responses to mitogens when subsequently tested *in vitro* (Monnai *et al.*, 1998). Casein has also been found to have varying effects on macrophage function.

Undigested α- and κ-casein and trypsin or chymotrypsin digested α- and κ-casein suppressed the *in vitro* phagocytosis of latex microbeads by macrophages, whereas pepsin digested α- and κ-casein enhanced phagocytic uptake (Otani & Futakami, 1996). The immunosuppressive capacity of casein was again shown when β-casein suppressed the chemotaxis of ovine neutrophils in response to the recombinant chemokinetic cytokine (interleukin-8) (Wong *et al.*, 1996a), and suppressed both respiratory burst responses and the bactericidal capacity of bovine neutrophils *in vitro* (Cooray, 1996).

1.3.4 Whey

Initial studies by Bounous and colleagues found an enhanced immune response to T-dependent antigens in mice fed bovine whey protein (predominantly lactalbumin) as a sole protein source at 12%, in comparison to the response of mice fed a reference diet supplemented with casein (12%) (Bounous *et al.*, 1981; Bounous & Kongshavn, 1982; Bounous *et al.*, 1983a). An examination of B-lymphocyte ontogeny found that the observed enhancing effect of lactalbumin was not due to an alteration in the rate of primary B-lymphocyte production in the marrow (Bounous & Kongshavn, 1985; Bounous *et al.*, 1985). It was postulated that the enhancing effect was due to changes in the functional responsiveness of the B lymphocytes or in the processes leading to B-lymphocyte activation, and the differentiation in the peripheral lymphoid tissues. Therefore, while determining the effect of various types of proteins on immune reactivity, these early investigations found a protein source that could not only adequately support the immune system, but apparently enhance the immune response (Bounous *et al.*, 1981; Bounous & Kongshavn, 1982; Bounous *et al.*, 1983a). The immunomodulatory effects of this bovine protein, namely bovine whey, are reviewed further in the latter sections of this chapter.

1.4 Proteins Contained In Bovine Whey

Bovine milk contains on average 87% water, 3.9% fat, 3.3% protein, 5.0% lactose, 0.8% ash and vitamins (Wong *et al.*, 1996b). The principal proteins in milk from the domestic cow (*Bos taurus*) are α_{s1}-caseins, α_{s2}-caseins, β-caseins, κ-caseins, β-lactoglobulins, α-lactalbumins, serum albumin and immunoglobulins (IgG_1, IgG_2, IgA and IgM) (Jenness, 1979). Expressed in grams per litre, the total protein content of bovine milk is approximately 28 g casein and 5.6 g whey proteins (Bounous & Amer, 1988). In the manufacture of cheese or casein from milk, casein-containing curds are formed by the action of rennet-type enzymes and/or acids. Whey is the liquid remaining after the recovery of these curds (Bounous *et al.*, 1988a). Hence, whey is a by product of cheese manufacture and the term whey proteins is used to describe the group of milk proteins that remain soluble in 'milk serum' or whey after the precipitation of caseins at pH 4.6 and 20°C (Eigel *et al.*, 1984). The immunomodulatory properties of whey proteins are evaluated throughout this thesis.

Whey contains high concentrations of water and lactose and low concentrations of proteins and residual lipids. Bovine whey contains on average 4 to 7 grams of protein per litre, however the concentration of individual whey proteins can vary depending on the type of whey, stage of lactation and health status of the cow, and on the processing conditions during the manufacture of cheese or casein (Korhonen *et al.*, 1998). In practice, the composition of cheese whey can differ from that of milk serum and varies from lot to lot. For example, rennet or sweet whey (pH 6 to 6.3) contains small but variable concentrations of fine cheese curd particles, and small milk fat globules from cheese milk, the concentrations of which are a function of the processing conditions used to manufacture it (Morr & Ha, 1993). Sweet whey also contains significant and variable concentrations of the inorganic minerals (Ca^{+2}, Mg^{+2}, citrate and phosphate ions) that are derived from casein micelles. On the other hand, acid whey (pH 4.4 to 4.6), is derived from cottage cheese or casein manufacture and contains small curd particles and higher concentrations of the inorganic minerals than sweet whey (Morr & Ha, 1993).

Whey contains a multitude of proteins that remain soluble after the precipitation of casein during the manufacture of cheese (Eigel *et al.*, 1984) providing a heterogeneous

mixture of proteins with wide ranging functional attributes for nutritional, biological and food purposes (Smithers *et al.*, 1996). The major constituents of whey include β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins. The minor components of whey include lactoferrin, lactoperoxidase and various growth factors. Many of these components possess immunomodulating properties (Ogra & Ogra, 1978; Juto, 1985; Stoeck *et al.*, 1989; Mincheva-Nilsson *et al.*, 1990; Barta *et al.*, 1991; Watson *et al.*, 1992). A few of the biological and immunomodulating properties of the major bovine milk proteins, including casein, are mentioned in Table 1.2 with a particular focus on the protein components in whey. In addition to the major milk components, bovine milk is also known to contain a number of peptides derived (usually by enzymatic hydrolysis) from the individual milk proteins, which can also modulate immune function. Consequently, the enzymatic digestion of bovine milk proteins provides a valuable source of biologically active peptides including those with immunostimulatory activity. The immunomodulatory effects of these 'bioactive' peptides have been reviewed by Werner *et al.* (1986), Jolles *et al.* (1993), Korhonen (1995), Gill *et al.* (2000b) and Shah (2000) and in Section 1.3.1 and 1.4.1.

The putative biological and physiological activities of whey protein components offer several opportunities for the food industry. Firstly, they provide the rationale for the thorough scientific evaluation of potential health benefits elicited by these proteins when included in the diet. Secondly, they provide the basis (once substantiated in human trials) for the development of valuable whey protein based food ingredients targeted at non-traditional dairy markets, such as the health-promoting food market (McIntosh *et al.*, 1998).

TABLE 1.2

Concentration and summary of the biological functions of the major milk proteins

Protein or peptide	g/L[1]	Function/putative activity	Reference
Casein (α, β and κ)	**28**	Ion carrier, precursors of bioactive peptides, immunostimulatory	(Migliore-Samour & Jolles, 1988; Migliore-Samour *et al.*, 1989; Coste & Tome, 1991; Otani *et al.*, 2000)
Lysozyme		Antimicrobial	(Steinhoff *et al.*, 1994)
Proteose-peptones	**1.2**	Uncharacterised	(Coste & Tome, 1991)
Total whey protein	**5.6**	Anti-carcinogenic	(Bounous *et al.*, 1988a; Bounous *et al.*, 1991; McIntosh *et al.*, 1998)
		Immunostimulatory	(Bounous & Amer, 1988; Bounous *et al.*, 1989a; Bounous & Kongshavn, 1989)
		Longevity	(Bounous *et al.*, 1989b)
β-lactoglobulin	3.3	Retinol carrier, binding fatty acids, possible anti-oxidant, digestive function	(Perez *et al.*, 1992; Korhonen, 1995)
β-lactorphin		Opioid agonist peptide	(Meisel & Schlimme, 1990)
α-lactalbumin	1.2	Lactose synthesis in mammary gland, Ca carrier, immunomodulation, anti-carcinogenic	(Rejman *et al.*, 1992a) (Swaisgood, 1996) (Bounous & Kongshavn, 1982) (Hakansson *et al.*, 1995)
α-lactorphin		Opioid agonist peptide	(Yoshikawa *et al.*, 1986; Meisel & Schlimme, 1990)
Lactoferrin	0.1	Anti-microbial peptide, iron transport, regulation, immunostimulatory, anti-inflammatory, cell growth, proliferation, anti-carcinogenic	(Kanyshkova *et al.*, 2001) (Lonnerdal, 1996) (Lima & Kierszenbaum, 1985; Lima & Kierszenbaum, 1987) (Hanson *et al.*, 1985) (Guimont *et al.*, 1997) (Fleet, 1995)
Lactoferricin		Anti-microbial peptide	(Meisel & Schlimme, 1990)
Immunoglobulins	0.7	Passive immunity	(Kobayashi *et al.*, 1991)
Lactoperoxidase	0.03	Anti-microbial, immunomodulatory	(Kanyshkova *et al.*, 2001) (Wong *et al.*, 1997b)
Growth factors		Cell growth, differentiation	(Francis *et al.*, 1995)
Serum albumin			
Serorphin		Opioid agonist peptide	(Yoshikawa *et al.*, 1986; Meisel & Schlimme, 1990)
Glycomacropeptide[2]	1.2	Antiviral, immunomodulatory	(Korhonen, 1995)

[1] Concentration g/L in whole milk, concentrations not listed are less than 0.03 g/L (Eigel *et al.*, 1984)
[2] A κ-casein derived peptide present in sweet whey, but absent in acid whey (McIntosh *et al.*, 1998)

β-Lactoglobulin is the most predominant whey protein with a molecular weight of 18.3 kDa (Morr & Ha, 1993), and makes up 47% of the total protein content of whey (Sadler, 1992). Bovine β-lactoglobulin is a 162 amino acid residue protein (Eigel *et al.*, 1984) with the composition: Asp_{10}, Asn_5, Thr_8, Ser_7, Glu_{16}, Gln_9, Pro_8, Gly_4, Ala_{15}, Cys_5, Val_9, Met_4, Ile_{10}, Leu_{22}, Tyr_4, Phe_4, Lys_{15}, His_2, Trp_2, and Arg_3, from which bioactive peptides may be derived by *in vitro* or *in vivo* enzymatic proteolysis (reviewed in Section 1.4.1). Seven genetic variants of β-lactoglobulin originating from different families of cattle are currently known. β-Lactoglobulin is a protein found in bovine milk but absent from the milks of certain other mammals, such as human and guinea pig milks (Jenness, 1979). β-Lactoglobulin appears to be produced by species that transfer large amounts of immunoglobulins to their young via colostrum, however the involvement of β-lactoglobulins in this process is unclear (Jenness, 1979).

Another major whey protein, α-lactalbumin makes up 13% of the total protein content of whey (Sadler, 1992). The complete amino acid sequence of bovine α–lactalbumin consists of 123 amino acid residues with the composition: Asp_9, Asn_{12}, Thr_7, Ser_7, Glu_8, Pro_2, Gly_6, Ala_3, Cys_8, Val_6, Met_1, Ile_8, Leu_{13}, Try_4, Phe_4, Lys_{12}, His_3, Trp_4 and Arg_{10}, from which bioactive peptides may be derived by *in vitro* or *in vivo* enzymatic proteolysis (reviewed in Section 1.4.1). α-Lactalbumin has a molecular weight of 14 kDa (Morr & Ha, 1993) and has three known genetic variants, A, B and C. The B variant occurs in Western cattle, whereas both A and B occur in milk from African Fulani and African and Indian Zebu cattle (Eigel *et al.*, 1984). α-Lactalbumin is a calcium binding protein (Wong *et al.*, 1996b) and is known to play an essential role in the biosynthesis of lactose (Jenness, 1979).

Over the past decade a number of pilot or industrial scale methods have been developed for the enrichment or isolation of biologically active minor whey proteins such as lactoferrin (reviewed by Maubois & Ollivier, 1997). Lactoferrin has been isolated from the milk of most mammalian species (Masson & Heremans, 1971) and is also found in exocrine fluids such as tears, saliva, bile and pancreatic fluid and is a major component of polymorphonuclear neutrophils (Vorland, 1999). Lactoferrin (or lactotransferrin) is an iron-binding (Wong *et al.*, 1996b) multifunctional glycoprotein with two carbohydrate groups attached (Reiter, 1985) and a molecular weight range of 80 to

92 kDa (Morr & Ha, 1993). In its native form, lactoferrin is only partly saturated with iron (8 - 30%) and this is physiologically important because iron can be chelated and thus inhibit bacteria by depriving them of iron which is essential for growth (Reiter, 1985). Similar to lactoperoxidase, lactoferrin has been proposed to have several physiological functions, such as enhancing iron absorption, and acts as a bacteriostatic/bactericidal agent and a growth factor (Reiter, 1985; Lonnerdal, 1996). Lactoferrin also plays a key role in host defences against infection and inflammation in mammals (reviewed by Ward *et al.*, 2002). The immunoregulatory properties of the minor whey protein, lactoferrin (making up approximately 1.8% of the total protein content of whey), will also be discussed throughout this review and investigated in this thesis because of its physiological and nutritional importance (Reiter, 1985; De Sousa *et al.*, 1988; Balmer *et al.*, 1989; Hambraeus & Lonnerdal, 1994; Lonnerdal, 1996; Kanyshkova *et al.*, 2001).

1.4.1 Whey protein-derived bioactive peptides

Hydrolysis of proteins generally results in the loss or alteration of its native structure, a reduction in the molecular weight of the peptides and enhanced interaction of peptides with both themselves and the environment (Kilara & Panyam, 2003). The most common methods used to produce peptides with biological activity include the processing of foods using hot alkali or acids to hydrolyse proteins, enzymatic hydrolysis of food proteins, and/or microbial fermentation (Korhonen *et al.*, 1998). For example, Bouhallab *et al.* (1992) reported a method of continuous tryptic hydrolysis of caseinomacropeptide (derived by the action of chymosin on κ-casein and recovered as a by product in cheese whey) to produce bioactive peptides. The purification of betacellulin (a major growth factor) from a growth factor enriched whey fraction of bovine milk by a combination of ion-exchange chromatography, gel-filtration affinity and reversed phase high pressure liquid chromatography has also been reported (Dunbar *et al.*, 1999). Similar to the antimicrobial peptides derived from the chymosin digestion of casein (reviewed by Kilara & Panyam, 2003) lactoferricin, a peptide cleaved from lactoferrin by pepsin, has also been shown to interact with microbial surfaces and exert an antimicrobial effect (Tomita *et al.*, 1991).

Although less extensively studied than the bioactive peptides derived from casein (Section 1.3.1), the physiological effects of several whey protein-derived bioactive

peptides have been documented. Yoshikawa *et al.* (1986) were the first to study the bioactive peptides present in whey proteins by synthesising α-lactorphin (Try-Gly-Leu-Phe, residues 50 - 53 of α-lactalbumin) and β-lactorphin (Tyr-Leu-Leu-Phe, residues 102 - 105 of β-lactoglobulin), on the basis of opioid-like fragments. Subsequent studies have demonstrated that α-lactorphin exerts opioid receptor binding and opioid properties in smooth muscle and β-lactorphin, despite demonstrating a similar receptor binding affinity, exerts a non-opioid stimulatory effect on guinea pig ileum (Paakkari *et al.*, 1994). Yamauchi (1992) reported that peptides derived from serum albumin and β-lactoglobulin induced the contraction of guinea pig ileum and referred to these peptides as albutensin A and β-lactotensin. Similar to β-lactorphin, the stimulatory effect of β-lactotensin exerts a non-opioid mediated stimulatory effect on smooth muscle hence the mechanisms of this effect remain unclear (Korhonen *et al.,* 1998). ACE is part of the renin-angiotensin system and has been implicated in blood pressure regulation and hypertension (Mullally *et al.,* 1996) and whey protein derived peptides such as α-lactorphin, β-lactorphin and β-lactotensin have been found to possess ACE-inhibitory activity *in vitro*, however further research is needed to show the activities of these peptides/hydrolysates *in vivo* (Korhonen *et al.,* 1998). Bovine lactoferricin B (residues 17 - 41 of lactoferrin) has also been found to suppress interleukin-6 production by a human monocytic cell line in response to lipopolysaccharide stimulation *in vitro* (Mattsby-Baltzer *et al.,* 1996) and stimulate the release of interleukin-8 from human polymorphonuclear leucocytes (Shinoda *et al.,* 1996). A recent study by Mercier *et al.* (2004) further demonstrated that the fractionation (by isoelectric focusing) of peptide hydrolysates released by the trypsin/chymotrypsin digestion of a microfiltered-whey protein, stimulated the proliferation of murine splenic lymphocytes at low concentrations (0.5 - 500 μg/mL) in *in vitro* cell culture. Collectively, these findings indicate that the enzymatic digestion of bovine whey proteins can release bioactive peptides that, once fully identified and isolated, could be used in the preparation of immunomodulatory products.

1.4.2 Whey protein products

The majority of milk products including whey protein concentrate (WPC) and whey protein isolate (WPI) are derived directly or indirectly from pasteurised skim milk (Wong *et al.*, 1996b). The product obtained from heat precipitation of whey is

commonly marketed as 'lactalbumin' and is in fact an insoluble mixture of whey protein aggregates (Figure 1.1) (Wong *et al.*, 1996b). Since the 1970s, several industrial scale technologies have been developed for isolating whey proteins. The advent of membrane separation techniques, in particular, has contributed to the commercial production of whole whey protein products, for example soluble WPC with 30 - 80% protein content (Korhonen *et al.*, 1998).

The development of industrial scale gel filtration and ion exchange chromatography techniques have also made it possible to manufacture high-quality pure whey protein products, referred to as WPIs or whey protein fractions, with 90 - 99% protein content (Korhonen *et al.*, 1998; Etzel, 2004). Briefly, WPCs are produced by the ultrafiltration and diafiltration (removal of salts, solvents and exchange buffers) of whey, and WPIs are produced by ion-exchange recovery (Figure 1.1) (Wong *et al.*, 1996b). WPC consists of a mixture of individual whey proteins such as β-lactoglobulin, α-lactalbumin, immunoglobulins, serum albumin and lactoferrin in decreasing amounts and may also contain small amounts of intact caseins, as well as their proteolytic products such as macropeptides and proteose-peptone fraction (Eigel *et al.*, 1984).

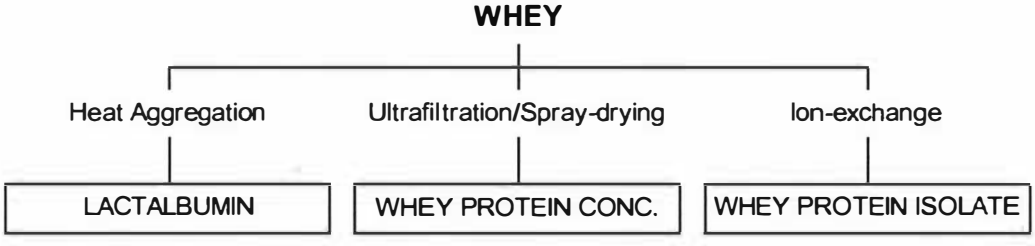


Figure 1.1 Processing of whey into whey protein products. Variations in the processing of whey produces lactalbumin, whey protein concentrate or whey protein isolate. Adapted from Wong *et al.* (1996b).

At high concentrations lactalbumin possesses a sandy and gritty mouth feel, therefore is most useful when used in small amounts as a protein fortification product in pasta, cereal, biscuits and bakery items (Wong *et al.*, 1996b.) WPCs or WPIs are currently very desirable as nutritional ingredients because of their excellent amino acid balance, including high concentrations of both essential and sulphur containing amino acids (Swaisgood, 1996). In the past, whey protein products had limited functionality because

of excessive protein denaturation or the presence of precipitating reagents; however modern technology has resulted in the development of a variety of products with varied functionality. A major proportion of commodity WPC (containing 35 to 55% protein) is used in animal feed manufacture, while speciality WPC and WPI (containing ≥ 70% protein) is used extensively as a functional and nutritional ingredient in medical, pharmaceutical and human food products, such as infant formula, gel product applications and frozen foods (Morr & Ha, 1993). Although WPCs and WPIs may be used in food product applications solely for their high nutritional quality, current applications of whey protein ingredients in formulated foods also include yoghurts, soft drinks, fruit juices, milk-based flavoured beverages, ice cream and meat based products (Swaisgood, 1996). WPCs are also used as a fat replacer, texturiser for use in dry sauce blends and as an ingredient in restructured meat and bakery products (Morr & Ha, 1993).

Functionality is a term used to describe the physico-chemical properties, such as hydrophilicity and hydrophobicity (influencing water protein interactions) interfacial (air-water and oil-water interfaces) intermolecular interactions (viscosity, fibre formation, film formation, gelation, cohesion, adhesion), and sensory (taste and smell) properties of a protein. The functionality of whey proteins can be improved or modified by a variety of enzymatic, chemical and physical methods (de Wit, 1989). Physico-chemical properties are influenced by the nature and the type of protein (i.e. the amino acid composition, conformation and bonds) and processing factors (i.e. source and variety, extraction processes including time, temperature, pH, ionic strength, impurities, conditions of storage and the method of use as well as the other ingredients present in the food formulation) (Kilara & Panyam, 2003). Processing can also remove the lactose and minerals which tend to reduce protein functionality due to complex formation and protein-ion interactions (Wong *et al.*, 1996b). While chemically modified whey proteins already have some use in foods, enzymatic hydrolysis and physical modification (by heat or pressure) offers a wide range of innovative possibilities for extending their use (reviewed by Kilara & Panyam, 2003).

A major application for whey protein hydrolysates today is in the manufacture of hypoallergenic infant formulas (Bahana, 1991). Furthermore, bovine α-lactalbumin and lactoferrin both possess a similar amino acid composition and structural homology to

their human counterparts, strengthening the argument for their use in dietary media such as infant formulae (Regester *et al.*, 1996). In these products, whey proteins are partially hydrolysed with digestive/microbial enzymes, sometimes followed by membrane separation technology to achieve a specific molecular mass distribution for the hydrolysate that significantly reduces the allergenicity of the proteins. Cow milk allergy is an important consideration when providing milk proteins as a food source for human consumption and is hence an area of continual research (Hernandez-Trujillo *et al.*, 2004; Peng *et al.*, 2004; reviewed by Kilara & Panyam, 2003). While further research is required on how to optimise the properties of bovine whey proteins for human consumption it is known that whey proteins are easily digested and have a high metabolic efficiency, giving these proteins a high biological value. In addition, total whey protein is available in concentrated powdered form and is one of the most economical, quality protein sources available commercially (Regester *et al.*, 1996). Collectively, these properties highlight the nutritional quality and cost effectiveness of whey protein, both as a direct dietary supplement, and as an ingredient in a variety of formulated foods and beverages. Indeed, several food companies are targeting vegetarians, athletes, active people, body builders, the elderly and those recuperating from illness in an attempt to utilise these features (Regester *et al.*, 1996). For example, Immunocal®, produced by a Canadian based company (Immunotec Research) is currently available as a pharmaceutical grade whey protein concentrate dietary supplement (90% protein, lactose- and fat-free) and is promoted to share many of the immune-promoting and enhancing properties of 'Mother's milk'. This Food and Drug Administration (US) approved food supplement claims to contribute to several body processes (such as improving immune function, liver function, wound healing, creation of lean muscle mass and inhibition of harmful bacteria) and may be beneficial in the treatment of cancer via the elevation of levels of tissue glutathione (Bounous *et al.*, 1989a; Bounous *et al.*, 1991; Bounous *et al.*, 1993; Kennedy *et al.*, 1995; Bounous, 2000). However, since the chemical composition and functionality of whey protein products are largely affected by the methods used during processing (Morr & Ha, 1993), the immunomodulatory properties of different whey protein concentrates and isolates can ultimately vary.

Advances in processing technologies over the past two decades have subsequently led to the accumulation of scientific data on the functional, biological and immunomodulatory

properties of whey proteins and contributed to the growing commercial value of a protein previously considered to be a waste product (Brown, 1984). New membrane separation and chromatographic techniques have made it possible to fractionate and enrich various components of whey even more effectively than before (Korhonen *et al.*, 1998), allowing extensive investigation into the immunomodulatory properties of highly purified whey protein products and components, with a view to developing possible industrial and biomedical applications (Wong *et al.*, 1997b).

1.4.3 Variation in composition, functionality and bioactivity of different whey protein preparations

Milk and whey protein concentrates have been classified as 'Generally Recognised As Safe' and therefore have been used as ingredients in formulated food products for human consumption (Hugunin, 1987). Billions of metric tons of whey and milk protein concentrates and isolates have been produced annually by the dairy industry for use as functional ingredients in a wide range of formulated food products (Morr, 1985). However, within the dairy industry considerable variation in the composition and functionality of whey protein products exists (de Wit *et al.*, 1986). In 1973, Morr *et al.* reported that the functional properties and composition of whey can be affected by its processing method and source (breed of cow), hence much of the variability of whey protein products can be attributed to use of different whey sources and the lack of standardised pre-treatment and processing conditions for the manufacturing and handling of whey proteins (Morr & Foegeding, 1990). For example, current whey protein preparation methods include pasteurisation, vacuum evaporation, ultrafiltration, reverse osmosis, ion exchange, gel filtration, electro-dialysis, crystallisation and spray drying (Spreer & Mixa, 1998) and the denaturation of α-lactalbumin and β-lactoglobulin has been found to increase with increasing pressure time, treatment time/temperature and milk pH (Huppertz *et al.*, 2004). The denaturation of whey proteins during processing is discussed further in Section 1.4.4.

In a study conducted to survey commercial WPCs and WPIs (produced by ion-exchange adsorption or ultrafiltration) around the world, three commercial WPIs, eight commercial WPCs and one commercial milk protein isolate were obtained from manufacturers in Denmark, United States, United Kingdom, West Germany and New Zealand (Morr and Foegeding, 1990). In addition to variations in the solubility, gelation,

foaming and turbidity, Morr and Foegeding (1990) found a significant variation in the composition of the whey protein products surveyed. Across all of the whey products assessed, protein components ranged from 72.0% to 92.7%, non-protein components from 0.34% to 4.56%, lactose from 0.42% to 5.22%, total lipids from 0.03% to 6.07% and pH from 6.15 to 7.39. The types of minerals in the ash component and the proportion of individual proteins were also found to vary in the samples tested (Morr and Foegeding, 1990). Consequently a concern of whey protein processors is to produce whey protein products with consistent food functionality; however the specific factors responsible for the variability of the products are not completely understood. The chemical characterisation of whey proteins has been found to be difficult in the less pure and damaged whey proteins which are frequently produced by commercial and semi-commercial manufacturing methods. Holt *et al.* (1999a) reported significant problems in determining the chemical composition (β-lactoglobulin, α-lactalbumin, bovine serum albumin, casein (glyco) macropeptide and triglycerides) of nine commercial whey protein products produced by three European companies. Reasons for these difficulties, such as the low and variable protein recoveries achieved by the analytical methods, were postulated to have been due to the presence of a heterogeneous population of aggregated, lactosylated and misfolded protein molecules in the samples, resulting from damage during processing. Physico-chemical and molecular structure studies confirmed that although the overall fold of the β-lactoglobulin molecule in the whey protein samples was preserved, all samples contained some misfolded and/or aggregated and /or lactosylated forms of the molecule (Holt *et al.*, 1999b), demonstrating that processing conditions can affect the physico-chemical properties of whey. The importance of the structure of whey proteins and its functional properties has been indicated by several researchers (Morr, 1984; Schmidt *et al.*, 1984; Wong *et al.*, 1996b). The composition and physico-characteristics such as solubility, foaming and emulsifying properties of WPCs have been found to be related to free fat, bound fat, ash, calcium and denaturation temperature (Kilara, 1994).

There are several reports on the composition of bovine milk (Eigel *et al.*, 1984, Barnes *et al.*, 1989) with the average composition of milk influenced by factors such as breed, stage of lactation, age, feed, season and general health of the cow. For example, Jersey cows have been reported to have higher amounts of total solids and less variation in protein content than the Holstein breed (Ji & Haque, 2003). The impact of protein

source (Jersey cattle herd or mixed herd) and whey protein processing (dehydration by four different processes involving ultrafiltration and/or vacuum-evaporation followed by lactose crystallisation and/or spray drying) on whey protein functionality was investigated in a recent study by Ji & Haque (2003). Moisture, crude fat, ash, calcium and crude protein were analysed and emulsifying activity index, emulsion stability, thermostability, oil holding capability and solubility were determined. Both whey protein processing and whey protein source were found to have an effect on these properties of whey and whey protein concentrates.In terms of composition, whey protein source had a significant impact on the crude protein content of the whey protein concentrates produced. Both whey protein source and whey protein processing methods either increased or decreased ash and calcium content, and of the five functional attributes studied thermostability and solubility were significantly affected by whey protein source. Emulsifying activity index and emulsion stability were affected by both whey protein source, and oil-holding capacity was not affected by either whey processing or whey protein source (Ji & Haque, 2003).

Differences in the bioactivity of bovine milk protein originating from different sources (breeds of cow) has been further highlighted in recent reports pertaining to β-casein A1 in milk obtained from Friesian/Holstein cattle (predominantly *Bos taurus*) and β-casein A2 in milk obtained from Jersey and Guernsey cattle (predominantly *Bos indicus)*. Several studies (epidemiological, *in vivo* and *in vitro)* have linked the consumption of A1 milk to Type 1 diabetes, vascular /heart disease and neurological disorders (Elliot & Martin, 1984; Laugesen & Elliot, 2003). The amino acid sequence of β-casein A2 contains proline in the 67^{th} position while β-casein A1 has histidine in the 67^{th} position; during digestion β-casein A1 is cleaved on the N terminal side of the histidine at position 67, releasing a fragment of seven amino acids termed β-casomorphin-7 (BCM-7). *In vivo* and *in vitro* studies have found BCM-7 to be an immunoregulatory peptide that exerts opioid-like activity (Teschemacher *et al.*, 1997) and influences factors related to heart disease such as platelet aggregation (Fiat *et al.*, 1989) and low-density lipoprotein oxidation (Torreilles & Guerin, 1995). Consequently it has been postulated that the consumption of the β-casein variant A1 yields the bioactive peptide BCM-7, which may promote physiological imbalances and lead to a predisposition for Type 1 diabetes and an increased risk of stroke and cardiovascular disease in humans, while the consumption of the β-casein variant A2 does not. Heart disease and the consumption of

liquid milk have not only been attributed to the β-casein A1 molecule, but previously to homogenised milk, via increased availability of xanthine oxidase (Oster, 1971) and cyanobacteria contamination of milk (Rank, 1986). While epidemiological studies indicate an association between milk consumption and diseases such as heart disease (Laugesen & Elliot, 2003), no such relationship has been reported between heart disease and processed or cultured dairy products (cheese, yoghurt and butter) possibly due to the killing of cyanobacteria and the destruction/denaturation of xanthine oxidase activity and the BCM-7 molecule during processing. Hence three factors appear to significantly influence the bioactivity and immunomodulatory potential of different bovine milk preparations, firstly the breed of cow the original milk was obtained from, secondly the composition of the milk protein and thirdly the treatments and processing techniques implemented to produce the milk protein preparations.

1.4.4 The denaturation of whey proteins during processing

Whey proteins obtained from dairy processing are an important source of functional ingredients used in many formulated foods, including processed meat, bakery, and dairy products because of their high nutritional value and functionality (e.g. their ability to stabilize emulsions and form gels) (Kinsella & Whitehead, 1989). Consequently, the heat-induced denaturation, aggregation, and gelation of WPCs and WPIs have been studied extensively (Dickinson, 1995; Huffman, 1996). Protein denaturation occurs by unfolding (involving the disruption of molecular interactions such as hydrogen bonds and hydrophobic interactions) and aggregation steps (de Wit, 1981). The denaturation of bioactive peptides during processing may reduce or eliminate the bioactive properties of proteins in their native state. For example, denaturation may cause the generation of peptide bonds resistant to cleavage during digestion or the breakage of peptide bonds that may release (or prevent the release) of bioactive peptides. Hence the state of the bioactive fraction (i.e. denatured or undenatured) is an important consideration.

The functional and bioactive attributes of whey proteins are determined by their molecular structure and interactions and can be altered by changes in environmental conditions, such as solvent composition, temperature and mechanical forces (Damodaran, 1996). Several processing treatments can cause proteins to denature including mechanical treatment such as high pressure, high temperature and pH changes (Demetriades & McClements, 1998). As discussed in Section 1.4.3, commercial whey

protein ingredients can vary significantly in their properties depending on their origin and the method used to isolate them (Huffman, 1996). This variability could therefore not only be attributed to differences in the amount of protein, lactose, fat, and minerals, differences in the relative amounts of the major whey proteins (e.g. β-lactoglobulin, α-lactalbumin, and bovine serum albumin), but also to differences in the degree of protein denaturation and aggregation caused by processing (Swaisgood, 1996). Other ingredients in food products may also interact with whey proteins and alter their functional characteristics, such as polysaccharides, sugars, vitamins, lipids, surfactants, and flavours (Kulmyrzaev *et al.*, 2000). The molecular basis for the functionality of whey proteins is, however, not yet completely defined due to the variability of whey protein ingredients and the use of whey proteins in a wide variety of different food products, each with its own unique composition, structure and processing requirements.

The heating of liquid milk (pasteurisation) destroys pathogenic micro-organisms and spoilage bacteria and deactivates enzymes without causing major changes to its constituents and is currently the most effective and suitable method of preservation (Walstra & Jenness, 1984). Consequently, pasteurisation is an important process used to not only inactivate bacteria but also to inactivate viruses in milk available for human consumption (Tomita *et al.*, 2002). A recent study reported an outbreak of *Campylobacter jejuni* enteritis in 13 out of 20 people after the consumption of raw (non-pasteurised) milk (Peterson, 2003); hence some whey protein products manufactured from raw milk may be unsuitable for human consumption.

There are several forms of pasteurisation including batch or vat (treatment at 63°C for 30 minutes), high temperature short-time (treatment at 72°C for 15 seconds), ultra pasteurisation (similar to high temperature short-time with higher temperatures and longer times) and ultra high temperature pasteurisation (treatment at $\geq$ 93°C for 2 seconds). Additional heating may also be necessary at different stages in the manufacture of various products such as whey protein powders (Law & Leaver, 2000; Section 1.4.1). Heating can however cause milk components to undergo modifications that affect the quality of the final product, such as lactose isomerisation to lactulose and degradation to organic acids, denaturation and loss of solubility of whey proteins such as β-lactoglobulin, destruction of some vitamins, hydrolysis of proteins and lipids and the disturbance of the mineral balance (Elliott *et al.*, 2003). Moreover, lactose interacts

with lysine residues on proteins and initiates a series of chemical changes known as the Maillard reaction that can result in changes in colour, flavour and nutritional value (O'Brien & Morrissey, 1989). The extent of these changes is related to the severity of the treatment i.e. the higher the temperature and/or the longer the heating time, the greater the impact (Elliott *et al.*, 2003).

On heating above 60°C, proteins tend to lose their globular conformation, and undergo aggregation (de Wit, 1981; Dannenberg & Kessler, 1988). Whey proteins are reasonably stable to pH precipitation but are susceptible to heat-induced denaturation (Morr, 1992). Heating can cause the unfolding and disulphide exchange of whey proteins and severe heating (above 75°C) can cause permanent denaturation or cause unfolded whey proteins to become associated with other whey proteins or casein micelles, however, changes in conformation resulting from mild heat are reversible (Mottar *et al.*, 1989). At lower pasteurisation temperatures and times (such as 72°C for 15 seconds) some whey proteins are considered to be quite resistant to denaturation, for example α-lactalbumin will unfold during the heating process but refold upon cooling (M. Pritchard, NZDRI, *pers. comm.*). Lactoferrin has also been found to be stable against heat treatment under acidic conditions and heating at pH 4, 90 - 100°C for 5 - 10 minutes and ultra heat treatment methods are considered suitable and practical pasteurisation methods for lactoferrin (Tomita *et al.*, 2002).

The extent of unfolding or aggregation is also dependant on the pH of heating, protein concentration, and ionic strength (de Wit, 1981; Dannenberg & Kessler, 1988). For example, rates of denaturation of the immunoglobulins and the serum albumin/lactoferrin fraction are highest at the lower end of the pH range, whereas rates of denaturation of β-lactoglobulin and α-lactalbumin increases over most of the pH range (Law & Leaver, 2000). Studies on reconstituted and fresh milk samples have also found that the association of whey proteins with casein micelles is markedly affected by the pH at which heating is carried out (Anema & Li, 2003). It is thought that this association may be due to hydrophobic interactions and the subsequent formation of disulfide bonds with other whey proteins or κ-casein (Haque & Kinsella, 1988). Heat treatment of lactoferrin at a neutral pH causes denaturation and the adjustment of pH and excessive heat treatment are known to result in the loss of particular amino acids

present in whey, such as cysteine and methionine (Damodaran, 1996). Hence, continued research is required to determine the optimal conditions required during heating to produce milk products with high amounts of undenatured protein. The use of alternative sterilisation methods (not requiring heat treatment) such as hydrostatic pressure and bacteriolytic enzymes to produce milk products suitable for human consumption also warrants further attention (reviewed by Gould, 2000).

β-Lactoglobulin (the major protein in whey) tends to dominate the behaviour of the total whey protein system. β-Lactoglobulin is a globular protein with a monomeric molecular structure that exists in various oligomeric states (dimers and octamers) as a function of pH, temperature, concentration, ionic strength, and genetic variant (Wong *et al.*, 1996b). The equilibrium between the monomeric and oligomeric forms of β-lactoglobulin is shifted to the monomeric form when the ionic strength is decreased and/or the temperature is increased (Renard *et al.*, 1998). β-Lactoglobulin denatures at temperatures above 65°C, and with mild heating the loss of the compact globular conformation may be reversible, but on more severe heating β-lactoglobulin tends to become aggregated through disulphide linkages and hydrophobic interactions (de Wit, 1990; Regester *et al.*, 1992). The concentration of milk solids has also been found to influence the denaturation and the rate of disulphide aggregation of β-lactoglobulin (Anema, 2000). Although β-lactoglobulin is often considered to determine the characteristics and behaviour of WPCs and WPIs, bovine serum albumin has also been shown to form disulfide-linked aggregates which may also influence the functionality and bioactivity of whey protein products (Havea *et al.*, 2000).

Commercially available whey protein supplements are commonly produced by filtration and/or ion exchange and WPCs are extracted on an industrial scale by ultra/diafiltration or by ion-exchange chromatography. While these processes give large yields of protein, they can cause irreversible conformational changes with undesired effects such as loss of protein solubility and functionality (Palazolo *et al.*, 2000). Large scale ion exchange processing involves the separation of proteins based on their electrical charge using hydrochloric acid and sodium hydroxide which can damage pH sensitive fractions and denature some proteins and peptides such as glycomacropeptides, immunoglobulins, lactoferrin, growth factors and α-lactalbumin. The elimination or reduction of these fractions can result in a higher percentage of other fractions such as β-lactoglobulin (a

reasonably stable fraction) that can account for up to 75% of the protein fraction present in whey protein preparations manufactured by ion-filtration. Hence, the ratios of individual whey proteins in whey protein preparations can be altered by processing techniques. Ion-exchange processing, involving alkaline (0.1 M NaOH) and heat treatment (75°C for 3 hours), has also been found to increase the amount of lysinoalanine in untreated lactalbumin from 0.10 g to 4.42 g/100 g protein (Sarwar *et al.*, 1999). The formation of lysinoalanine (a bonded amino acid compound) in treated proteins was accompanied by the loss of cysteine (73 - 77%), threonine (35 – 45%), serine (18 - 30%) and lysine (19 – 20%) and has been shown to adversely affect growth, protein digestibility, protein quality and mineral bioavailability and digestibility when fed to rats (Sarwar *et al.*, 1999). Thermal treatments and chemical modification have also been shown to significantly affect the enzymatic digestibility of whey proteins prepared from skim milk (pasteurised and non-pasteurised) (Sudharmarajan *et al.*, 2001), hence processing-based strategies provide opportunities not only to manufacture whey proteins with biologically beneficial ratios of whey protein fractions but also to modulate the digestibility of whey proteins and subsequent generation of bioactive peptides. In summary, processing can potentially affect the protein structure and composition of whey. Consequently, whey preparations produced under different conditions are likely to be subtly different from one another, hence the evaluation of bioactivity in WPCs and WPIs from different production streams is warranted.

1.5 The Modulation Of Lymphocyte Responses By Whey Proteins

1.5.1 Hypersensitivity

Research has shown that bovine milk proteins, including bovine whey proteins, can modulate (heighten or ameliorate) hypersensitivity responses (an excessive and adverse immune response involving IgE antibodies and allergen-specific T cells (Roitt, 1996; Janeway *et al.*, 1999)). While Bounous and Kongshavn (1985) found dietary lactalbumin had no ameliorating effect on *in vivo* delayed-type hypersensitivity responses in mice, Wong and Watson (1995) found that mice immunised with a subcutaneous injection of SRBC and fed a whey protein-enriched diet had significantly heightened delayed hypersensitivity responses to intradermally-injected SRBC as assessed by the foot pad assay. Furthermore, lactoferrin and κ-casein were found to ameliorate antigen-induced immediate (Type I), but not immune-complex mediated (Type III) or delayed-cellular (Type IV), skin hypersensitivity responses in guinea pigs, possibly by limiting local histamine release from sensitised mast cells when injected into the skin (Otani & Yamada, 1994; 1995).

1.5.2 Cytokine production and surface receptor expression

Cytokines (sometimes called lymphokines or interleukins) are small soluble proteins secreted by cells that can alter the behaviour or properties of the cells themselves or of other cells (Janeway *et al.*, 1999). Several researchers have demonstrated that bovine whey proteins can modulate lymphocyte cytokine production and surface marker expression when included in *in vitro* cell culture. Lactoferrin and lactoperoxidase, but not β-casein, have been shown to suppress the secretion of the cytokine interferon-γ by mitogen-stimulated ovine lymphocytes *in vitro* by Wong *et al.* (1997b). Similarly, Cross & Gill (1999) found that a modified whey protein concentrate suppressed the secretion of interferon-γ and surface expression of interleukin-2 receptors by mitogen-stimulated murine lymphocytes *in vitro*. The T cell surface expression of the interleukin-2 receptor by mitogen-stimulated murine lymphocytes has also been shown to be suppressed by kappa-casein-derived CGP in *in vitro* cell culture (Otani *et al.*, 1996). Bovine milk proteins have also been shown to modulate cytokine production in several human cell lines. An *in vitro* study using a human fibroblast cell line found that several milk-derived proteins (including kappa-casein-derived CGP, lactoferrin and a casein (κ)) suppressed production of the cytokine interferon-β while α- and β-casein enhanced

cytokine production (Yamada *et al.*, 1991). When included in *in vitro* cell culture bovine lactoferrin has also been found to suppress the secretion of the cytokine interleukin-6 by mitogen-stimulated human monocytic cells (THP-1 cell line) (Mattsby-Baltzer *et al.*, 1996).

1.5.3 Lymphocyte proliferation *in vitro*

The proliferation (clonal expansion) of antigen-specific lymphocytes in response to antigenic stimulation (preceding their differentiation into effector cells) is an essential step in specific adaptive immunity. This process allows rare antigen-specific cells to increase in number so that they can effectively combat the antigen that elicited the response (Janeway *et al.*, 1999). Whole casein, alpha-, beta-, and kappa-casein (including derivatives thereof), whole whey protein, lactoferrin, lactoperoxidase, and milk growth factor have all been found to modulate the *in vitro* proliferation of lymphocytes in a number of ruminant and non-ruminant species (including cattle, sheep, rabbits, and mice). These findings suggest that even though the exact effects of milk components can vary from species to species, the immunomodulatory potential of bovine milk is not limited by phylogenetic classification.

Due to their ability to stimulate a number of cell lines in culture, milk fractions or by-products have been used in culture media or as a substitute for foetal calf serum (Guimont *et al.*, 1997). In addition to the basic nutritional medium, whey contains growth factors, adhesion factors and transport proteins. The growth factors present in whey have a noticeable impact on cell growth by promoting synthesis of DNA and protein, and by inhibiting degradation of protein (Smithers *et al.*, 1996). Whey protein prepared by dialysis from WPC (New Zealand Dairy Board, Wellington, New Zealand) has been found to stimulate the proliferation and differentiation of an osteoblastic cell line (MC3T3-E1) when included in *in vitro* cell culture (Takada *et al.*, 1996) and lactoferrin and a mixture of caseins and whey proteins have been found to stimulate the proliferation of human and murine hybridomas and immunoglobulins (Murakami, 1989). Serum albumin and casein alone have also been shown to stimulate proliferation, but to a lesser extent. Polet and Spieker-Polet (1976) successfully used plasma-derived serum albumin *in vitro* as a growth factor for mitogen-activated lymphocytes. In addition to its ability to support cell growth in culture, the immunomodulatory effects of bovine whey preparations on lymphocyte function *in vitro* has been demonstrated by

several researchers. Mercier *et al.* (2004) demonstrated that microfiltered-whey protein isolates (at a concentration of 100 μg/mL) can significantly increase the *in vitro* proliferation of murine splenic lymphocytes without mitogen stimulation. A previous study by Wong *et al.* (1998) also found that a whey mixture consisting of α-lactalbumin, bovine serum albumin, bovine gamma globulin and β-lactoglobulin (10^{-5} M total milk protein) significantly increased cell proliferation and production of IgM by murine splenocytes in culture (Wong *et al.*, 1998). In the same study, while the commercially available whey protein dietary supplement Immunocal® (Immunotec Research, Canada) (10^{-5} M total milk protein) had no stimulatory effect on murine lymphocyte proliferation or IgM in culture, β-lactoglobulin (10^{-5} M total milk protein) containing both variants A and B was found to increase cell proliferation and production of IgM more than the β-lactoglobulin containing purified variant A or purified variant B alone (Wong *et al.*, 1998). Brix *et al.* (2003) found that a commercially available β-lactoglobulin preparation (Sigma-Aldrich) not only induced significant proliferation of spleen and mesenteric lymph node cells but also increased the production of tumour necrosis factor-α, interleukin-6, -1β and -10 and the level of cellular glutathione in spleen cell cultures. Tumour necrosis factor-α, interleukin-6, -1β and -10 production was also induced in murine marrow derived dendritic cells by β-lactoglobulin (Sigma-Aldrich). However, in the same study, purified β-lactoglobulin did not exhibit the same immunomodulatory activity as the commercial β-lactoglobulin (Sigma-Aldrich) preparations, leading the authors to conclude that endotoxin contamination was the main immunomodulatory component present in the commercial β-lactoglobulin (Sigma-Aldrich) preparations assessed in their study, rather than the β-lactoglobulin protein itself (Brix *et al.*, 2003). A bovine milk-derived whole whey protein (at a concentration of 1.1 ng/mL) has also been found to suppress T-lymphocyte mitogenesis *in vitro* (Torre & Oliver, 1989). Hence, whole whey derived from lactating dairy cattle has been postulated to be a potent suppressant of bovine T- and B-lymphocyte proliferation *in vitro*, however Barta *et al.* (1991) showed this activity was directly associated with the prior history of inflammatory mastitis among the individual animals, suggesting suppression may have been caused by an immunosuppressant resulting from the inflamed tissue rather than the milk protein itself. Different whey protein preparations have therefore been found to have a stimulatory effect (Smithers *et al.*, 1996; Takada *et al.*, 1996; Guimont *et al.*, 1997; Wong *et al.*, 1998; Mercier *et al.*, 2004), no effect (Brix

et al., 2003) or a suppressive effect (Torre & Oliver, 1989) on cellular proliferation *in vitro*.

Bovine lactoferrin has been shown to suppress the proliferation of T cells from cows, mice and sheep, when included in *in vitro* culture with lymphocytes stimulated or not stimulated by mitogens. For example, lactoferrin inhibited the *in vitro* proliferation of non-stimulated (without mitogen) murine splenic lymphocytes (Mercier *et al.*, 2004) and suppressed the mitogen-stimulated proliferation of peripheral blood T lymphocytes, as well as the interleukin-2-stimulated proliferation of mammary gland lymphocytes *in vitro* (Rejman *et al.*, 1992a; Rejman & Oliver, 1993). However, lactoferrin was found to have a bimodal effect on the interleukin-2 dependent proliferation of a bovine T-cell line, in that it suppressed proliferation at high doses but enhanced proliferation at low levels in *in vitro* culture. Otani & Odashima (1997) demonstrated that lactoferrin could suppress mitogen-induced proliferation of murine B and T lymphocytes, while Wong *et al.* (1997b) indicated that lactoferrin only suppressed T-cell (not B-cell) mitogenesis in sheep. These results imply that there may be some species-specific differences in the action of lactoferrin on different lymphocyte types.

Highly purified whey components have also been found to possess the ability to affect lymphocyte function. For example, Stoeck *et al.* (1989) found that a growth factor, isolated from bovine milk through multi-step chromatography, had a potent suppressive effect against human T-lymphocyte proliferation *in vitro*. The milk growth factor shared partial amino acid sequence homology with the endogenous cytokine transforming growth factor-β, and had the ability to suppress human lymphocyte proliferation at picomolar quantities following substantial purification. In addition, Wong *et al.* (1997b) showed that bovine lactoperoxidase can suppress ovine T-cell mitogenesis *in vitro,* although comparable to lactoferrin, it had no measurable effect on B-cell proliferation. Interestingly, Zavizion *et al.* (1993) and Stelwagen *et al.* (1994) used a bovine mammary epithelial cell line (MAC-T) to characterise a 13 kDa protein from bovine milk (mammary-derived growth inhibitor), which could inhibit cell proliferation at low picogram levels when included in *in vitro* cell culture.

Studies by Kulczycki & MacDermott (1985) and Kulczycki *et al.* (1987) demonstrated that milk-derived IgG (at levels as low as 0.3 mg/mL) could suppress both the

production of human IgG-, IgA- and IgM-class antibodies (at concentrations as low as 40 μg/mL), and the proliferative responses of T cell and T/B cell mitogen stimulated human peripheral blood lymphocytes when included in *in vitro* cell culture. Since cow's milk frequently contains IgG at concentrations between 0.6 and 0.9 mg/mL, it was concluded that endogenous bovine IgG may have the potential to modulate human immune function *in vivo*.

As technology allows individual milk components to be more effectively purified from whole milk protein the immunomodulatory effects of individual milk proteins become more apparent. In some cases the immunomodulatory effect of individual milk components have been found to be undetectable or diminished when assayed in its whole milk form (Wong *et al.*, 1997b). For example the purified whey proteins lactoferrin or lactoperoxidase alone have been shown to be potent modulators of ovine lymphocyte proliferation, however when combined into a single fraction their effects were diminished (Wong *et al.*, 1997b). In fact, Wong *et al.* (1997b) found that intact whey protein concentrate had no modulatory effect on mitogen-induced proliferation of ovine lymphocytes. A modified whey protein concentrate (i.e. one which had been partially depleted of extraneous non-immunomodulating whey proteins) has been shown by Cross and Gill (1999) to suppress mitogen- and alloantigen-induced proliferation when included in *in vitro* culture with murine lymphocytes. These findings suggest that the protein components in whey may counteract each other with or without the influences of other trace factors and that certain components may regulate the immunomodulatory functions of others (Wong *et al.*, 1997b).

1.5.4 Lymphocyte function *in vivo*

The effects of bovine whey proteins on lymphocyte function *in vivo* have also been investigated in several studies. The majority of these studies have reported an enhancement of lymphocyte function after *in vivo* exposure to bovine whey proteins. Splenic lymphocytes obtained from mice fed α-lactalbumin and primed with a T cell stimulator (*Mycobacterium bovis,* strain BCG) showed enhanced proliferative responses to T cell mitogens (Bounous *et al.*, 1983a). Further study of BCG-primed mice showed that increasing concentrations of dietary lactalbumin hydrolysates promoted enhanced splenic lymphocyte responses to T and B cell mitogens (Bounous & Kongshavn, 1982). Similarly, Wong & Watson (1995) found that compared to soy- or wheat-based diets,

diets enriched with whey protein increased splenocyte proliferative responses to T and B mitogens in mice. The antigen-specific response of lymphocytes derived from Peyer's patches and spleen were also found to be enhanced in mice fed lactoferrin (Debabbi *et al.*, 1998) suggesting that the immunomodulation of lymphocyte function by bovine whey protein is experienced both systemically and locally.

1.5.5 Antibody production

Antibodies are produced by plasma cells and bind to and neutralise pathogens or prepare them for uptake and destruction by phagocytes in response to infection or immunisation (Janeway *et al.*, 1999). Therefore, in some instances, lymphocyte activation can be measured by the *in vivo* production of a specific antibody.

Dietary bovine whey protein has been found by several researchers to modulate antibody responses in mice after antigenic challenge. For example, Bounous *et al.* (1981, 1983a) and Bounous & Kongshavn (1982) demonstrated that the dietary inclusion of lactalbumin (a hydrolysed whey protein aggregate, rich in α-lactalbumin) could enhance antibody responses (measured by plaque-forming cell response to SRBC) in mice inoculated intraperitoneally with a foreign antigen (BCG) after the commencement of dietary treatment. This immunomodulatory effect could be achieved using lactalbumin in both the native and hydrolysed state (Bounous & Kongshavn, 1982). In a 1988 report, Bounous and co-workers fed a commercially available lactalbumin (LACPRODAN–80, Denmark Protein, Worthington, Ohio, USA) to mice and found the humoral immune response measured by a plaque-forming cell response to SRBC was enhanced (by 168 - 927%) in lactalbumin-fed animals compared to those fed casein, soy, wheat, corn protein, egg albumin, fish protein, *Spirulina maxima, Scenedesmus* algae protein or Purina mouse chow (Bounous & Amer, 1988; Bounous *et al.*, 1988a). This effect was not associated with pre-sensitisation of the lactalbumin fed group, since only very low numbers of plaque-forming cells per spleen were found in non-immunised mice, and animals fed on all diets had similar responses at the pre-immunised stage (Bounous & Amer, 1988). All dietary proteins sustained similar growth, with comparable food consumption and serum protein levels. Mixing lactalbumin with other protein sources also resulted in enhanced humoral immune responses. For example, a 50:50 mix with soy resulted in a 4 fold increase in immune response compared to a pure soy diet, an 80:20 mix with casein induced a 3 fold

increase and a 20:80 mix resulted in a 2 fold increase over the response of a pure casein diet (Bounous & Amer, 1988). The characteristics of the immune enhancing effect of dietary whey protein found in several studies conducted by Bounous and colleagues are summarised in Table 1.3.

The immune enhancing effect of dietary whey protein was later confirmed in a study by Wong and Watson. In 1995, Wong and Watson found that, compared to other protein sources such as soybean protein isolate and ovine colostral whey proteins, the dietary intake of crude WPC enhanced IgG responses to ovalbumin in mice. Some reports have however indicated that proteins isolated from bovine whey can suppress antibody responses in mice. In 1992, Watson *et al.* found that a bioactive fraction, isolated from bovine colostral whey by cation exchange and reverse phase chromatography, could suppress systemic IgE antibody responses in mice. Similarly, Monnai *et al.* (1998) found that dietary kappa-casein-derived CGP (a major constituent of sweet whey) suppressed IgG (but not IgM, IgA or IgE) antibody responses to systemically administered β-lactoglobulin. The study by Monnai *et al.* (1998) also found that dietary CGP suppressed the production of IgG antibody against an orally administered antigen. This finding suggested that bovine milk may directly affect the gastrointestinal immune system *in vivo.* A recent study has also found that the mode of oral bovine whey protein administration may be an important consideration when investigating the regulation of immune responses by food proteins. Sfeir *et al.* (2004) found that while the administration of lactoferrin daily for 6 weeks by buccal dose, intragastric gavage and *ad libitum* (100 mg lactoferrin/diet) to mice stimulated immune function (measured by systemic and intestinal antibody responses against lactoferrin, total immunoglobulins, proliferation of spleen or Peyer's patch cells and Th1, Th2 cytokine responses), the addition of lactoferrin to the drinking water had no effect on immune status.

TABLE 1.3

Characteristics of the modulation of humoral immune responses by dietary whey

Characteristic	Observation
Mouse Strains	DBA/2J;C3H/HeN or C3H/HeJ;C57BL/6 or C57BL/6NIA;BALB/c and BALB/cj;CBA/N or CBA/NxDBA/2J or DBA/2/2JxCBA/N; A/J. These findings imply the immunoenhancing effect of dietary whey on the humoral immune response is not murine strain specific.
Age of mice	The age of mice showing enhancement ranged from 7 weeks up to 6 months of age.
Dose	Murine B lymphocytes showed a maximal antibody response, five times higher than normal levels, to antigenic challenge when fed a 20 g lactalbumin/100 g diet.
Nutritional quality	Body weight increase and feed consumption were similar for all diets that contained adequate vitamin supplements. Thus, this immune-enhancing effect is independent of protein energy level.
Kinetics	The immune enhancing effect of lactalbumin was seen after 2 weeks and persisted for approximately 8 weeks, as long as the dietary treatment continued.
Antigenic stimulus	In the absence of antigenic stimulus, dietary protein type had little or no effect on the parameters examined. Body growth, food consumption, serum protein, minerals, trace metals; circulating lymphocytes; mitogenic responsiveness and formation of bone marrow B lymphocytes were within normal limits.
Total spleen cell number	The immune enhancing effect was not associated with total spleen cell number.
Plasma amino acid profile	A change in plasma amino acid profile was the only significant effect that could be related to protein type. This profile conformed to the amino acid composition of the ingested protein.
Heat labile	The immunoenhancing activity of lactalbumin was subject to heat-induced denaturation and most effective in an un-denatured state.
Whey protein components	Individual major components of whey increased humoral responses, but were less effective than the total lactalbumin mixture.
Free amino acid mix	Free amino acid mix duplicating the specific amino acid profile of the various proteins maintained immune enhancing effects, but elicited a lower response than the intact proteins. Thus, the immune enhancing effect of dietary lactalbumin is associated with the overall amino acid profile resulting from the contribution of all protein components.
B-cell function	The antibody response to a T-independent antigen was increased by dietary WPC, indicating whey proteins may exert their immunoenhancing effect by enhancing B-cell function.

(Bounous & Kongshavn, 1982; Bounous *et al.*, 1983a; Bounous *et al.*, 1983b; Bounous & Kongshavn, 1985; Bounous *et al.*, 1985; Bounous *et al.*, 1988a; Bounous *et al.*, 1988b; Bounous *et al.*, 1988c; Bounous & Kongshavn, 1989; Papenburg *et al.*, 1990)

1.6 The Modulation Of Non-Lymphoid Functions By Whey Protein

1.6.1 NK and Neutrophil function

NK cells are large granular lymphocyte-like cells with important functions in innate immunity such as participating in cellular cytotoxicity against cells infected with a virus or tumour cells (Janeway *et al.*, 1999). Wong *et al.* (1996a, 1997b) were unable to demonstrate a significant effect of bovine lactoferrin, lactoperoxidase or β-casein on NK cell cytotoxicity against viral-infected target cells *in vitro.* However, 1 mg/g body weight of lactoferrin was found to protect mice from cytomegalovirus infection by enhancing T cell-mediated NK function *in vivo* (Shimizu *et al.*, 1996), demonstrating that bovine whey components can have a direct impact on immune-mediated health.

Bovine whey proteins have also been shown to modulate neutrophil function. Neutrophils are major circulating phagocytic polymorpho-nuclear granulocytes that enter tissues early in immune responses and mediate antibody dependent cell-mediated phagocytosis (Roitt, 1996). Several studies have found that neutrophil oxidative responses can be affected by bovine whey proteins when included in *in vitro* cell culture (Wong *et al.*, 1996a; Shinoda *et al.*, 1996; Wong *et al.*, 1997b; Miyauchi *et al.*, 1997). The generation of the superoxide anion by ovine neutrophils was enhanced by whey protein concentrate, lactoferrin/lactoperoxidase (mixed) and β-casein *in vitro* (Wong *et al.*, 1996a; Wong *et al.*, 1997b). Miyauchi *et al.* (1997) confirmed the ability of bovine whey-derived proteins to enhance *in vitro* neutrophil function in heterologous species, when human neutrophils showed enhanced phagocytic capacity following culture with lactoferrin or lactoferricin (derived from pepsin hydrolysis of lactoferrin). Shinoda *et al.* (1996) also found that the induction of interleukin-8 by lactoferrin affected the chemotactic function of human neutrophils in culture. However, Wong *et al.* (1997b) found that lactoferrin, lactoperoxidase, α-lactalbumin or whey protein concentrate had no effect on ovine neutrophil chemotaxis in response to serum-derived complement chemotaxins, while whole bovine whey protein inhibited neutrophil chemotaxis. These findings suggest firstly that there may be species-specific differences in the modulatory capacity of bovine whey protein with respect to neutrophil function and secondly that fractionated or concentrated proteins may not have the same immunomodulatory effects as the parent (native) product.

1.6.2 Macrophage function

One important role of macrophages is to function as effector cells in humoral and cell-mediated immunity by processing and presenting antigens in association with cell surface major histocompatibility (MHC) class II molecules, to responsive T cells (Janeway *et al.*, 1999). To date research has not demonstrated a measurable effect of milk proteins (β-casein, lactoferrin, lactoperoxidase or α-lactalbumin) on expression of MHC antigen-presenting molecules by macrophages (Politis *et al.*, 1991; Wong *et al.*, 1996a; Wong *et al.*, 1997b). Bovine milk components, including whey protein components have, however, been found to have a heterogeneous effect on the phagocytic function of murine macrophages *in vitro*. α-Lactalbumin (and β-casein) has been found to enhance phagocytic function, while lactoferrin (and α- and κ-casein) reduced phagocytic function of murine macrophages in culture (Otani & Futakami, 1994). Phagocytic function was assessed by determining the number of target microparticles phagocytosed per cell and the number of cells that were actively phagocytic. Interestingly, studies by Otani and colleagues (Otani & Futakami, 1994; Otani & Futakami, 1996) on enzymatically (trypsin, chymotrypsin and pepsin) digested bovine milk components (caseins) found that the enzyme treated bovine milk had a different immunomodulatory effect on the phagocytic function and nitrite production of murine macrophages compared to intact native bovine milk. This finding may explain, in part, some of the varying immunomodulatory effects of bovine milk protein components observed in *in vitro* and *in vivo* studies.

Activated macrophages also play an important role in innate immunity by producing cytokines and by producing microbicidal reactive oxygen and nitrogen intermediates. Several *in vitro* studies have shown that bovine proteins can modulate these functions. Interleukin-1 (but not tumour necrosis factor) production by lipopolysaccharide-stimulated ovine macrophages was found to be enhanced by β-casein (Wong *et al.*, 1996a) and α-lactalbumin (Wong *et al.*, 1997b). However, the production of interleukin-1 cytokines by lipopolysaccharide-activated murine macrophages and a macrophage cell line (P388D1) was found to be blocked by κ-casein-derived CGP (Otani & Monnai, 1995; Monnai & Otani, 1997). The production of nitrite (an index of reactive nitrogen intermediate production) by murine macrophages was also found to be suppressed by lactoferrin, α- and κ-casein (Otani and Futakami, 1994; 1996).

1.7 Mechanisms Of Action

The precise mechanisms by which bovine whey proteins act on the immune system have not been fully elucidated. It has been suggested that the immunoenhancing ability of milk proteins may in part be due to its unique overall amino acid profile. Substitution of the intact milk proteins with an equivalent free amino acid mix which duplicated the specific amino acid profile of the proteins, elicited lower responses than the intact proteins, but still enhanced humoral immune responses above their basal level (Bounous & Kongshavn, 1989). There is also strong evidence for the involvement of glutathione (L-gamma-glutamyl-L-cysteinyl-glycine, GSH) in immune enhancement by dietary whey proteins (which are rich in precursors required for glutathione synthesis, such as cysteine (Parodi, 1998)). Glutathione is a ubiquitous tripeptide residing in the cell membrane bi-layer that functions directly or indirectly in many biological phenomena such as protecting cells against oxidative damage by superoxide radicals and peroxides (reviewed by Meister & Anderson (1983); Fidelus & Tsan, 1987; Lacey & Wilmore, 1990). The functions of glutathione are particularly important in actively metabolising cells such as lymphocytes (Sadler, 1992) and adequate levels of glutathione have been shown to be necessary for lymphocyte proliferation in the development of the immune response (Noelle & Lawrence, 1981). Glutathione is the most abundant thiol reducing agent in mammalian tissues and is not only central in cellular protection against oxygen radials, but also maintains normal tissue hydration, detoxifies a variety of harmful compounds and xenobiotics and is important in the binding and elimination of transitional metals (reviewed by Bounous *et al.*, 1989b).

A high concentration of glutathione in the spleen during antigen driven clonal expansion of B lymphocytes has been found in young adult C3H/HeN mice fed a lactalbumin diet for 3 weeks, whereas, mice fed denatured WPC, casein, egg white or cysteine-enriched casein as a protein source exhibited low concentrations of glutathione in the spleen. Dietary cysteine is the rate limiting substrate for glutathione synthesis and the efficiency of dietary cysteine in inducing higher than normal glutathione levels is greater when it is delivered as a whey protein than as free cysteine (Bounous & Gold, 1991). Furthermore, the administration of L-buthionine-sulfoximine, which inhibits gamma-glutamyl cysteine synthetase (a precursor for glutathione synthesis) significantly reduces humoral immune responses (Bounous *et al.*, 1989b). The unique immune enhancing

characteristic of WPC is therefore thought to reside in its high cysteine content and the occurrence of glutamyl-cysteine groups within its amino acid sequence, which act as precursors in glutathione synthesis.

An increased level of glutathione is also seen in the heart, liver and lymphocytes of animals fed WPC over 3 - 4 months (Bounous *et al.*, 1989a; Bounous *et al.*, 1989b). This implies that WPC can increase the rate of synthesis, replenishment and/or the concentration of glutathione in a number of tissues. Cole and Ketterer (1990) demonstrated that the presence of high levels of glutathione in tissues suppresses tumour development at various sites in the body, possibly by reducing free radical- and oxidant-induced damage to chromosomal DNA. Moreover, glutathione transferase enzymes catalyse the conjugation of potentially damaging chemical mutagens and carcinogens which can then be eliminated from the body (Parodi, 1998). McIntosh *et al.* (1995) also measured tissue glutathione levels in their study on the inhibitory effect of WPC on colon tumourgenesis, finding that the liver glutathione concentration was the lowest in soybean protein-fed rats and highest in WPC- and casein-fed rats. The results indirectly support a role for whey proteins in enhancing tissue glutathione levels and the provision of a degree of protection against tumour development. In humans, supplementation with whey proteins has been found to increase plasma glutathione levels in glutathione-deficient patients with advanced infection, warranting clinical trials to investigate the biochemical efficacy of whey proteins in benefiting HIV-infected patients (Micke *et al.*, 2001). Bennett & Davis (1981) and Faith *et al.* (1984) have also demonstrated receptors for milk proteins and peptides on immuno-competent cells of humans, therefore it is possible that some milk components may exert their immunomodulatory effects by acting directly on immuno-competent cells.

1.8 Anti-cancer Properties

Cancers are caused by the progressive growth of the progeny of a single transformed cell; therefore curing cancer requires the removal or destruction of the malignant cells without killing the patient (Janeway *et al.*, 1999). Studies in animals have shown that T cells are a critical mediator of anti-tumour immunity and advances in the understanding of antigen presentation and the molecules involved in T-cell activation have provided new immunotherapeutic strategies including the enhancement of the immune response against tumours by dietary proteins.

The ability of whey protein to protect against chemically-induced carcinogenesis has been clearly demonstrated in several rodent models. In 1990, Papenburg *et al.* found that the incidence of dimethylhydrazine (DMH)-induced tumours in whey protein-fed mice was significantly less than in casein- and chow-fed mice. Further study by Bounous *et al.* (1991) also demonstrated that dietary WPC has potent anticancer properties against colon cancer induced by DMH in mice. McIntosh *et al.* (1995) found a protective role for dietary dairy proteins against DMH-induced intestinal tumour development in rats. The results of the study by McIntosh and colleagues illustrated that dietary whey protein and casein were more protective against the development of intestinal cancers in rats than red meat or soy bean protein. This finding not only suggests that major dietary proteins differ in their ability to protect against cancer development, but also that the proteins in dairy foods (particularly whey proteins) may play a significant role in cancer prevention (McIntosh *et al.*, 1995). There is also some evidence from controlled clinical studies of the effectiveness of WPC in limiting metastasis during anti-cancer therapy in humans (Kennedy *et al.*, 1995).

It is postulated that low antioxidant status and high levels of fat in the faeces represent risk factors for colon tumourgenesis (Samelson *et al.*, 1985; Potter, 1996). Therefore in addition to investigating the effects of dietary whey protein on the incidence of DMH-induced tumours in rats, McIntosh *et al.* (1995) also investigated the effect of different protein diets on antioxidant status and faecal fat. In their study, rats that were fed whole whey protein had the highest antioxidant status and the lowest content of faecal fat.

The anti-cancer potential of individual whey protein components such as lactoferrin has also been investigated by several researchers, as reviewed by Lonnerdal and Iyer (1995). The iron-binding properties of lactoferrin are postulated to contribute the most significantly to the anti-cancer properties of this whey protein component. Free iron is thought to act as a mutagenic promoter by inducing oxidative damage to the nucleic acid structure; hence the binding of iron by lactoferrin in tissues may reduce the risk of oxidant-induced carcinogenesis (Lonnerdal and Iyer, 1995).

The oral administration of lactoferrin and hydrolysed lactoferrin significantly inhibited the metastasis of colon 26 carcinoma in the lungs of mice before and after tumour transplantation (Kuhara *et al.*, 2001). Orally-administered lactoferrin also augmented $CD4^+$ and $CD8^+$ T cells in the spleen and peripheral blood by enhancing their cytotoxic activities against Yac-1 and colon-26 carcinoma cells. In the small intestinal epithelium $CD4^+$ and $CD8^+$ cells were markedly increased and the simultaneous enhancement of interleukin-18 production was confirmed in the intestinal epithelial cells. In this model, intravenous injection of murine interleukin-18 showed significant inhibition of lung colonisation by colon-26 carcinoma cells. These results suggest that inhibition of experimental metastasis by oral administration of lactoferrin may be due to enhanced cellular immunity, presumably mediated by enhanced interleukin-18 production. The effect of orally-administered lactoferrin on leucocyte populations in the peripheral blood of mice was later confirmed by Wakabayashi *et al.* (2002), who also found significant increases in $CD4^+$ T cells, $\gamma\delta$(T-cell receptor)$^+$ T cells, NK cells and granulocytes after 1 day of administration.

1.9 Resistance To Infection

Several studies by Bounous and colleagues have demonstrated that dietary lactalbumin can increase resistance to bacterial infection and subsequently reduce the mortality of mice (Bounous & Amer, 1988; Bounous & Kongshavn, 1989). The overall mortality in C3H/HeJ mice intravenously challenged with pneumococcus type 3 bacteria (10^3) was 36% in casein fed (20% w/w) mice and 7% in lactalbumin fed (20% w/w) mice (Bounous & Kongshavn, 1989). In the same study, the similarity of animal growth of the two groups of mice prior to inoculation indicated that resistance to infection was independent of weight at the time of infection and weight gained before infection. At a higher dose (10^4) of pneumococcus type 3 bacteria, no difference in mortality was seen (Bounous & Kongshavn, 1989). The increased resistance to infection in lactalbumin fed mice was postulated to be associated with increased production of anticapsular antibodies, as the acquired immunity to *Streptococcus pneumoniae* is largely dependent on the humoral immune response (Bounous & Amer, 1988). Conversely, an earlier study by Bounous *et al.* (1981) found the effect of lactalbumin on orally-administered *S. typhimurium* infection was short term and overall mortality was not altered. However, Bounous & Amer (1988) later concluded that dietary lactalbumin had no likely influence on resistance to *S. typhimurium* infection because resistance to this facultative intracellular bacterium is primarily cell-mediated.

Bounous *et al.* (1988a) observed that aging mice were more prone to intravenous *Staphylococcus* infection and subsequent death. This decline in resistance to intravenous staphylococcal infection in aging mice correlated with a significant decrease in intracellular glutathione levels, splenic glutathione, and the progressive failure of T lymphocytes to respond to proliferating stimuli (Bounous *et al.*, 1988a). The feeding of lactalbumin delayed the aging process and mortality of mice by increasing resistance to spontaneous infections and decreasing tumour growth (Bounous *et al.*, 1988a). Very old (20 month old) mice fed a whey-based diet exhibited higher levels of glutathione in heart and liver tissues than mice fed casein or Purina mouse chow. Furthermore, glutathione supplementation has been shown to reverse the age-associated decline in immune responsiveness (Furukawa *et al.*, 1987). Therefore it is possible that dietary whey protein may preserve superior immune capability in older animals by maintaining high glutathione levels rendering the host more resistant to spontaneous infection.

The effect of dietary protein type on parasitic infection and their interaction with the immune system has also received research attention. While whole milk protein has been demonstrated to exacerbate the clinical condition of rats infected with malaria (van Doorne *et al.*, 1998), several studies have demonstrated that lactoferrin can protect mice against infection from micro organisms such as *Toxoplasma gondii, Giardia lamblia* and *Helicobacter pylori* (Turchany *et al.*, 1995; Isamida *et al.*, 1998; Wada *et al.*, 1999). Additionally, compared to mice fed casein and soy proteins, dietary bovine whey protein (α-fraction) caused the most significant stimulatory impact on the immune responsiveness (total white blood cell count, lymphocyte count, interferon production) in mice infected with the parasite *Eimeria vermiformis* (Ford *et al.*, 2001). In a recent study by Dial & Lichtenberger (2002) human lactoferrin was also found to be active against *Helicobacter felis* infection in mice. The oral administration of recombinant human lactoferrin not only significantly reduced the gastritis caused by *Helicobacter felis* compared to untreated mice but also improved gastric surface hydrophobicity (a measure of mucosal integrity) (Dial & Lichtenberger, 2002).

1.10 Introduction To The Current Study

There is currently a large body of literature supporting the immunoenhancing ability of bovine whey proteins in several heterologous species, and steps are being undertaken by several research teams to produce bovine milk products with proven *in vitro* and/or *in vivo* immunomodulatory activity with, in many cases, the end product (following clinical trials) being a dietary supplement designed to promote health in humans. Whey protein preparations investigated in previous studies have, however, been demonstrated to either have no effect, a suppressive effect or a stimulatory effect on several different parameters of the immune system (described in Section 1.5 and 1.6), indicating that different whey protein preparations can exhibit different immunomodulatory abilities, possibly due to differences in the preparation techniques (Section 1.4.3). Hence, this thesis aims to contribute to the current knowledge of the immunomodulatory properties of whey protein preparations by evaluating different whey protein preparations whose immunomodulatory properties are currently unknown.

The immunomodulatory ability of previously untested bovine whey products (dairy-derived whey protein fractions and whey protein concentrate) on general immunoresponses will be investigated and described for the first time in this thesis. The *in vitro* immunomodulatory abilities of these dairy-derived whey proteins obtained from the New Zealand Dairy Board will also be directly compared to commercially prepared (ICN) whey protein fractions, whose effect on the immune system have not been extensively described. In many cases a different immunomodulatory effect has been realised when milk products have been tested both *in vitro* and *in vivo* (Otani *et al.*, 1995; Wong & Watson, 1995; Monnai *et al.*, 1998). Therefore, the immunomodulatory ability of the previously untested whey proteins will be investigated both *in vitro* and *in vivo*.

The ability of a dietary bovine whey-derived protein to enhance immune responses to specific antigens and to be of use as a dietary adjuvant or an immunopotentiator of immune responses to vaccines is particularly relevant to modern human health. However, research investigating the ability of bovine whey proteins to enhance orally and parentally–administered antigens is limited. Debabbi *et al.* (1998) has found that the oral administration of bovine lactoferrin stimulates both mucosal and systemic antibody

responses in mice. Additionally, He *et al.* (2001) observed a trend towards a greater increase in specific IgA among human subjects receiving *Salmonella typhi* (*S. typhi*) oral vaccine in conjunction with orally administered liquid bovine colostral whey, suggesting that bovine colostral whey may possess some potential to enhance human humoral immune responses. In contrast, Penttila *et al.* (2001) found that the oral administration of a growth factor derived from bovine whey suppressed the immune activation to a specific antigen (ovalbumin). Collectively these findings emphasise that current information regarding the effect of bovine whey proteins on immune responses against orally and parentally–administered antigens is conflicting and far from exhaustive. The ability of bovine whey proteins to modulate murine immune responses to vaccines currently in routine medical use is currently unknown. Consequently, this thesis also aims to address this gap in the current knowledge of the effect of bovine whey proteins on immune responses against orally and parentally-administered antigens, and investigate the ability of bovine whey proteins to modulate murine immune responses to vaccines currently in routine medical use.

The results of this study are arranged into 6 chapters representing the progression of research. Initially in Chapter 3, individual whey proteins (α-lactalbumin, β-lactoglobulin and lactoferrin) were screened for their *in vitro* effects on lymphoid cell function. The experiments in this chapter examined the mitogenic activity of whey proteins on lymphocytes stimulated or unstimulated by mitogens and compared these effects to other commercially available whey proteins. Results suggested that individual whey proteins (β-lactoglobulin, α-lactalbumin and lactoferrin) could enhance lymphoid cell function *in vitro* and therefore have significant mitogenic effects. These findings led to further *in vitro* screening of the effects of these isolates on other immune functions, namely the phagocytic activity of non-lymphoid cells. The results of these experiments are presented in Chapter 4. In this chapter individual whey proteins were screened for *in vitro* effects on the phagocytic activity of murine peritoneal cells and a human monocytic cell line (THP-1), by investigating the ability of these cells to phagocytose *Escherichia coli* (*E. coli*) and unopsonised latex beads after incubation with various concentrations of whey proteins. These effects were compared to other commercially available whey proteins. The results of these experiments suggested that the individual whey proteins (β-lactoglobulin, α-lactalbumin and lactoferrin) could enhance

phagocytic cell function of both murine peritoneal cells and a human monocytic cell line (THP-1) *in vitro*. The positive immunoenhancing effect of α-lactalbumin, β-lactoglobulin and lactoferrin found *in vitro* in both Chapters 3 and 4 provided evidence that these components have the potential to modulate immune function. The investigation of the immunomodulatory capabilities of these individual whey proteins in the *in vivo* murine model was consequently undertaken.

The objective of Chapter 5 was to examine the *in vivo* effect of the short term feeding of individual whey proteins on a variety of immune functions in the murine model using a panel of *in vivo* and *ex vivo* assays. The results of these experiments suggested that short term feeding (up to 6 weeks) of individual whey proteins (β-lactoglobulin, α-lactalbumin and lactoferrin) had immune enhancing properties *in vivo* in the murine model. Previous research by Bounous & Amer (1988), Bounous *et al.* (1988a) and Wong & Watson (1995) indicated that WPCs (containing a mixture of β-lactoglobulin, α-lactalbumin and lactoferrin) have the potential to exhibit immunostimulatory effects and possibly exert stronger immune enhancing effects *in vivo* than individual whey protein components such as those tested in Chapter 5. The availability of a promising (previously untested) WPC (ALACEN 472) provided an opportunity to evaluate the WPC for immunomodulatory capacity *in vivo*. In Chapter 6, the effect of this WPC on mucosal antibody responses to orally administered T-dependent antigens was investigated. Furthermore, the concurrent effect of this dietary WPC on several lymphocyte functions in the murine model was also examined. The results of these experiments suggested that this dietary WPC could enhance *in vivo* mucosal antibody responses after 3 immunisations of orally administered cholera toxin and ovalbumin. These findings implied that this WPC might be of use in human health as a dietary adjuvant or immunopotentiator to increase immune responses to commonly-administered vaccines. To further investigate this implication, the effect of the short term feeding of the WPC on immune responses to vaccines commonly used to immunise humans was evaluated in the murine model. The results of this investigation are presented in Chapter 7. Additionally, the immunomodulatory effects of feeding lactoferrin enriched WPC on several parameters of immune function were also evaluated, as lactoferrin alone has been shown by several researchers to possess immunomodulatory properties. The results of these experiments found the short term

feeding of both the WPC and the lactoferrin enriched WPC enhanced mucosal and systemic antibody responses to orally and systemically administered vaccines commonly used to immunise humans.

The objective of Chapter 8 was to further investigate the potential of the WPC as a dietary adjuvant to enhance immune responses to vaccines commonly used to immunise humans. In this chapter experiments examining the effect of the long-term-feeding (up to 12 weeks) of WPC on antibody responses to T-dependent vaccines, administered according to schedules recommended for human vaccination, in the murine model were conducted. Additionally, the impact (if any) of pre-feeding WPC for 4 weeks prior to commencement of immunisation schedule was evaluated. The results of this final chapter confirmed that the long term feeding of WPC can enhance both mucosal and systemic antibody responses to commonly used oral and systemic human vaccines administered according to recommended immunisation schedules irrespective of when the dietary regime commenced.

CHAPTER 2

General Materials And Methods

2.1 Whey Proteins

All of the dairy whey protein powders tested in this study were produced by the New Zealand Dairy Board (NZDB), Wellington, New Zealand (now known as Fonterra) and supplied by the New Zealand Dairy Research Institute (NZDRI), Palmerston North, New Zealand (now known as Fonterra Marketing and Innovation, Palmerston North, New Zealand). All commercially available whey protein powders tested in this study were obtained from ICN (Nutrition Biomedical, Cleveland, Ohio) or Tatua Biologics (Morrinsville, New Zealand). All whey proteins used in *in vitro* studies were solubilised in complete RPMI-1640 (RPMI-1640 supplemented with 10% v/v heat inactivated foetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin sulphate, 2 mM L-glutamine, 50 μM 2-mercaptoethanol (all reagents from Gibco, Poole, UK)) at room temperature and filter sterilised (0.22 μm, Millipore, MA, USA) prior to addition to cell cultures. In *in vivo* studies whey proteins were supplied in powder form as part of a complete diet (Bounous *et al.*, 1983a) as described in Table 2.1. Components of complete test diets were combined by gentle mixing using a Tyrone Planetary Mixer (Model TR203, Robot Coupe, North Bridge, New South Wales, Australia) and refrigerated between feedings. Complete test diets were continuously available to mice (in plastic feeders designed to minimise spoilage and spillage) and refreshed three times a week. Drinking water was provided *ad libitum*. Specific details of filter sterilised whey proteins used in cell culture or test diets are given in the Materials and Methods section of each chapter.

Table 2.1

Composition of complete test diets

	Total percent
Protein (whey protein)	**18 - 20%**
Carbohydrate (corn flour[1])	**68%**
Fibre (cellulose[2])	**1%**
Fat (corn oil[1])	**8%**
Vitamin and mineral mix[3]	**5%**

[1] Davis Trading, Palmerston North, New Zealand
[2] 'Vitacel', Swift New Zealand Limited, Auckland, New Zealand
[3] Crop & Food Research, Palmerston North, New Zealand

2.2 Mice

All the mice used in this study were male BALB/c mice supplied by the Small Animal Production Unit (Massey University, Palmerston North, New Zealand). All mice were bred, raised and housed (under conventional conditions) at the Small Animal Production Unit (Massey University, Palmerston North, New Zealand). Standard (control) murine pellet diets consisted of a ground cow's-milk-free mouse chow (Table 2.2) comprising 18% w/w total protein of dry weight (Sharps, Lower Hutt, New Zealand) and water *ad libitum*. For all procedures mice were euthanased via an overdose of inhalation anaesthetic (Forthane, Abbott Laboratories, Abbott Park, IL, USA). Approval for research has been obtained from the Massey University Animal Ethics Committee for all of the experiments described in this thesis.

Table 2.2

Composition of Standard Mouse Chow Diet

	Pellet (control)	
Protein	Meat meal	6.0%
	Lucerne meal	5.0%
	Fish meal	7.0%
	Soybean meal	1.5%
Total Protein Content	**18 g/100 g dry weight**	
Total Fat Content	**7 g/100 g dry weight**	

2.3 Preparation Of Splenic Lymphocyte Suspensions

Spleens were removed aseptically from euthanased mice, and single cell suspensions were prepared in complete RPMI–1640 medium by breaking up the spleen tissue using a sterile 1 mL syringe plunger. The resulting suspension was centrifuged at 180 x gravity for 10 min. Erythrocytes were lysed using a buffered ammonium chloride solution (8.29g NH_4Cl, 0.07g $KHCO_3$, 2 mL Na_2EDTA total volume to 1 L using dd(H_2O) pH 7.4, adjusted with HCl (all reagents from Sigma, St Louis, MO, USA)) and the remaining leucocyte suspensions were washed twice (180 x gravity, 10 minutes) in complete RPMI-1640 medium. Using Becton Dickinson Immunocytometry systems "Cell Quest" software, viable lymphocytes were identified and counted on a FACSCalibur flow cytometer (Becton Dickinson) by gating around the mixed lymphocyte cell population (Figure 2.1). Spleen cell numbers were adjusted to a final concentration of 2 x 10^6 or 4 x 10^6 mononuclear lymphocyte-like cells/mL in complete RPMI-1640, immediately prior to use.

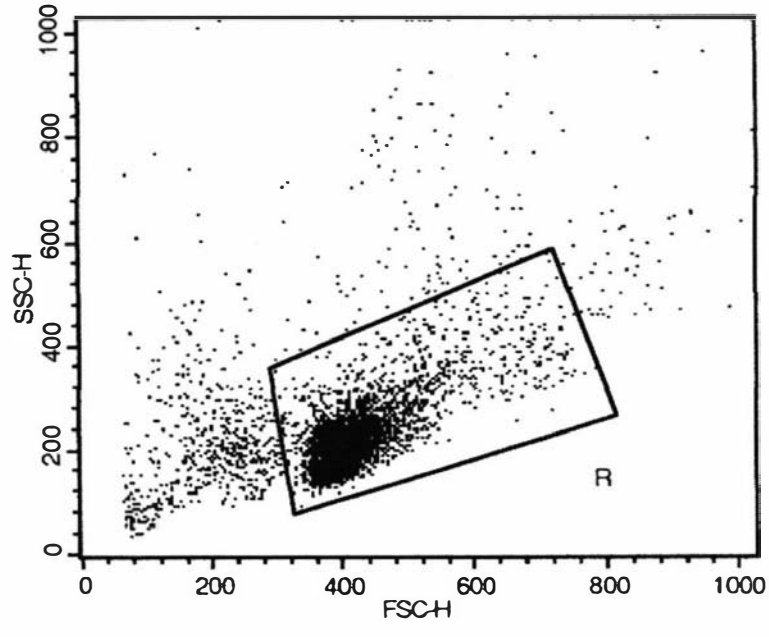


Figure 2.1 Murine splenic lymphocytes. Murine splenic lymphocytes were counted on a FACSCalibur flow cytometer (Becton Dickinson) using Becton Dickinson Immunocytometry systems "Cell Quest" application. The population of cells that were determined as the lymphocyte cell population was located within Gate **R.**

2.4 Lymphocyte Cultures And Proliferation Assay

In vitro proliferation responses of splenic lymphocytes to mitogens were determined using an enzyme-based colourimetric cell proliferation kit (Cell Proliferation Enzyme Linked Immunosorbent Assay BrdU (colourimetric) Kit, Boehringer Mannheim, Mannheim, Germany) as previously described (Cross & Gill, 1999). Briefly, 10^5 cells in 50 μL of complete RPMI-1640 medium were added in triplicate to the wells of a 96-well flat-bottomed tissue culture plate (Nunc, Roskilde, Denmark) and cultured in the presence or absence of the following optimal (determined in preliminary experiments) amounts of mitogen diluted in complete RPMI-1640: 2.5 μg/mL Concanavalin A (Con A) (Sigma, USA); 5 μg/mL lipopolysaccharide (LPS) (Sigma, USA); 20 μg/mL pokeweed mitogen (PWM) (Gibco, UK) or 13.3 μg/mL phytohaemagglutinin (PHA) (Sigma, USA). Control wells received complete RPMI-1640 medium in place of mitogen. The cells were then cultured for 96 hrs at 37°C in a 5% humidified CO_2-air atmosphere, and cell proliferation over the final 18 hrs of culture was determined by measuring the incorporation of 5-bromo-2'deoxyuridine (BrdU) using peroxidase-conjugated anti BrdU antibodies and a peroxidase substrate system. The absorbance of each well was read at 450nm (reference wavelength 650nm) using a CERES 900C Bio-Tek microtitre plate reader (Biotek Instruments Incorporated, Winooski, VT, USA) and results expressed as a mean (± SEM) differential optical density ($OD_{MITOGEN} - OD_{NO\ MITOGEN}$) of triplicate samples of each animal.

2.5 Blood

Mice were euthanased as described in Section 2.2 and blood collected by cardiac puncture using a hypodermic needle into vacutainer tubes (Becton Dickinson, New Jersey, USA) containing EDTA (for preparing live cells) or EDTA-free tubes (for preparing blood serum via centrifugation (180 x gravity/10 minutes) after 4 hours).

2.6 Preparation Of Murine Peritoneal Macrophages

Resident peritoneal cells were collected from euthanased mice (Section 2.2) by washing the peritoneal cavity of each mouse with 5 mL sterile complete RPMI-1640. The cells were washed twice (centrifuged 180 x gravity, 10 minutes) in complete RPMI-1640 medium and resuspended in fresh medium at 10^6 cells/mL. Murine peritoneal cell populations were identified on a FACSCalibur flow cytometer (Becton Dickinson Instruments, Cambridge, MA) using Becton Dickinson Immunocytometry systems "Cell Quest" Application (Figure 2.2) by gating around the macrophage cell population.

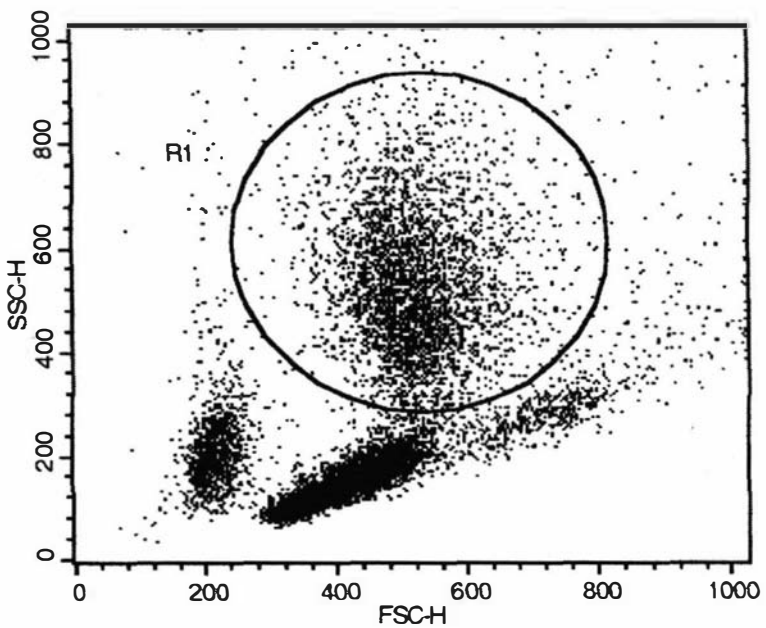


Figure 2.2 Murine peritoneal cells. Murine peritoneal macrophages were identified on a FACSCalibur flow cytometer (Becton Dickinson) using Becton Dickinson Immunocytometry systems "Cell Quest" application. The population of cells that were determined as the macrophage cell population was located within Gate **R1.**

2.7 Preparation Of Murine Peripheral Blood Cells

Peripheral blood lymphocytes were collected from euthanased mice as described in Section 2.5. Murine blood cell populations were identified on a FACSCalibur flow cytometer (Becton Dickinson Instruments, Cambridge, MA) using Becton Dickinson Immunocytometry systems "Cell Quest" Application (Figure 2.3) by gating around the macrophage cell population.

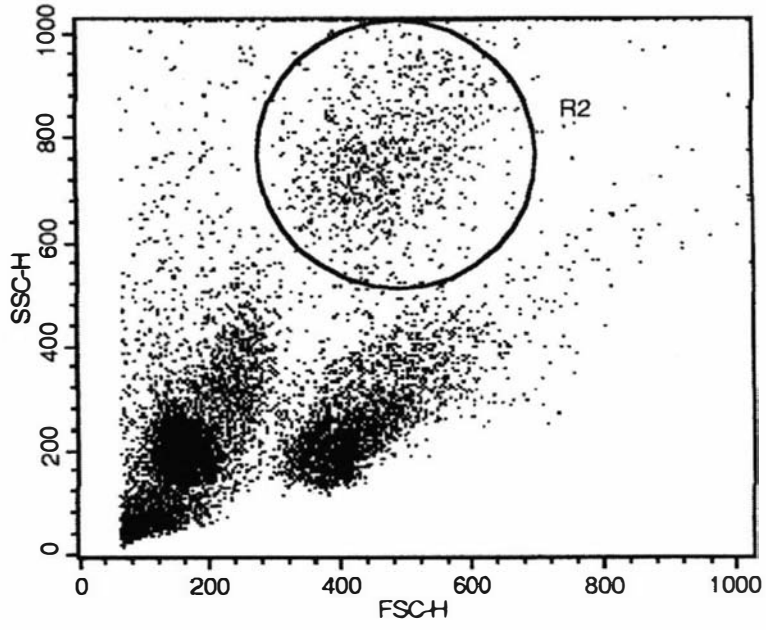


Figure 2.3 Murine peripheral blood cells. Murine peripheral blood macrophages were identified on a FACSCalibur flow cytometer (Becton Dickinson) using Becton Dickinson Immunocytometry systems "Cell Quest" application. The population of cells that were determined as the macrophage cell population was located within Gate **R2.**

2.8 Phagocytosis Assay To Assess The Phagocytic Ability Of Murine Peritoneal Cells Or Peripheral Blood Cells

To assess the phagocytic capacity of macrophages a rapid and sensitive assay based on the method of Wan *et al.* (1993) was developed. Twenty μL of fluorescein isothiocyanate (FITC)-labelled *E. coli* (Molecular Probes Incorporated, Oregon, USA) (8 x 10^8/mL, stored at -20°C) was added to 100 μL murine peritoneal cells (Section 2.6), peripheral blood cells (Section 2.7) or human THP-1 cells (10^6 cells/mL) and the suspension incubated for 20 - 30 minutes at 37°C/5% CO_2. Optimal incubation times were determined in preliminary experiments. Immediately following incubation, 0.5 mL of ice cold phosphate buffered saline (PBS) was added to stop the phagocytosis and 50 μL of 0.4% Trypan blue was added to quench the fluorescence of unphagocytosed particles. The level of phagocytic activity was determined using Becton Dickinson Immunocytometry systems "Cell Quest" application (Becton Dickinson Instruments, Cambridge, MA). Results were expressed as the proportion (percentage) of the macrophage population (see Figures 2.2 and 2.3) which contained FITC-labelled *E. coli* based on a minimum of 10,000 gated events (lymphocytes, platelets and erythrocytes excluded).

2.9 Assessment Of The Effects Of Dietary Whey Proteins On Systemic And Mucosal Antibody Responses To Various Vaccines In Mice

An enzyme linked immunosorbent assay (ELISA) was used to determine systemic serum antibody responses to influenza vaccine (Flu), tetanus toxoid (TT) and diphtheria toxin vaccine (Dip); and gut mucosal antibody responses to cholera toxin vaccine (CT), ovalbumin (OV) and polio (myelitis) vaccine (polio), as described elsewhere (Gill *et al.*, 2000a). Details of the vaccines used in this thesis are given in Table 2.3. Vaccination schedules are outlined in the Materials and Methods section of each chapter. To assess systemic antibody responses blood was collected via cardiac puncture using a hypodermic needle into EDTA-free tubes and serum separated by centrifugation (180 x gravity, 10 minutes) after 4 hours. To assess mucosal antibody responses the small intestine was removed and intestinal fluid samples were obtained by flushing the intestines with 1.5 mL PBS. Samples were then clarified by centrifugation (180 x gravity, 10 minutes) and immediately stored at -18°C. Prior to assay; samples were

diluted in sample buffer (PBS containing 5% heat inactivated foetal calf serum) as required. Optimal sample dilutions were determined in preliminary experiments. Samples were added in triplicate to antigen coated wells of a ninety-six well maxisorp microtitre plate (Nunc, Roskilde, Denmark) and specific antibody binding was visualised using alkaline phosphatase-conjugated rat anti-mouse whole Ig (Silenus, Boronia, Victoria, Australia). The colour was allowed to develop for 30 minutes before the absorbance was read at 405 nm using a CERES 900C BioTek microtitre plate reader. Serial dilutions of hyperimmune serum (Section 2.9.1), obtained from mice repeatedly immunised with Flu, TT, Dip, CT, OV or polio antigens systemically, were included on each plate as standards as described else where (Gill *et al.*, 2000a) and results expressed as antibody units (units/mL) (Section 2.9.2).

Table 2.3

Details of Vaccines

Vaccine	Administration Route	Dose/mouse	Supplier
Influenza (Flu)	Subcutaneous	1.75 μg in 25 μl PBS solution	CSL Limited (Victoria, Australia)
Aluminium-phosphate-absorbed diphtheria and tetanus toxoid (ADT)	Subcutaneous	0.1 IU diphtheria toxoid, 1 IU tetanus toxoid in 25 μl PBS solution	CSL Limited (Victoria, Australia)
Polio (myelitis) Sabin (polio)	Oral	70,000 $TCID_{50}$ in 6 μl 1 M magnesium chloride solution	SmithKline Beecham Biologicals (Auckland, New Zealand)
Ovalbumin (OV)	Oral	1 mg in 25 μl 0.5 M NaH_2CO_3	CSL Limited (Victoria, Australia)
Cholera toxin (CT)	Oral	10 μg in 25 μl 0.5 M NaH_2CO_3	Sigma (St Louis, USA)

2.9.1 Preparation of hyperimmune serum standards

To prepare hyperimmune serum, twenty five, 8-week old male BALB/c mice were immunised by subcutaneous injection (25 μL/mouse) of absorbed diphtheria and tetanus vaccine (ADT) (CSL, Victoria, Australia) and inactivated influenza vaccine (Fluvax) (CSL). Mice also received oral inoculations of cholera toxin (10μg/mouse) (Sigma, St

Louis, MO, USA) and ovalbumin (1 mg/mouse) (CSL, Victoria, Australia) in 25μL 0.5 M NaH_2CO_3 pH 8.2, and live attenuated polio vaccine (6 μl/mouse) (SmithKline Beecham Biologicals, Mt Wellington, Auckland). Each mouse received 3 doses of both orally administered and systemically administered vaccines with one week between each dose. At the end of the immunisation regime, the mice were euthanased and blood was collected as described in Section 2.5. Serum was separated by centrifugation (180 x gravity, 10 minutes) after 4 hours. Hyperimmune serum was stored (for no longer than 3 months) in 200 μL aliquots at -18°C. Serum samples were defrosted immediately prior to use.

2.9.2 Assignment of antibody units (units/mL)

Optimal standard dilutions were determined in preliminary experiments. To evaluate whole Ig (Silenus, Boronia, Victoria, Australia) systemic or mucosal responses the highest standard was prepared by a 1/200 (Flu, Dip), 1/10000 (CT), 1/40000 (OV) or 1/10 (polio) dilution of suitable hyperimmune serum (Section 2.9.1) and assigned an arbitrary value of 2000 units/mL. To evaluate whole Ig (Silenus, Boronia, Victoria, Australia) systemic responses to TT the highest standard was prepared by a 1/200 dilution and assigned an arbitrary value of 2000 units/mL (Chapter 8) or 8000 units/mL (Chapters 5 and 7). To evaluate specific mucosal IgG (Silenus) responses to CT and OV the highest standard was prepared by using undiluted hyperimmune serum and assigned an arbitrary value of 2000 units/mL. To evaluate specific mucosal IgA (Silenus) responses the highest standard was prepared by 1/4 (CT) or 1/2 (OV) dilution of suitable hyperimmune serum and assigned an arbitrary value of 2000 units/mL.

2.10 Analysis Of Blood Leucocyte Subsets

Flow cytometric analysis was used for monitoring the expression of $CD4^+$ (helper T cells), $CD25^+$ (interleukin-2 receptor α chain, activated T cells) and $CD40^+$ (accessory cells and B cells) antigens on blood leucocytes. Staining of peripheral blood leucocytes (PBLs) was performed using the method of Lloyd *et al.* (1995) with some modifications. Briefly, 100 μL of whole blood was incubated with 5 μL of fluorescein-conjugated monoclonal antibodies to CD4 (Serotec, Raleigh, NC, USA) or phycoerythrin-conjugated monoclonal antibodies to CD25 or CD40 (Serotec, Raleigh, NC, USA). Samples were incubated on ice for 20 minutes and then washed twice with

PBS. PBLs were then fixed with 100 μL of 8% paraformaldehyde for 1 minute and the red blood cells lysed in 1 mL of sterile water for 10 minutes. After washing twice in PBS, cells were resuspended in PBS and stored in the dark (for a maximum of 2 hrs) until analysed using a FACSCalibur flow cytometer (Becton Dickinson Instruments, Cambridge, MA) (Figure 2.4) and Becton Dickinson Immunocytometry systems "Cell Quest" Application using an excitation wavelength of 488 nm and an emission wavelength of 525 nm for CD4 or 575 nm for CD25 and CD40. Results were expressed as the proportion (percentage) of positively labelled cells of each phenotype in each leucocyte population based on a minimum of 10,000 gated events (granulocytes, platelets and erythrocytes excluded).

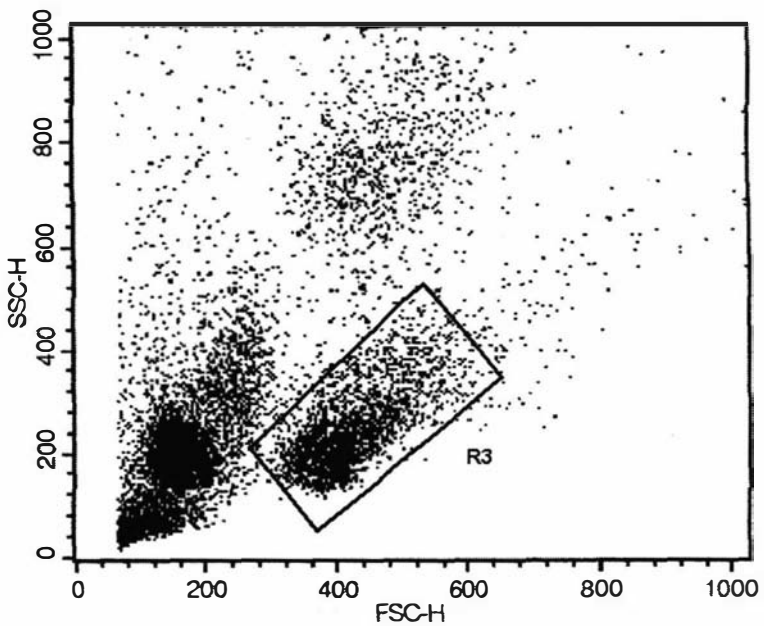


Figure 2.4 Murine peripheral blood leucocytes. Murine peripheral blood leucocytes (PBLs) were identified on a FACSCalibur flow cytometer (Becton Dickinson) using Becton Dickinson Immunocytometry systems "Cell Quest" application. The population of cells that were determined as the PBLs was located within Gate **R3.**

2.11 Statistical Analysis

Unless stated otherwise, statistical analysis of the results discussed in this study were carried out using the statistical program GraphPad Prism® Version 3.00 (GraphPad Software Incorporated, USA, 1999).

CHAPTER 3

Effect of Whey Proteins on Murine Splenic Lymphocyte Proliferation *In Vitro*

3.1 INTRODUCTION

The specific/adaptive arm of the immune response is governed by antigen–reactive lymphocytes. Because each lymphocyte has a different antigen-binding specificity, the fraction of lymphocytes that can bind and respond to any given antigen is very small. To generate sufficient specific effector lymphocytes to fight an infection, an activated lymphocyte must undergo mitosis and clonal proliferation before its progeny finally differentiate into effector cells. This clonal expansion is a feature of all adaptive immune responses (Janeway *et al.*, 1999). Therefore, the proliferation of antigen-specific lymphocytes in response to antigenic stimulation is an essential step in adaptive immunity allowing antigen-specific cells to increase in number so that they can effectively combat the antigen that originally elicited the response (Roitt, 1996). In the absence of diseases where an enhanced proliferative response would be undesirable (such as autoimmune disease), improving the ability of these cells to proliferate and differentiate into effector cells could feasibly enhance the immune system's response to foreign antigenic challenge and hence (potentially) reduce susceptibility to infection.

Extracts of bovine whey protein (isolated by ultrafiltration) have been found to have an ability to induce or enhance mitosis in lymphocytes of many different specificities or clonal origins, and thus have mitogenic capabilities *in vitro* (Francis *et al.*, 1995). β-Lactoglobulin and the hydrophobic fraction of proteose-peptones have been demonstrated to support the short-term growth of murine hybridomas *in vitro* (Mati *et al.*, 1993; Capiaumont *et al.*, 1994), and a glycophosphopeptide isolated by ultrafiltration from bovine cheese whey protein concentrate has been found to have strong mitogenic activity in murine splenocytes *in vitro* (Yun *et al.*, 1996). The whey fractions from raw or ultrafiltrated pasteurised milk have also been shown to stimulate DNA synthesis in hybridomas and fibroblasts (Damerji *et al.*, 1988). Fractions of dried whey have been shown to facilitate the growth of hybridomas and increase their antibody production by 20% compared to hybridomas cultured with foetal calf serum (Legrand *et al.*, 1993). Similar results concerning the proliferation of a CHO-K1 cell line in a medium supplemented with industrial whey was also reported by Capiaumont *et al.* (1996). Furthermore, a minor protein of whey, lactoferrin, has been shown to have the ability to bind to human promonocytic cells and enhance their proliferation *in vitro*. This activity could be maintained even in the presence of other milk proteins or after

mild heat treatment of the type used in milk processing (Oria *et al.*, 1993). Such findings strengthen the possibility of adding whey proteins in a biologically active form to dietary formulations based on cow's milk, suitable for both animal and human consumption. Notably, Baruchel & Viau (1996) found that a bovine whey protein concentrate (0.01 - 100 μg/mL), exerted a dose-dependent stimulatory effect on the cell proliferation of normal human peripheral blood leucocytes *in vitro.* On the contrary, when incubating human cancer cell lines (MATB or Jurkat T cells) with bovine whey protein concentrate (0.01 - 100 μg/mL), cellular proliferation was inhibited (Baruchel & Viau, 1996). This finding demonstrates that bovine whey protein concentrate has a differential effect on the proliferation of cells *in vitro* depending on whether normal human or human cancer cells are used. This result is significant as the enhancement of the proliferation of cancer cells by whey proteins (*in vitro* or *in vivo*) would be an undesirable modulatory effect.

A further study (Wong *et al.*, 1998) has provided additional evidence that bovine casein and a whey mixture (consisting of α-lactalbumin, bovine serum albumin, bovine gamma globulin and β-lactoglobulin) can stimulate IgM production and cell proliferation of normal murine (BALB/c) spleen cells *in vitro*. β-Lactoglobulin was found to be the most stimulatory of the proteins tested (Wong *et al.*, 1998). Reports of immunostimulation *in vitro* by major whey proteins (such as β-lactoglobulin (Mati *et al.*, 1993; Capiaumont *et al.*, 1994; Wong *et al.*, 1998)) and minor whey proteins (such as lactoferrin (Mincheva-Nilsson *et al.*, 1990; Oria *et al.*, 1993)) support the screening of previously untested dairy-derived whey protein fractions for their immunomodulating properties *in vitro.* Therefore, the aim of this chapter was to determine the immunomodulatory potential of a previously untested non-commercially available β-lactoglobulin (NZDB, Wellington, New Zealand) and α-lactalbumin (NZDB, Wellington, New Zealand) using the mitogenic activity of murine splenic lymphocytes as an index, both in the presence or absence of concanavalin A or phytohaemagglutinin (T cell mitogens) and lipopolysaccharide (a B cell mitogen). Evidence of immunomodulatory function by these whey proteins *in vitro* may support the notion that these whey proteins may also have significant immunomodulatory potential *in vivo*.

Different bovine whey protein fraction preparations have however been shown to have varying immunomodulatory effects (such as no effect, a stimulatory effect, or a suppressive effect, reviewed in Section 1.5 and 1.6), possibly due to their different processing treatments. Depending on origin and the methods used to isolate them, the properties of commercial whey proteins vary appreciably (reviewed in Section 1.4.3). Several factors may contribute to the variability in functionality and immunomodulatory properties of different whey protein preparations. For example, whey protein preparations may contain different amounts of protein, lactose, fat and minerals. Differences in the ratios of the major proteins and the degree of protein denaturation and aggregation caused by processing may also contribute to the variability of commercially prepared whey protein. Commercially manufactured and dairy-derived (non-commercial) whey protein fractions are rarely directly compared in the same study. Hence, this chapter also sought to directly compare the immunomodulatory effects of commercially prepared whey protein fractions (obtained from ICN) to dairy-derived whey protein fractions (obtained from NZDB).

3.2 MATERIALS AND METHODS

3.2.1 Whey proteins

Two (non-commercially available) whey protein preparations derived from pasteurised milk (treated at 72°C for 15 seconds; M. Pritchard, NZDRI, Palmerston North, New Zealand, *pers. comm.*) were used in this study. The β-lactoglobulin (PT3886, NZDB, Wellington, New Zealand) was supplied as a β-lactoglobulin-enriched whey protein isolate, prepared by selective elution of α-lactalbumin from a standard whey protein isolate (M. Pritchard, NZDRI, *pers. comm.*). The α-lactalbumin (PT5714, NZDB, Wellington, New Zealand) was supplied as an α-lactalbumin enriched isolate, prepared by loose ultra-filtration of a whey protein isolate (M. Pritchard, NZDRI, *pers. comm.*). The total protein content and purity of the β-lactoglobulin and α-lactalbumin sources used in this study are summarised in Table 3.1. The typical microbiological estimates of the whey proteins evaluated in this study are given in Table 3.2. The following commercially available whey proteins prepared from pasteurised milk (treated at 140°C for a minimum of 8 hours and then shocked at 110°C for 15 seconds; Z. Dokonal, MP Biomedicals, Australia, *pers. comm.*) were also tested in this study; β-lactoglobulin (Catalogue number 151536, ICN, Nutrition Biomedical, Cleveland, Ohio) isolated by chromatography (Z. Dokonal, MP Biomedicals, Australia, *pers. comm.*) and α-lactalbumin (lactalbumin hydrolysate) (Catalogue number 102129, ICN) isolated by ion exchange chromatography (Z. Dokonal, MP Biomedicals *pers. comm.*) and the minor whey protein, lactoferrin (Catalogue number 151535, ICN). All whey proteins were diluted to required concentrations in sterile complete RPMI-1640 medium (Section 2.1) and freshly prepared prior to use. All whey proteins were readily soluble in sterile complete RPMI-1640 and sterile filtered using a 0.22 μm filter (Millipore, MA, USA) prior to addition to cell cultures.

TABLE 3.1

Protein analysis of whey proteins evaluated in this study

Whey Protein Isolates	Protein content (TN*6.38)	% β-lac (% of TN*6.38)	% α-lac (% of TN*6.38)	% LF
β-lactoglobulin (NZDB)	94%	85%	9%	-
α-lactalbumin (NZDB)	92%	18%	71%	-
β-lactoglobulin (ICN)	99%	-	-	-
α-lactalbumin (ICN)	95 - 98%	-	80%	-
Lactoferrin (LF) (ICN)	94.5%	-	-	94%

Note:
1. – indicates that the parameter was not analysed
2. TN = Total Nitrogen
3. NZDB analyses conducted by reversed phase high pressure liquid chromatography (M. Pritchard, NZDRI)
4. ICN product details obtained from ICN Biomedicals, Sydney, Australia

TABLE 3.2

Typical microbiological estimates for the whey proteins evaluated in this study

Whey Protein Isolates	Standard plate count (non-specific bacteria)	Coliforms	Yeasts & moulds	*S. aureus*	*Salmonella*
β-lactoglobulin (NZDB)	730/g	Neg/g	<10/g	Neg/g	Neg/750 g
α-lactalbumin (NZDB)	1000/g max	Neg/g	<10/g	Neg/g	Neg/750 g
β-lactoglobulin (ICN)	1000/g max	Neg/0.1 g	50/g max	Neg/0.1 g	Neg/5 g
α-lactalbumin (ICN)	1000/g max	Neg/0.1 g	50/g max	Neg/0.1 g	Neg/5 g
Lactoferrin (ICN)	1000/g max	Neg/0.1 g	50/g max	Neg/0.1 g	Neg/5 g

Note:
1. All counts calculated from number of colony forming units
2. Negative (Neg)
3. *Staphylococcus aureus (S. aureus)*
4. NZDB product details obtained from NZDB, Wellington, New Zealand
5. ICN product details obtained from ICN Biomedicals, Sydney, Australia

3.2.2 Preparation of immune cell suspensions

Six to eight week old BALB/c mice were euthanased as described in Section 2.2. Spleens were removed aseptically from the mice, and single cell suspensions were prepared in complete RPMI–1640 medium as described in Section 2.3. For each individual whey protein the spleens of 12 BALB/c mice were pooled to provide sufficient cell numbers to assess splenocyte proliferative potential either in the presence or absence of mitogens.

3.2.3 Lymphocyte proliferation assay to assess the mitogenic activity of whey proteins

The *in vitro* mitogenic effect of whey proteins was determined by incubating spleen lymphocytes in the presence or absence of various concentrations of whey proteins. Cell proliferation of splenic lymphocytes was measured using an enzyme-based colourimetric cell proliferation kit (Boehringer Mannheim, Mannheim, Germany), as described in Section 2.4 with the following modification. After 50 μL of cells at a concentration of 4 x10^6cells/mL in complete RPMI-1640 medium were added to the wells of a 96-well flat-bottomed tissue culture plate (Nunc, Roskilde, Denmark) containing 100 μL of complete RPMI-1640, 50 μL of the required concentration of whey protein or complete RPMI-1640 (control) was then added to the wells, resulting in a final volume of 200 μL/ well. No known mitogens (such as Con A, PHA and LPS) were added to the cell cultures.

Two fold serial dilutions of β-lactoglobulin and α-lactalbumin were added per well at concentrations ranging from 0.06 – 1.88 μg/mL or 3.75 – 120 μg/mL. Two fold serial dilutions of lactoferrin were added per well at concentrations ranging from 0.003 – 0.1 μg/mL or 0.06 – 1.88 μg/mL. Control wells received 50 μL complete RPMI-1640 in place of whey protein (0 μg/mL). The BrdU incorporation over the final 18 hours of a 96 hour culture period was measured as described in Section 2.4. The results were expressed as mean (± SEM) absorbance of triplicate samples. Eight independent experiments were performed.

3.2.4 Lymphocyte proliferation assay to assess the effect of whey proteins on the activity of various mitogens

The *in vitro* proliferative response of splenic lymphocytes to whey proteins and mitogens was determined by incubating mitogen-stimulated splenic lymphocytes with various concentrations of whey proteins. Lymphocyte proliferation was measured using an enzyme-based colourimetric cell proliferation kit (Boehringer Mannheim, Mannheim, Germany), as described in Section 2.4 with the following modifications; 100 μL of mitogen diluted in sterile RPMI-1640 was added to each well at the following final concentrations; 5 μg/mL LPS (B cell mitogen); 2.5 μg/mL Con A (T cell mitogen); or 13.3 μg/mL PHA (T cell mitogen) resulting in a final volume of 200μL/well. The BrdU incorporation over the final 18 hours of a 96 hour culture period was measured as described in Section 2.4. The results were expressed as mean (± SEM) absorbance of triplicate samples. Six independent experiments were performed.

3.2.5 Statistical analysis

The effects of each whey protein on mean splenic cell proliferation compared to the control group (no whey protein) were tested using analysis of variance (GraphPad Prism®, USA, 1999). Individual protein concentrations were compared to controls (no whey protein) using a Dunnett's multiple comparison post-hoc test (GraphPad Prism®, USA, 1999). Individual protein concentrations were compared to other protein concentrations using a Tukey's post-hoc test (GraphPad Prism®, USA, 1999).

3.3 RESULTS

3.3.1 The effect of β-lactoglobulin (NZDB) on murine splenic lymphocyte proliferation

To investigate the mitogenic effect of β-lactoglobulin (NZDB), the proliferation of murine spleen lymphocytes incubated with various concentrations of β-lactoglobulin was measured. The β-lactoglobulin used in this study exerted a dose-dependent stimulatory effect on the growth of splenic lymphocytes as shown in Figure 3.1. Lymphocyte proliferation was 30 - 90% higher than the control ($P < 0.05$, 0.01 respectively) at concentrations ranging between 0.53 - 1.88 μg/mL (Figure 3.1 A) and 250 - 300% greater than the control ($P < 0.01$) at higher concentrations ranging between 7.5 - 120 μg/mL (Figure 3.1 B). Maximum enhancement of splenic lymphocyte proliferation occurred when lymphocytes were incubated with 15 - 120 μg/mL of β-lactoglobulin (Figure 3.1 B).

3.3.2 The effect of α-lactalbumin (NZDB) on murine splenic proliferation

To investigate the mitogenic effect of α-lactalbumin (NZDB), the proliferation of murine spleen lymphocytes incubated with various concentrations of α-lactalbumin was measured. The α-lactalbumin used in this study had no significant stimulatory effect on splenic lymphocytes at concentrations ranging between 0.06 - 1.88 μg/mL (Figure 3.1 A). However, across the higher concentrations evaluated (3.75 - 120 μg/mL) the proliferation of splenic lymphocytes appeared to decline and at 120 μg/mL α-lactalbumin significantly suppressed proliferation compared to the control ($P < 0.01$) (Figure 3.1 B).

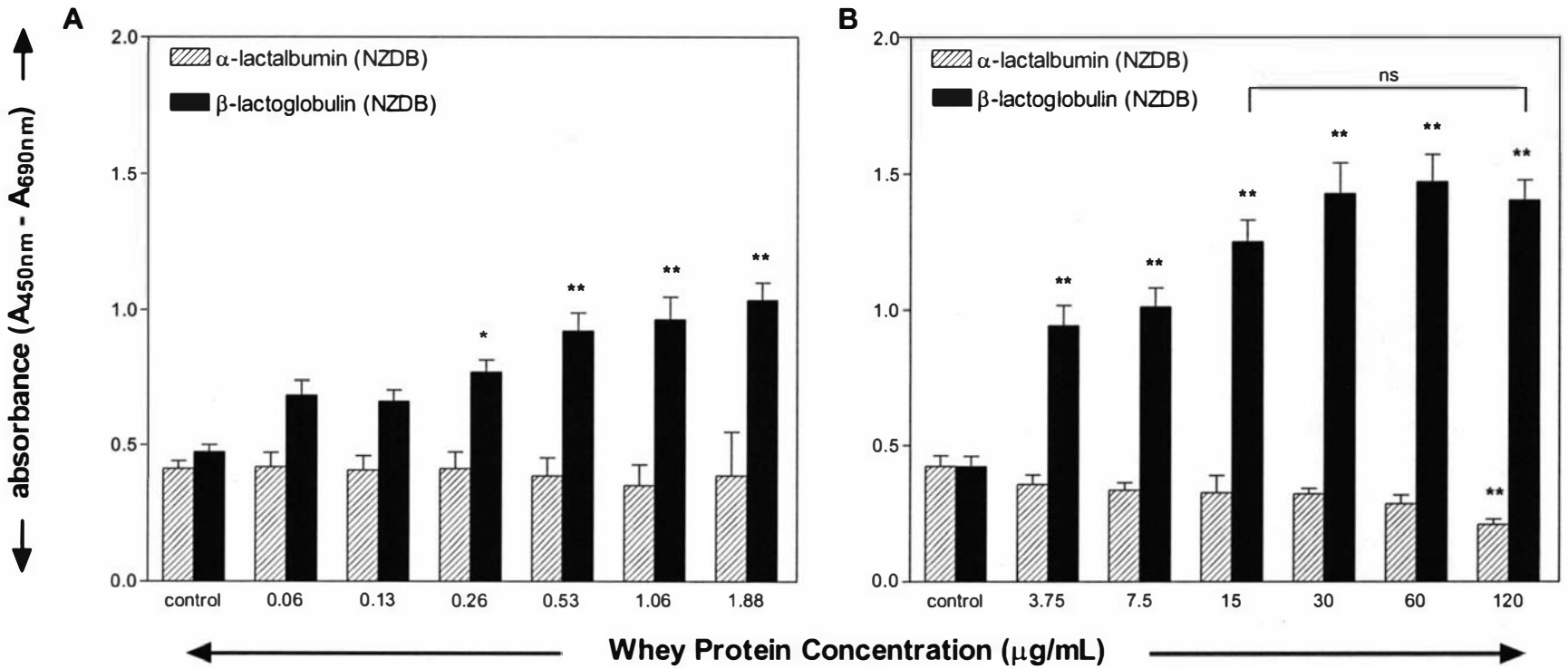


Figure 3.1 β-Lactoglobulin (NZDB), but not α-lactalbumin (NZDB), enhances the proliferation of murine splenic lymphocytes at concentrations ranging between 0.06 - 1.88 μg/mL (A) and 3.75 - 120 μg/mL (B) compared to control. The mitogenic effect of β-lactoglobulin and α-lactalbumin was measured by a colourimetric assay based on the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into splenic lymphocytes pooled from 12 BALB/c mice. Results are expressed as mean absorbance ± SEM (n = 8 mean results obtained from independent experiments). * $P < 0.05$, ** $P < 0.01$.

3.3.3 The effect of β-lactoglobulin (NZDB) on the proliferation of LPS-, Con A- and PHA-induced murine splenic lymphocytes

To evaluate the effect of β-lactoglobulin (NZDB) on mitogen-induced cell proliferation, the proliferation of LPS-, Con A- or PHA-stimulated murine lymphocytes incubated with various concentrations of β-lactoglobulin was measured (Table 3.3). At concentrations ranging between 0.06 - 1.88 μg/mL, β-lactoglobulin had no significant effect on the proliferation of LPS (B cell mitogen)-stimulated lymphocytes compared to the control (no added whey protein, 0 μg/mL). However, at higher concentrations ranging between 3.75 - 30 μg/mL β-lactoglobulin significantly increased the proliferation of LPS-stimulated splenic lymphocytes by up to 70% compared to the control ($P < 0.05$). At concentrations greater than 30 μg/mL significant enhancement of LPS-induced cell proliferation was no longer observed and a dose dependent decline in cell proliferative potential appeared to occur that did not decline below the control value (at the concentrations tested). In the presence of Con A (T cell mitogen), the β-lactoglobulin used in this study significantly ($P < 0.01$) increased the proliferation of splenic lymphocytes at concentrations ranging between 0.06 - 1.88 μg/mL by 6 - 15% compared to the control. At higher concentrations (3.75 - 120 μg/mL) no significant enhancement of Con A-induced cell proliferation was observed compared to the control. At the lower concentrations of 0.06 - 1.88 μg/mL β-lactoglobulin had no significant effect on the proliferation of PHA (T cell mitogen)-stimulated lymphocytes, however at higher concentrations ranging between 3.75 - 120 μg/mL, PHA-stimulated cell proliferation was significantly enhanced by 13 - 56% ($P < 0.05$) compared to control.

TABLE 3.3

At concentrations between 0.06 - 1.88 and 3.75 - 120 μg/mL, β-lactoglobulin (NZDB) modulates the proliferation of mitogen-stimulated splenic lymphocytes *in vitro*[1]

Protein Concentration (μg/mL)	Mitogen		
	LPS (5 μg/mL)	**Con A** (2.5 μg/mL)	**PHA** (13.3 μg/mL)
	β-lactoglobulin (NZDB)		
Control	2.15 ± .19	3.15 ± .04	1.73 ± .16
0.06	2.20 ± .16	↑3.37[b] ± .13	1.70 ± .12
0.13	2.18 ± .17	↑3.42[b] ± .14	1.76 ± .14
0.26	2.16 ± .14	↑3.50[b] ± .15	1.72 ± .14
0.53	2.13 ± .13	↑3.64[b] ± .16	1.88 ± .15
1.06	2.16 ± .14	↑3.67[b] ± .14	1.84 ± .16
1.88	2.14 ± .12	↑3.65[b] ± .15	1.83 ± .15
Significance[2]	NS	$P < 0.0001$	NS
Control	2.13 ± .22	3.20 ± .18	1.41 ± .05
3.75	↑2.78[b] ± .11	3.21 ± .10	↑1.59[a] ± .11
7.5	↑2.97[b] ± .14	3.49 ± .11	↑2.02[b] ± .12
15	↑3.15[b] ± .16	3.49 ± .13	↑2.28[b] ± .11
30	↑3.08[b] ± .17	3.50 ± .40	↑2.17[b] ± .17
60	2.45 ± .16	3.19 ± .19	↑2.32[b] ± .15
120	2.19 ± .15	3.13 ± .20	↑2.20[b] ± .17
Significance[2]	$P < 0.0001$	NS	$P < 0.0001$

[1] Cell proliferation of splenocytes pooled from 12 BALB/c mice expressed as mean absorbance (A_{450nm} - A_{650nm}) ± SEM (n = 6 mean results obtained from independent experiments)

[2] ANOVA main effect of protein concentrations (NS = not significant)

[a] Means differ from control (0 μg/mL) using Dunnett's multiple comparison post-hoc test ($P < 0.05$)

[b] Means differ from control (0 μg/mL) using Dunnett's multiple comparison post-hoc test ($P < 0.01$)

↑ Indicates a significant increase compared to the control

3.3.4 The potential binding of BrdU to β-lactoglobulin (NZDB) residue

It was possible that the increase in absorbance values observed in this study may have been due to the binding of BrdU to whey protein residue rather than an increase in BrdU incorporation into dividing murine splenocytes. To confirm that this was not occurring, the lymphocyte proliferation assay was repeated as described Section 3.2.3 and Section 3.2.4 with no cells in culture. The absorbance values (A_{450nm} - A_{650nm}) of β-lactoglobulin (0.06 - 1.88 μg/mL) cultured in the absence of cells did not differ significantly from the absorbance value of the control (no cells, no added β-lactoglobulin) (Table 3.4). The absorbance values of β-lactoglobulin (NZDB) incubated with mitogen only and without cells were not significantly different from the control group (no protein, only mitogen) (Table 3.4). This indicates that the significant increase in absorbance values as the concentration of β-lactoglobulin increased, is not due to the binding of BrdU to the whey protein residue, but is indeed a result of increased BrdU incorporation into dividing splenic lymphocytes.

TABLE 3.4

The absorbance values of β-lactoglobulin (NZDB) cultured in the absence of cells is not significantly different from controls[1]

Protein Concentration (μg/mL)	No mitogen	Mitogen
Control	0.221 ± .016	0.221 ± .016
0.06	0.196 ± .002	0.229 ± .010
0.13	0.203 ± .003	0.242 ± .012
0.26	0.195 ± .003	0.236 ± .008
0.53	0.202 ± .003	0.242 ± .007
1.06	0.197 ± .003	0.233 ± .010
1.88	0.210 ± .010	0.254 ± .006
Significance[2]	NS	NS

[1] mean absorbance (A_{450nm} - A_{650nm}) ± SEM (n = 6 mean results obtained from independent experiments)

[2] ANOVA main effect of protein concentrations (NS = not significant)

3.3.5 The effect of α-lactalbumin (NZDB) on the proliferation of splenic lymphocytes induced by LPS, Con A and PHA

To evaluate the effect of α-lactalbumin (NZDB) on mitogen-induced cell proliferation, the proliferation of LPS-, Con A- or PHA-stimulated murine lymphocytes incubated with various concentrations of α-lactalbumin was measured (Table 3.5). After incubation with α-lactalbumin, the proliferation of lymphocytes induced with all the mitogens used in this study declined in a dose dependent manner. At concentrations between 1.88 μg/mL and 120 μg/mL, α-lactalbumin significantly reduced the proliferation of splenic lymphocytes stimulated with LPS by 20 - 74% compared to the control ($P < 0.05$, 0.01 respectively). Similarly, Con A-induced proliferation was 10 - 27% lower than the control when cells were incubated with 1.06 - 1.88 μg/mL of α-lactalbumin ($P < 0.05$, 0.01 respectively) and 30 - 94% lower than the control when incubated with higher concentrations of 3.75 - 120 μg/mL of α-lactalbumin ($P < 0.01$). In the presence of PHA, concentrations of α-lactalbumin lower than 15 μg/mL had no significant effect on lymphocyte proliferation, however at higher concentrations ranging between 15 - 120 μg/mL, α-lactalbumin significantly ($P < 0.01$) reduced the proliferation of splenic lymphocytes in a dose-dependent manner by 47% to 75%.

TABLE 3.5

At concentrations between 0.06 - 1.88 and 3.75 - 120 μg/mL, α-lactalbumin (NZDB) modulates the proliferation of mitogen-stimulated splenic lymphocytes *in vitro*[1]

Protein Concentration (μg/mL)	Mitogen		
	LPS (5 μg/mL)	**Con A** (2.5 μg/mL)	**PHA** (13.3 μg/mL)
	α-lactalbumin (NZDB)		
Control	2.18 ± .13	3.18 ± .17	1.73 ± .17
0.06	2.17 ± .15	3.14 ± .13	1.46 ± .19
0.13	2.09 ± .16	3.04 ± .18	1.60 ± .10
0.26	2.07 ± .13	3.00 ± .16	1.47 ± .11
0.53	2.11 ± .11	2.97 ± .14	1.40 ± .16
1.06	1.97 ± .16	↓2.93[a] ± .14	1.37 ± .13
1.88	↓1.8[a] ± .14	↓2.58[b] ± .16	↓0.99[a] ± .27
Significance[2]	$P < 0.005$	$P < 0.0001$	NS
Control	2.14 ± .11	3.23 ± .06	1.53 ± .14
3.75	↓1.68[b] ± .13	↓2.25[b] ± .15	1.48 ± .17
7.5	↓1.85[b] ± .17	↓2.01[b] ± .15	1.37 ± .17
15	↓1.56[b] ± .16	↓2.03[b] ± .16	↓1.06[b] ± .12
30	↓1.28[b] ± .13	↓1.42[b] ± .14	↓0.80[b] ± .13
60	↓1.04[b] ± .16	↓0.40[b] ± .14	↓0.67[b] ± .11
120	↓0.85[b] ± .40	↓0.20[b] ± .20	↓0.38[b] ± .11
Significance[2]	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

[1] Cell proliferation of splenocytes pooled from 12 BALB/c mice expressed as mean absorbance (A_{450nm} - A_{650nm}) ± SEM (n = 6 mean results obtained from independent experiments)

[2] ANOVA main effect of protein concentrations (NS = not significant)

[a] Means differ from control (0 μg/mL) using Dunnett's multiple comparison post-hoc test ($P < 0.05$)

[b] Means differ from control (0 μg/mL) using Dunnett's multiple comparison post-hoc test ($P < 0.01$)

↓ Indicates a significant decrease compared to control

3.3.6 The effect of β-lactoglobulin (ICN) on murine splenic cell proliferation without or with mitogen stimulation

To compare the mitogenic ability of the β-lactoglobulin obtained from NZDB (Wellington, New Zealand) (Section 3.3.1 and 3.3.3) with a commercially available whey protein, the mitogenic activity of β-lactoglobulin (ICN) was also evaluated over the lower concentration range tested in this study. At concentrations of 1.06 and 1.88 μg/mL β-lactoglobulin (ICN) significantly enhanced the proliferation of murine splenic lymphocytes by approximately 80% ($P < 0.05$, 0.01 respectively) (Figure 3.2). At concentrations ranging between 0.06 and 1.88 μg/mL, β-lactoglobulin (ICN) had no significant effect on LPS- or PHA-induced lymphocyte proliferation (Table 3.6). However, at a concentration of 1.88 μg/mL β-lactoglobulin (ICN) significantly enhanced the proliferation of splenic lymphocytes in the presence of Con A by 24% ($P < 0.05$) (Table 3.6). Therefore, at the lower concentrations evaluated in this study, the commercially available β-lactoglobulin (ICN) and the β-lactoglobulin enriched whey protein (NZDB) exhibited similar effects on the proliferation of splenic lymphocytes both with and without mitogen stimulation.

Whey proteins from ICN were tested in this study to determine whether or not the previously untested β-lactoglobulin obtained from NZDB had a similar effect on cell proliferation as β-lactoglobulin obtained from a different source. From these results it was concluded that the NZDB derived β-lactoglobulin had a similar mitogenic activity *in vitro* as the commercially available β-lactoglobulin at the lower concentrations evaluated (0.06 - 1.88 μg/mL). An evaluation of the mitogenic effect of the ICN β-lactoglobulin at higher concentrations (3.75 - 120 μg/mL) was not conducted due to a limited supply of ICN β-lactoglobulin at the time of this study.

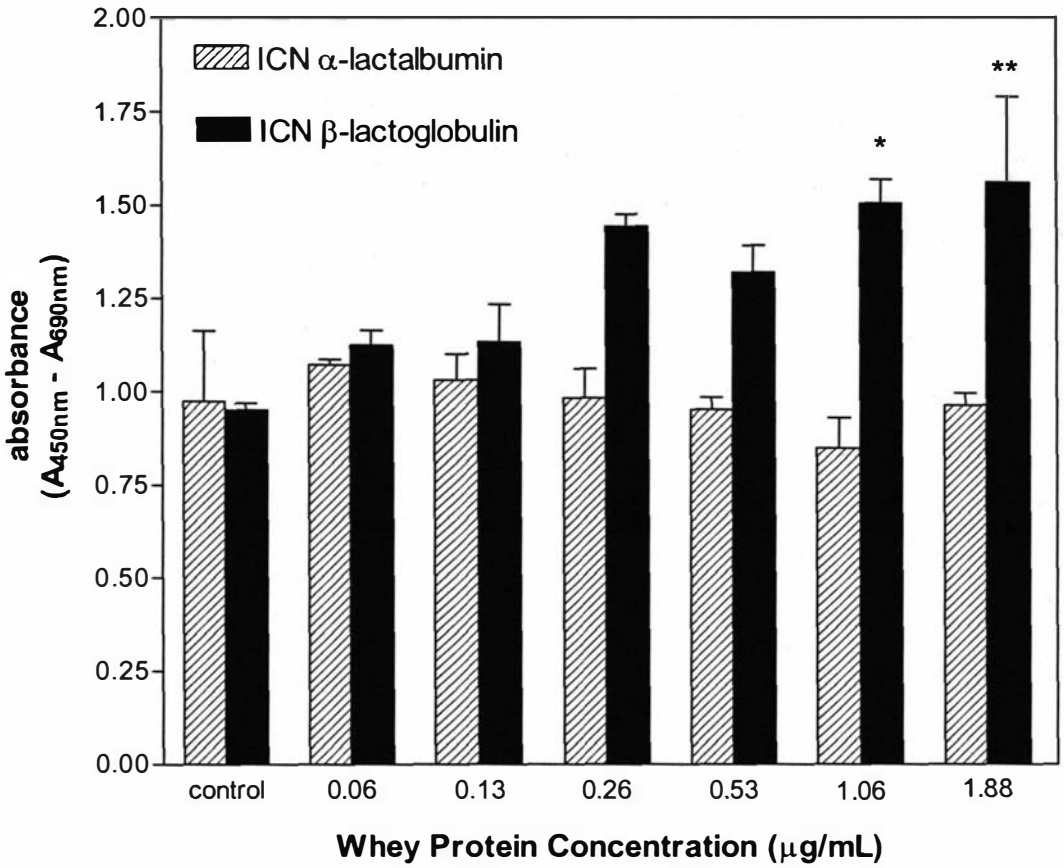


Figure 3.2 β-lactoglobulin (ICN), but not α-lactalbumin (ICN), stimulates the proliferation of murine splenic lymphocytes at concentrations ranging between 1.06 - 1.88 μg/mL. The mitogenic effect of ICN α-lactalbumin and β-lactoglobulin (0 μg/mL - 1.88 μg/mL) was measured by a colourimetric assay based on the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into splenic lymphocytes pooled from 12 BALB/c mice. Results are expressed as mean absorbance ± SEM (n = 8 mean results obtained from independent experiments). * $P < 0.05$, ** $P < 0.01$.

TABLE 3.6

At concentrations between 0.06 - 1.8 μg/mL, β-lactoglobulin (ICN) and α-lactalbumin (ICN) modulate the proliferation of mitogen-stimulated splenic lymphocytes *in vitro*[1]

Protein Concentration (μg/mL)	Mitogen		
	LPS (5 μg/mL)	**Con A** (2.5 μg/mL)	**PHA** (13.3 μg/mL)
	β-lactoglobulin (ICN)		
Control	3.27 ± .46	2.21 ± .10	2.45 ± .17
0.06	3.61 ± .14	2.65 ± .18	2.54 ± .19
0.13	3.31 ± .12	2.29 ± .16	2.70 ± .14
0.26	3.32 ± .15	2.30 ± .21	2.73 ± .11
0.53	2.93 ± .18	2.22 ± .14	2.66 ± .13
1.06	3.60 ± .19	2.52 ± .12	2.62 ± .15
1.88	3.57 ± .10	↑2.74[a] ± .17	2.24 ± .11
Significance[2]	NS	$P < 0.005$	NS
	α-lactalbumin (ICN)		
Control	3.63 ± .14	3.30 ± .16	2.45 ± .17
0.06	3.23 ± .13	3.38 ± .12	2.53 ± .13
0.13	3.06 ± .23	3.18 ± .12	2.50 ± .19
0.26	↓2.71[a] ± .26	3.13 ± .10	2.58 ± .10
0.53	↓2.68[a] ± .28	↓2.91[b] ± .16	2.60 ± .13
1.06	↓2.70[a] ± .11	↓2.91[b] ± .16	2.46 ± .12
1.88	↓2.76[a] ± .11	↓2.81[b] ± .15	2.29 ± .15
Significance[2]	$P < 0.05$	$P < 0.0001$	NS

[1] Cell proliferation of splenocytes pooled from 12 BALB/c mice expressed as mean absorbance (A_{450nm} - A_{650nm}) ± SEM (n = 6 mean results obtained from independent experiments)

[2] ANOVA main effect of protein concentrations (NS = not significant)

[a] Means differ from control (0 μg/mL) using Dunnett's multiple comparison post-hoc test ($P < 0.05$)

[b] Means differ from control (0 μg/mL) using Dunnett's multiple comparison post-hoc test ($P < 0.01$)

↑ Indicates a significant increase compared to control

↓ Indicates a significant decrease compared to control

3.3.7 The effect of α-lactalbumin (ICN) on murine splenic cell proliferation without or with mitogen stimulation

Similar to the α-lactalbumin obtained from NZDB (Wellington, New Zealand), α-lactalbumin (ICN) had no effect on the proliferation of splenic lymphocytes at the concentrations evaluated in this study (Figure 3.2), and reduced the proliferation of certain mitogen-stimulated cells (Table 3.6). α-Lactalbumin (ICN) significantly reduced the proliferation of splenic lymphocytes induced by LPS ($P < 0.05$) and Con A ($P < 0.01$) at concentrations greater than or equal to 0.26 μg/mL by 20 - 50% (Table 3.6). Therefore, it was concluded that the α-lactalbumin from NZDB tested in this study had a similar mitogenic activity *in vitro* as the commercially available α-lactalbumin at the concentrations evaluated. An evaluation of the mitogenic effect of the ICN α-lactalbumin at higher concentrations (3.75 - 120 μg/mL) was not conducted due to a limited supply of ICN α-lactalbumin at the time of this study.

3.3.8 The effect of lactoferrin (ICN) on the proliferation of splenic lymphocytes

The mitogenic effect of lactoferrin (ICN) on splenic lymphocytes was determined over a concentration range of 0.003 - 0.1 and 0.06 - 1.88 μg/mL. At a concentration of 0.1 μg/mL, lactoferrin significantly enhanced the proliferation of murine splenic lymphocytes by 50% compared to the control (Figure 3.3 A). In contrast, at the higher concentrations evaluated (0.26 - 1.88 μg/mL), lactoferrin appeared to gradually reduce the proliferation of splenic lymphocytes compared to the control in a dose-dependent manner, however this trend was not significant (Figure 3.3 B). Due to restrictions in the amount of lactoferrin available for testing, concentrations greater than 1.88 μg/mL were not evaluated in this study.

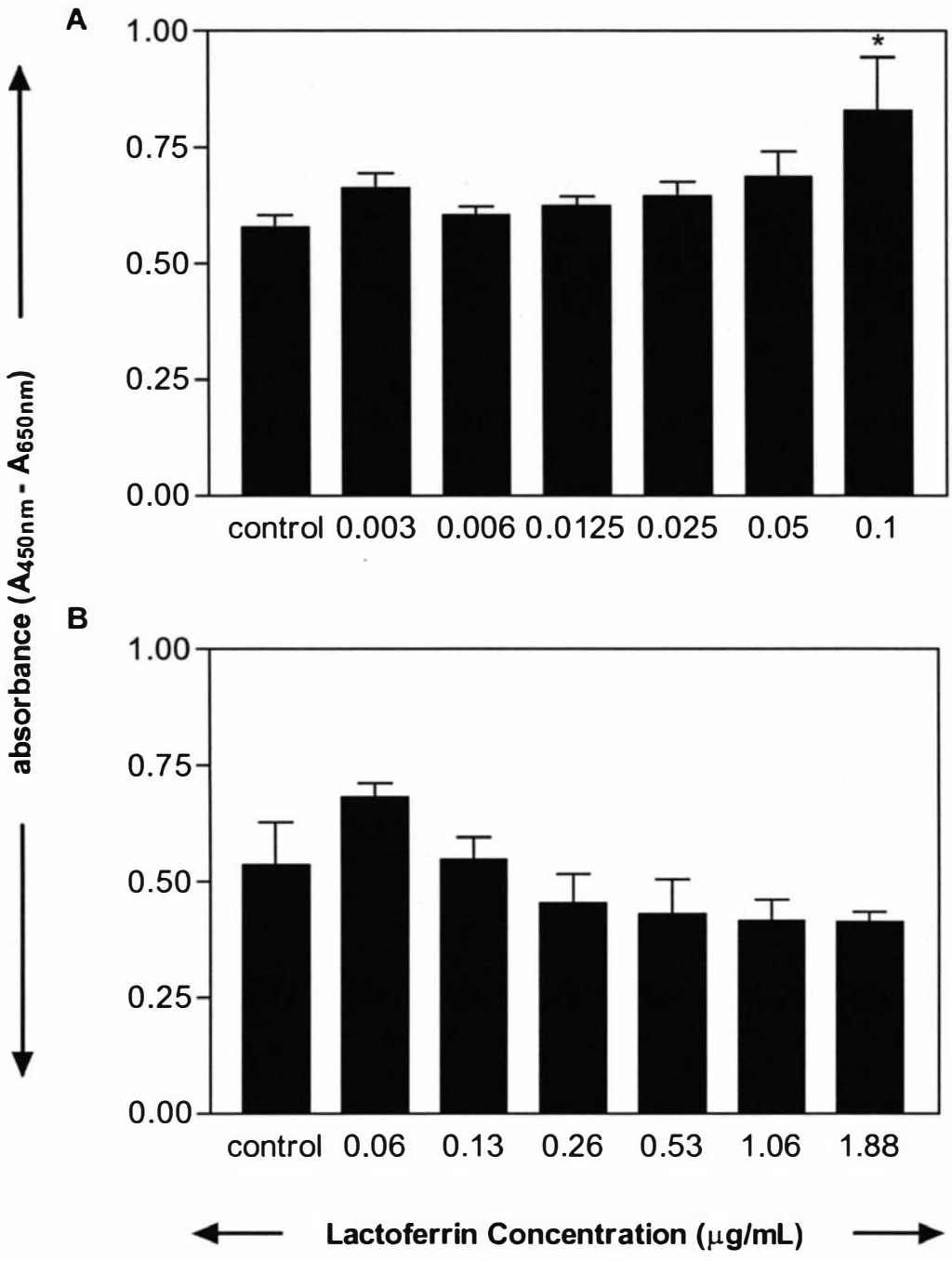


Figure 3.3 At 0.1 μg/mL, lactoferrin (ICN) enhances the proliferation of murine splenic lymphocytes. The mitogenic effect of lactoferrin at a concentration range of 0 μg/mL – 0.1 μg/mL (**A**) and 0 μg/mL – 1.88 μg/mL (**B**), was measured by a colourimetric assay based on the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into splenic lymphocytes pooled from 12 BALB/c mice. Results are expressed as mean absorbance ± SEM (n = 8 mean results obtained from independent experiments). * $P <$ 0.05.

3.3.9 The effect of lactoferrin (ICN) on the proliferation of splenic lymphocytes induced by LPS, Con A and PHA

To evaluate the effect of lactoferrin (ICN) on mitogen-induced cell growth, the proliferation of LPS-, Con A- or PHA-stimulated murine lymphocytes incubated with various concentrations of lactoferrin was measured (Table 3.7). At lower concentrations (0.003 - 0.1 and 0.06 - 1.06 μg/mL) lactoferrin had no significant effect on the proliferation of LPS-stimulated lymphocytes, however at a concentration of 1.88 μg/mL, lactoferrin significantly reduced LPS-induced proliferation of splenic lymphocytes by 60% ($P < 0.05$). In the presence of Con A, 0.003 - 0.1 μg/mL lactoferrin resulted in a dose-dependent decline in the proliferation of lymphocytes ($P < 0.05$) compared to the control. In fact, at concentrations ranging between 0.06 - 0.13 μg/mL the proliferation of Con A-stimulated lymphocytes declined to near negligible amounts ($P < 0.01$) compared to the control. PHA-induced cell proliferation was not affected by concentrations of lactoferrin ranging between 0.003 - 0.1 μg/mL and 0.06 - 0.53 μg/mL, however at the two highest lactoferrin concentrations (1.06 and 1.88 μg/mL), PHA-induced cell proliferation was 40 - 50% lower than the control ($P < 0.01$). Due to restrictions in the amount of lactoferrin (ICN) available for testing, concentrations greater than 1.88 μg/mL were not evaluated in this study.

TABLE 3.7

Lactoferrin (ICN) reduces the proliferation of splenic lymphocytes stimulated by LPS, Con A and PHA *in vitro*[1]

Protein Concentration (μg/mL)	Mitogen		
	LPS (5 μg/mL)	**Con A** (2.5 μg/mL)	**PHA** (13.3 μg/mL)
	Lactoferrin		
Control	2.69 ± .13	2.99 ± .17	1.76 ± .11
0.003	2.82 ± .15	3.37 ± .12	1.73 ± .14
0.006	2.70 ± .12	3.51 ± .12	1.88 ± .19
0.0125	2.64 ± .12	3.00 ± .12	1.81 ± .16
0.025	2.60 ± .18	2.86 ± .15	1.93 ± .10
0.05	2.90 ± .13	2.91 ± .16	2.08 ± .23
0.1	2.95 ± .15	↓2.22[a] ± .11	2.12 ± .11
Significance[2]	NS	$P < 0.005$	NS
Control	2.57 ± .14	3.06 ± .09	2.29 ± .17
0.06	2.35 ± .42	↓2.43[b] ± .15	2.81 ± .24
0.13	2.05 ± .28	↓0.67[b] ± .29	1.84 ± .17
0.26	2.40 ± .15	↓0.15[b] ± .11	1.87 ± .11
0.53	2.41 ± .09	↓0.17[b] ± .11	1.89 ± .16
1.06	2.11 ± .14	↓0.15[b] ± .11	↓1.44[b] ± .15
1.88	↓1.60[a] ± .11	↓0.14[b] ± .11	↓1.20[b] ± .11
Significance[2]	$P < 0.005$	$P < 0.0001$	$P < 0.0001$

[1] Cell proliferation of splenocytes pooled from 12 BALB/c mice expressed as mean absorbance (A_{450nm} - A_{650nm}) ± SEM (n = 6 mean results obtained from independent experiments)

[2] ANOVA main effect of protein concentrations (NS = not significant)

[a] Means differ from control (0 μg/mL) using Dunnett's multiple comparison post-hoc test ($P < 0.05$)

[b] Means differ from control (0 μg/mL) using Dunnett's multiple comparison post-hoc test ($P < 0.01$)

↓ Indicates a significant decrease compared to control

3.4 DISCUSSION

A whey protein (termed milk basic protein) prepared by dialysis from WPC (NZDB, Wellington, New Zealand) has previously been shown to dose dependently stimulate the proliferation (measured by the incorporation of ^{3}H-thymidine) and differentiation of a murine osteoblastic cell line (MC3T3-E1) when included in *in vitro* cell culture at concentrations ranging from 10 μg/mL to 100 μg/mL (Takada *et al.*, 1996). In the present study a β-lactoglobulin enriched whey protein (NZDB), which has not been tested for its immunomodulatory effects before, was shown to exert a dose-dependent stimulatory effect on the proliferation of murine splenic lymphocytes (Figure 3.1), with maximum enhancement occurring at 15 - 120 μg/mL (Figure 3.1). These results demonstrate that the β-lactoglobulin investigated in this study also possesses mitogenic activity *in vitro*. This finding is in agreement with that of Wong *et al.* (1998) who found that bovine β-lactoglobulin (containing β-lactoglobulin variants A and B) significantly increased cell proliferation and IgM production in murine spleen cell cultures. In fact, β-lactoglobulin was found to be more stimulatory compared to casein or whey mixtures (consisting of α-lactalbumin, serum albumin, gamma globulin and β-lactoglobulin) (Wong *et al.*, 1998). In addition, the results of the present study demonstrate that β-lactoglobulin (NZDB) is not only able to induce the proliferation of non-mitogen stimulated cells, but is also able to enhance the proliferation of both mitogen-activated T cells and mitogen-activated B cells. For example, at the lower concentrations evaluated in this study (0.06 - 1.88 μg/mL), β-lactoglobulin was able to enhance the proliferative response of T lymphocytes activated by Con A (Table 3.3). At the higher concentrations evaluated (3.75 - 120 μg/mL) β-lactoglobulin enhanced the proliferative responses of T lymphocytes activated by PHA, and B lymphocytes activated by LPS (Table 3.3). The increase in absorbance values observed in this study was not due to the binding of BrdU to β-lactoglobulin residue (Table 3.4).

Although Rejman *et al.* (1992a) found that a commercial β-lactoglobulin (Sigma, St Louis, USA) (19.5 - 635 μg/mL) did not enhance the *in vitro* proliferation of bovine mammary epithelial cells (in the absence and the presence of foetal bovine serum), the commercial β-lactoglobulin (ICN) evaluated in this study did demonstrate mitogenic activity when included in culture with murine splenic lymphocytes. The mitogenic activity demonstrated by the commercial β-lactoglobulin (ICN) evaluated in the present

study was, however, not as strong as the mitogenic activity demonstrated by the β-lactoglobulin obtained from the NZDB. β-lactoglobulin (NZDB) significantly increased lymphocyte proliferation at concentrations as low as 0.26 μg/mL (Figure 3.1), while the commercial β-lactoglobulin increased lymphocyte proliferation only at concentrations greater than or equal to 1.06 μg/mL (Figure 3.2). Similarly, β-lactoglobulin enhanced Con A-induced proliferation from 0.6 μg/mL (Table 3.3) while the commercial β-lactoglobulin only enhanced Con A-induced splenic lymphocyte proliferation at a concentration of 1.88 μg/mL (Table 3.6). These results suggest that the β-lactoglobulin investigated in this study has stronger mitogenic activity than the commercially available β-lactoglobulin tested *in vitro*.

Notably, the NZDB whey protein isolates were prepared from milk subjected to a relatively mild pasteurisation treatment (low heat and short-time); however the ICN whey protein fractions were prepared from milk subjected to a severe pasteurisation treatment (extreme heat and long-time). Heat treatment and variations in preparation techniques can result in whey protein denaturation, hydrolysis and changes in the mineral balance in the extracellular medium (Guimont *et al.*, 1997; reviewed in Section 1.4.4). Hence, the severe pasteurisation method used to treat the original milk may have caused functional changes in the milk protein that ultimately affected the immunoregulatory potential of the ICN whey protein products. Subsequent variations in the storage, treatment and isolation techniques of the commercial β-lactoglobulin (ICN) used in this study may also have altered the whey protein biochemically and physicochemically, reducing its mitogenic capability compared to the non-commercially available β-lactoglobulin used in this study. Alternatively, the methods used to manufacture the β-lactoglobulin enriched whey protein isolate (NZDB) may have enhanced its immunomodulatory potential. Another important consideration is that, in addition to the β-lactoglobulin protein itself, other factors (contaminants) present in the NZDB preparation of β-lactoglobulin may have been responsible for part (or all) of the observed stimulation. For example, in a study conducted by Brix *et al.* (2003), a commercial β-lactoglobulin preparation (Sigma-Aldrich, St Louis, Mo) induced pronounced proliferation of both murine spleen cells and cells from mesenteric lymph nodes (and increased levels of cytokines and intracellular glutathione) compared to other milk proteins (α-lactalbumin, bovine serum albumin) and ovalbumin, but a β-

lactoglobulin purified from raw milk (using ion-exchange and size exclusion chromatography methods) failed to exert the same immunostimulatory effect *in vitro*. This led the researchers to conclude that the immunostimulatory effect of the Sigma-Aldrich β-lactoglobulin preparation was not caused by β-lactoglobulin *per se* but by mitogenic contaminants (such as endotoxin) present in the commercial preparation. Whether or not, at the levels present (3.4 - 6486 endotoxin units) in the 5 commercial β-lactoglobulin preparations tested, endotoxin alone would exert a mitogenic effect when included in cell culture was however not assessed. It is also possible that while attempting to purify β-lactoglobulin from raw milk, and remove any microbiological contamination, Brix *et al.* (2003) inadvertently destroyed or removed the mitogenic activity of the protein.

Analysis of the β-lactoglobulin preparation tested in the present study (Table 3.2) revealed the original powder was essentially free of microbiological contamination prior to sterile filtration and addition to cell cultures, hence the presence of any residue endotoxin is unlikely or would probably be at biologically insignificant levels (V. Korolik, Microbiology, Griffith University, Australia, *pers comm.*). Furthermore, previous studies by Wong *et al.* (1998) have found that bacterial endotoxin was unlikely to be responsible for cell stimulation as the presence of the endotoxin inhibitor polymyxin B did not inhibit the stimulatory activity of β-lactoglobulin. Consequently, while a *Limulus* amoebocyte lysate assay (or similar analysis for the presence of endotoxin in the whey protein source) was not conducted in the present study, bacterial contamination and endotoxin release are considered to be unlikely sources for the observed effects on cell proliferation. Further studies determining the level (if any) of endotoxin present in the sterile filtered dairy-derived whey protein preparations and the determination of the effect of minute quantities endotoxin on murine splenic proliferation stimulated or not stimulated by mitogen *in vitro* would be advantageous.

In addition to the significant variation in the levels of endotoxin contamination present in different commercial β-lactoglobulin preparations, not all commercially available products tested induced murine cell proliferation when included in culture (β-lactoglobulin from Aria Foods Denmark failed to induce murine splenocyte or mesenteric lymph node proliferation *in vitro* (Brix *et al.*, 2003)). These observations highlight the differences (in content) of different commercial whey protein preparations

(reviewed in Section 1.4.3) and the variable immunomodulatory potential of different whey protein preparations. Therefore it appears that the variation in the mitogenic capability of β-lactoglobulin preparations *in vitro* reported by different research groups is most likely due to the differences in the β-lactoglobulin preparations themselves as a result of differing processing techniques, the origin and the composition of the milk.

A number of substances are known to co-purify with β-lactoglobulin, such as folate binding protein. However, no immunostimulatory activity by folate binding protein has been reported, instead an inverse relationship between cell replication and cellular concentration of folate binding protein has been found (Wong *et al.*, 1998). It is possible that the β-lactoglobulin (85% purity, Table 3.1) tested in this study may have contained traces of other proliferation-inducing milk protein fractions such as serum albumin, proteose-peptone 3 and lactoferrin (Guimont *et al.*, 1997), bioactive peptides or other potentially biologically active factors such as interleukins, interferon and tumour necrosis factor. However, studies by Wong *et al.* (1998) investigating the presence of other potentially biologically active substances contaminating β-lactoglobulin preparations have failed to detect (by ELISA) traces of interleukin-2, -4, -10, interferon-γ or tumour necrosis factor.

Although the possibility of immunostimulatory contaminants contributing to the activity of the β-lactoglobulin tested in this study cannot be completely eliminated, the findings of this study support the notion that β-lactoglobulin itself may be an immunostimulatory molecule. For instance, bovine whey fractions have been shown to contain various compounds able to promote cell growth such as numerous hormones, peptides, lipids, proteins and minerals (Derouciche *et al.*, 1990), and β-lactoglobulin exhibits structural homology with retinol-binding proteins and carries hydrophobic substances such as fatty acids, lipids and retinoic acid (Hemly *et al.*, 1979; Brown, 1984). Retinoic acid has been shown to enhance thymidine incorporation and ornithine deoxycarboxylase activity in PHA (T cell mitogen)-stimulated human lamina propria lymphocytes (Elitsur *et al.*, 1997). Therefore, the mitogenic activity of β-lactoglobulin may be attributed to its capacity to transport hydrophobic components and minerals (Baumy & Brule, 1988). Furthermore, β-lactoglobulin is particularly rich in glutamine (Eigel *et al.*, 1984) and there is strong evidence suggesting that glutamine is a nutrient necessary for cell

proliferation (Lacey & Wilmore, 1990); for example a decrease in the glutamine concentration in the cell culture medium greatly reduces the rate of proliferation of human lymphocytes *in vitro* (Parry-Billings *et al.*, 1990), hence β-lactoglobulin may be providing lymphocytes in culture with this amino acid. Unlike other edible plant and animal protein, whey proteins also contain substantial amounts of the precursors (glutamyl-cysteine) required for glutathione synthesis (Bounous & Gold, 1991). Glutathione is critical to the functional state and activation of many cells including T and B lymphocytes (Noelle & Lawrence, 1981) and has been found to increase lymphocyte responses to mitogen stimulation (Fidelus & Tsan, 1986). Therefore the immunoenhancing activity of β-lactoglobulin may be related, in part, to providing high levels of glutathione during lymphocyte proliferation.

At the concentrations evaluated in this study, both the α-lactalbumin (NZDB) and the commercially available α-lactalbumin (ICN) tested had no significant direct effect on the proliferative potential of murine splenic lymphocytes (Figure 3.1 and 3.2). This result is similar to that of Wong *et al.* (1998) who found that a commercially available bovine α-lactalbumin purchased from Sigma (St Louis, USA) alone did not stimulate murine spleen cell proliferation or IgM production, but is in contrast with the work of Rejman *et al.* (1992a) who observed a reduction in bovine mammary epithelial cell proliferation after incubation with α-lactalbumin (0 - 625 μg/mL). These discrepancies may be due to differences in the cell types tested and/or the α-lactalbumin tested. The proliferation of T cells activated by Con A or PHA, and B cells activated by LPS, was significantly inhibited by the α-lactalbumin (NZDB) tested in this study, at concentrations ranging between 0.06 - 1.88 μg/mL (Table 3.5) and 3.75 - 120 μg/mL (Table 3.5). Similarly, the commercial α-lactalbumin (ICN) investigated in this study also caused a significant reduction in the proliferation of lymphocytes induced by Con A over the concentration range evaluated (Table 3.6). α-lactalbumin is present in high concentrations in lactating bovine mammary glands therefore the effect of reduced proliferation by α-lactalbumin on cells such as epithelial cells may be a mechanism of maintaining cell number during lactation (Rejman *et al.*, 1992a). Bovine milk has been found to contain a mammary-derived growth inhibitor (Grosse *et al.*, 1991) that reduces the proliferation of both human and murine mammary tumour cell lines *in vitro* (Lehmann *et al.*, 1989). The addition of bovine milk whey has also been shown to

reduce the growth of human cancerous cells (MCF-7 and PC-3) when included in cell culture (Bourtourault *et al.*, 1991), therefore the ability of α-lactalbumin to reduce cellular proliferation may have positive implications where increased cellular proliferation is undesirable (e.g. cancer, autoimmune disease and allergy).

In contrast, Wong *et al.* (1997b) reported bovine α-lactalbumin to have no influence on the mitogenic responses of either ovine blood T or B lymphocytes stimulated with Con A and LPS respectively. Therefore, reports on the mitogenic effect of bovine α-lactalbumin on different cell types and different species are currently varied. Although α-lactalbumin is known to be an important component of the lactose synthetase enzyme complex and is believed to be involved in lactose synthesis (Rejman *et al.*, 1992a), α-lactalbumin has received limited attention in terms of its immunological properties *in vitro*. However, some dietary α-lactalbumin hydrolysates have been shown to have strong immunomodulatory properties *in vivo*, including the ability to enhance lymphocyte proliferation when fed to mice as a major dietary protein source (Bounous *et al.*, 1983a; Bounous & Kongshavn, 1985). Therefore, further *in vitro* studies on this protein are required to fully elucidate any direct *in vitro* effects on lymphocyte proliferation.

Lactoferrin (LF) has previously been shown to have a bimodal effect on cellular proliferation in several different systems by different groups (Mincheva-Nilsson *et al.*, 1990; Rejman & Oliver, 1993; Hambraeus & Lonnerdal, 1994). Bovine LF had an inhibitory effect on the proliferation of bovine MAC-T mammary cells (Rejman *et al.*, 1992a) and bovine peripheral blood mononuclear cell blastogenesis (Rejman *et al.*, 1992b) at concentrations as low as 19 μg/mL. In contrast, LF has been reported to enhance the *in vitro* proliferation of murine BALB/c lymphocytes (Azuma *et al.*, 1989) and human L6 myoblasts (Byatt *et al.*, 1990). Previous studies by Oria *et al.* have found that both bovine and human lactoferrin can bind to a murine macrophage cell line (Oria *et al.*, 1988) and human monocytes or macrophages (Oria *et al.*, 1993) and enhance monocyte proliferation *in vitro*. The results of the present study suggest that lactoferrin may also enhance the non-mitogen stimulated proliferation of murine splenocyte proliferation *in vitro*. In this study, at the very low concentration of 0.1 μg/mL, lactoferrin significantly increased murine splenic lymphocyte proliferation compared to

the control (Figure 3.3 A). However, when cultured with increasing concentrations of bovine lactoferrin (0.13 - 1.88 μg/mL) the proliferation of murine splenic lymphocytes tended to decline in a dose-dependent manner (Figure 3.3 B). Lactoferrin has also been shown to promote the *in vitro* proliferation of human PHA-stimulated peripheral blood lymphocytes and a human lymphocyte cell line (Mazurier *et al.*, 1989). Although the non-specific binding of BrdU to lactoferrin was not tested in this study, the fact that other groups reported a similar stimulation of cellular proliferation by lactoferrin *in vitro* suggests that the increase in absorbance values observed in this study was not due to the binding of BrdU to LF residue. The anti-oxidant properties of lactoferrin may play a role in the ability of lactoferrin to modulate splenic cell metabolism and proliferation (reviewed by Steijns & Hooijdonk, 2000). For example, lactoferrin has been found to limit the extent of oxygen-radical-mediated damage to cells by sequestration of free iron, thereby inhibiting the formation of free radicals (Leveugle *et al.*, 1993), therefore, LF may exert its stimulatory effect on cell proliferation via lactoferrin receptors present on the cell surface of lymphocytes (Tome & Debabbi, 1998).

While lactoferrin significantly enhanced murine splenocyte proliferation in the absence of mitogen, lactoferrin significantly reduced mitogen (LPS, Con A and PHA) induced lymphocyte proliferation at concentrations ranging between 0.06 - 1.88 μg/mL (Table 3.7). At lower concentrations (0.003 - 0.05 μg/mL), lactoferrin had no significant effect on the proliferation of lymphocytes stimulated with LPS and PHA (Table 3.7). Crago *et al.* (1981) observed similar results when human colostrum (containing approximately 7 mg/mL lactoferrin (Steijns & Hooijdonk, 2000)) was incubated with pokeweed mitogen (PWM, T cell-dependent B cell mitogen)-stimulated human PBLs. In the absence of PWM, colostrum did not stimulate the uptake of tritiated thymidine by PBLs, however in the presence of PWM, colostrum (at dilutions of up to 1:100) significantly inhibited the uptake of tritiated thymidine by human PBLs (Crago *et al.*, 1981). A reduction in the proliferative responses of bovine blood mononuclear cells induced by Con A has also been observed in the presence of apo- and Fe-lactoferrin (0.78 - 10 mg/mL) (Rejman *et al.*, 1992b). Human lymphocyte proliferation stimulated by PHA has been shown to be reduced by apo-lactoferrin and not affected by iron-saturated lactoferrin (Djeha & Brock, 1992). In addition, lactoferrin (6.25 - 400 μg/mL) and lactoperoxidase were

found to inhibit proliferation and interferon production of ovine blood lymphocytes in response to Con A stimulation, however they did not affect lymphocyte proliferative responses to LPS stimulation (Wong *et al.*, 1997b). Similar to these results, purified lactoferrin isolated from human milk inhibited human peripheral T-lymphocyte proliferative responses to PHA in a dose-dependent manner (Richie *et al.*, 1987). This suppressive effect was not due to lactoferrin-mediated cytotoxicity since proliferative activity could be restored by washing the cells that had been pre-incubated with lactoferrin. The reduction in the proliferation of mitogen-stimulated lymphocytes after incubation with the higher concentrations of lactoferrin (Table 3.7) used in the present study could be further investigated by assessing splenic leucocyte viability and metabolism (via trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, respectively). The mechanisms by which lactoferrin regulates cell function are not completely known, however, human lactoferrin has been shown to be a myelopoietic regulatory protein with suppressor activity on many cells types (activated lymphocytes, macrophages, monocytes and platelets) (Broxmeyer *et al.*, 1978; Bagby *et al.*, 1981; Mazurier *et al.*, 1989). The effect of lactoferrin on cell division and/or cell activation may be differentiated by further studies investigating the effect of lactoferrin on other aspects of lymphocyte function following mitogen activation (such as cytokine production and cell surface marker expression).

Clearly, the specific role of lactoferrin in cellular responses is multifaceted. Further research using lactoferrin in *in vitro* models may assist in the determination of potential *in vivo* roles for lactoferrin and define potential mechanisms of action. The same research strategy should also apply to the major whey proteins β-lactoglobulin and α-lactalbumin. Bovine and human α-lactalbumin share partial amino acid sequence and structural homologies, providing support for the use of whey proteins in formula suitable for human consumption (Heine *et al.*, 1991). Furthermore, β-lactoglobulin shows high solubility over a broad pH range and is stable following ultra high temperature (UHT) treatment (Smithers *et al.*, 1996). Therefore, the potential exists for the development of a whey protein-based immunoenhancing product capable of boosting the proliferative ability of specific immune cells in response to foreign antigenic challenge, thus reducing susceptibility to infection. An important consideration is

however the issue of 'food allergy', with cow milk protein intolerance being the most common form of food intolerance and allergy in children (reviewed by Kilara & Panyam, 2003). Although the extent of hydrolysis and heat denaturation during manufacture has been found to decrease and in some cases eliminate the allergenicity of whey proteins (Enomoto *et al.*, 1993), further work still is required to specifically assess the allergenicity potential of whey proteins such as β-lactoglobulin prior to their use as a food product.

The strong mitogenic activity of a whey protein (β-lactoglobulin), both in the absence and presence of mitogen stimulation, has been demonstrated in this chapter. Enhancement of murine splenic proliferative responses indicates that this whey protein has the potential to modulate immune function *in vitro*. This finding does not prove that these whey proteins will affect immune function in an *in vivo* model. When whey proteins are consumed other variables that may affect their immunomodulatory potential must be taken into account, such as the effects of digestion. However, the *in vitro* testing of these whey proteins on immune cells or cells lines allows for a far wider manipulation of experimental variables (such as dose and concentration) to be carried out repetitively, rapidly and economically. Therefore, further *in vitro* studies investigating how these whey proteins affect other indices of immunological function such as the phagocytic ability of monocytes and macrophages is warranted.

CHAPTER 4

Effect of Dairy-Derived Whey Proteins on Monocyte and Macrophage Function *In Vitro*

4.1 INTRODUCTION

The β-lactoglobulin-enriched whey protein isolate prepared by the NZDB has already been shown to have mitogenic properties *in vitro* (Chapter 3). To further elucidate the immunomodulatory potential of whey protein isolates (α-lactalbumin, β-lactoglobulin and lactoferrin) their effect on another immune parameter *in vitro* was evaluated, namely the phagocytic capacity of murine macrophages.

Macrophages are large mononuclear phagocytic cells important in innate immunity, in early phases of host defence as antigen presenting cells, and as effector cells in humoral and cell-mediated immunity. Macrophages play a crucial role in host defence by trapping, engulfing and destroying pathogens by a process of phagocytosis (internalisation of particulate matter by cells) (Janeway *et al.*, 1999). The ability of macrophages to phagocytose particles plays an important part in the elimination of intruding micro-organisms, cellular debris and tumour cells and is therefore the focus of this study. To evaluate the effect of bovine whey proteins on the percentage of cells with phagocytic capacity a rapid and sensitive flow cytometric method was developed.

An effect on phagocytic function by both bovine and human casein milk peptides has been demonstrated in several *in vitro* studies (Jolles *et al.*, 1981-1982; Parker *et al.*, 1984; Gattegno *et al.*, 1988; Migliore-Samour *et al.*, 1989). Migliore-Samour *et al.* (1989) have shown synthetic human and bovine casein-derived peptides (0.1 μM - 30 μM) can stimulate the phagocytosis of SRBC by murine and human macrophages in culture. An immunomodulatory fraction in a tryptic digest of human casein has been demonstrated to stimulate phagocytosis by murine macrophages *in vitro* (Jolles *et al.*, 1981-1982). In addition, Gattegno *et al.* (1988) found that peptides from human caseins (1 μM) were able to stimulate the *in vitro* binding and phagocytic ability of human monocytic cells towards homologous red blood cells after a co-incubation period of 2 hours at 37°C.

To date caseins have been the major bovine milk protein found to possess bioactive peptides with the ability to modulate phagocytic capacity (Otani & Futakami, 1994; Otani & Futakami, 1996, reviewed by Cross & Gill, 2000; Gill *et al.*, 2000b) although some fragments of bovine whey proteins have also been observed to have various

biological activities such as opioid, vaso-relaxing and ACE inhibitory capacity *in vitro* (Yoshikawa *et al.*, 1986). A glycopeptide prepared from bovine whey protein concentrate has also been found to modulate macrophage (murine macrophages and a human macrophage cell line) function by inducing the production of an interleukin-1 receptor antagonist-like component (Otani & Monnai, 1995; Monnai & Otani, 1997).

Lactoferrin (an iron binding glycoprotein), which is found not only in milk but also in the endocrine secretions of mammals and in the secondary granules of polymorphonuclear neutrophils, has a wide range of biological functions including iron metabolism, antimicrobial and antibacterial effects (reviewed by Vorland, 1999). The presence of exogenous lactoferrin in cell culture is, therefore, likely to have important anti-microbial and antibacterial effects. Lactoferrin is also known to be involved in immune function, particularly in phagocytic killing by macrophages (Ellison & Giehl, 1991). Part of the involvement of lactoferrin in immune function may be explained by the stimulatory effect of lactoferrin on cytokine release by cells (Crouch *et al.*, 1992). It is also known that lactoferrin binds strongly to DNA and may act as a transcription factor (Lonnerdal, 1996). Human lactoferrin has been suggested to be an important feed-back regulator of myelopoieses by inhibiting monocyte/macrophage production of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Broxmeyer *et al.*, 1978) or by inhibiting the production of a monokine that stimulates T lymphocytes to produce GM-CSF (Bagby *et al.*, 1981). Several effects of lactoferrin on the inflammatory and immunological response have also been demonstrated. For example, lactoferrin has been found to enhance neutrophil production of hydroxyl radicals (Ambruso & Johnston, 1981), possess anti-complement activity (Kijlstra & Jeurissen, 1982), modulate nuclear cell activities and suppress murine *in vitro* primary antibody responses (Duncan & McArthur, 1981) and enhance monocytic cytotoxicity (Nishiya & Horwitz, 1982). Furthermore, human lactoferrin has been shown to enhance the monocytic uptake and killing of intracellular parasites (Lima & Kierszenbaum, 1985). However, the ability of undigested bovine whey proteins (such as β-lactoglobulin and α-lactalbumin), and also bovine lactoferrin, to modulate phagocytic function *in vitro* has not yet been determined. Therefore, the objective of this study was to assess the ability of both murine peritoneal cells and a human monocytic cell line (THP-1) to phagocytose *E. coli* and inert particles (latex beads) following incubation with an intact whole β-lactoglobulin, α-lactalbumin or lactoferrin. The immunomodulatory effect of

these bovine whey proteins was also compared to commercially available whey proteins.

4.2 MATERIALS AND METHODS

4.2.1 Whey proteins

Two non-commercially available major whey proteins, β-lactoglobulin (NZDB) and α-lactalbumin (NZDB) were used in this study (previously described in Chapter 3, Section 3.2.1). These whey proteins were compared to the following commercially available whey proteins; β-lactoglobulin, α-lactalbumin and lactoferrin (all ICN, Nutrition Biochemical, Cleveland, Ohio). All whey proteins were freshly prepared prior to use and diluted in sterile complete RPMI-1640 to required concentrations. All whey proteins were readily soluble and sterile filtered using a 0.22 μm filter (Millipore) prior to addition to cell cultures.

4.2.2 Preparation of peritoneal macrophages and culturing of human monocytic THP-1 cells

Mice were euthanased as described in Section 2.2 and resident peritoneal cells were collected and prepared as described in Section 2.6. The human monocytic cell line THP-1 (American Type Culture Collection (ATCC), Manassas, VA, ATCC Number TIB-202) which originated from a human acute monocytic leukaemia (monocyte) were grown and maintained in complete RPMI-1640 medium at 37°C and 5% CO_2. THP-1 cells are phagocytic (for both latex beads and sensitised erythrocytes) and lack surface cytoplasmic immunoglobulin.

4.2.3 Phagocytosis assay to assess the phagocytic ability of murine peritoneal cells and human monocytic THP-1 cells

The phagocytic capacity of a human monocytic cell line (THP-1) and murine peritoneal macrophages was assessed by flow cytometry as described in Section 2.8 with the following modifications. One hundred μL of peritoneal macrophages or human monocytic cells in complete RPMI-1640 (10^6 cells/mL) were mixed and pre-incubated with 100 μL of various concentrations of whey protein at 37°C/5% CO_2 for 2 hours. Twenty μL of FITC-labelled *E. coli* (8 x 10^8/mL, stored at -20°C) or unopsonised fluorescent yellow-green latex beads (1 μm diameter, diluted to 2% in PBS, Sigma) was then added and the suspension incubated for 20 minutes (murine peritoneal macrophages) or 30 minutes (human monocytes) at 37°C/5% CO_2.

4.2.5 Statistical analysis

The effect of each whey protein on mean percentage of cells with phagocytic capacity compared to the control group (no whey protein) was tested using analysis of variance (GraphPad Prism®, USA, 1999). Individual whey protein concentrations were compared to controls (no added whey protein, 0 μg/mL) using a Dunnett's multiple comparison post-hoc test (GraphPad Prism®, USA, 1999).

4.3 RESULTS

4.3.1 The effect of β-lactoglobulin (NZDB) on the ability of murine peritoneal cells to phagocytose FITC-labelled *E. coli*

The β-lactoglobulin (NZDB) exerted a dose-dependent stimulatory effect on the ability of murine peritoneal cells to phagocytose *E. coli*. The ability of murine peritoneal cells to phagocytose FITC-labelled *E. coli* was 13 - 40% higher than the control after incubation with 0.09 - 1.5 μg/mL of β-lactoglobulin ($P < 0.01$) (Figure 4.1 A). β-Lactoglobulin continued to significantly enhance the phagocytic ability of murine peritoneal cells by up to 170% compared to the control, at concentrations ranging from 3 - 96 μg/mL ($P < 0.01$) (Figure 4.1 B). Furthermore, at concentrations ranging between 16 - 250 μg/mL, β-lactoglobulin increased phagocytosis by 80 - 150% compared to the control ($P < 0.01$) (Figure 4.1 C) with maximum enhancement occurring at 96 μg/mL. Phagocytic activity began to decline in a dose-dependent manner as concentrations of β-lactoglobulin increased above 96 μg/mL. At the highest β-lactoglobulin concentration tested (500 μg/mL), the percentage of cells with phagocytic capacity was no longer significantly higher than the control (Figure 4.1 C). The phagocytosis of FITC-labelled *E. coli* by murine peritoneal cells after incubation with β-lactoglobulin (0.05 - 500 μg/mL) is summarised in Figure 4.1 D.

4.3.2 The effect of α-lactalbumin (NZDB) on the ability of murine peritoneal cells to phagocytose FITC-labelled *E. coli*

Incubating murine peritoneal cells with 0.5 - 1.5 μg/mL of α-lactalbumin had no significant effect on their ability to phagocytose FITC-labelled *E. coli* (Figure 4.1 A). At concentrations ranging from 0 - 96 μg/mL (Figure 4.2 B) and 0 - 500 μg/mL (Figure 4.1 C) α-lactalbumin also had no significant effect on the ability of murine peritoneal cells to phagocytose FITC-labelled *E. coli*. At a concentration of 125 μg/mL (α-lactalbumin) a slight increase in the phagocytic capacity of murine peritoneal cells was observed, however this increase was not significant (Figure 4.1 C). The phagocytosis of FITC-labelled *E. coli* by murine peritoneal cells after incubation with α-lactalbumin (0.05 - 500 μg/mL) is also summarised in Figure 4.1 D.

FIGURE 4.1

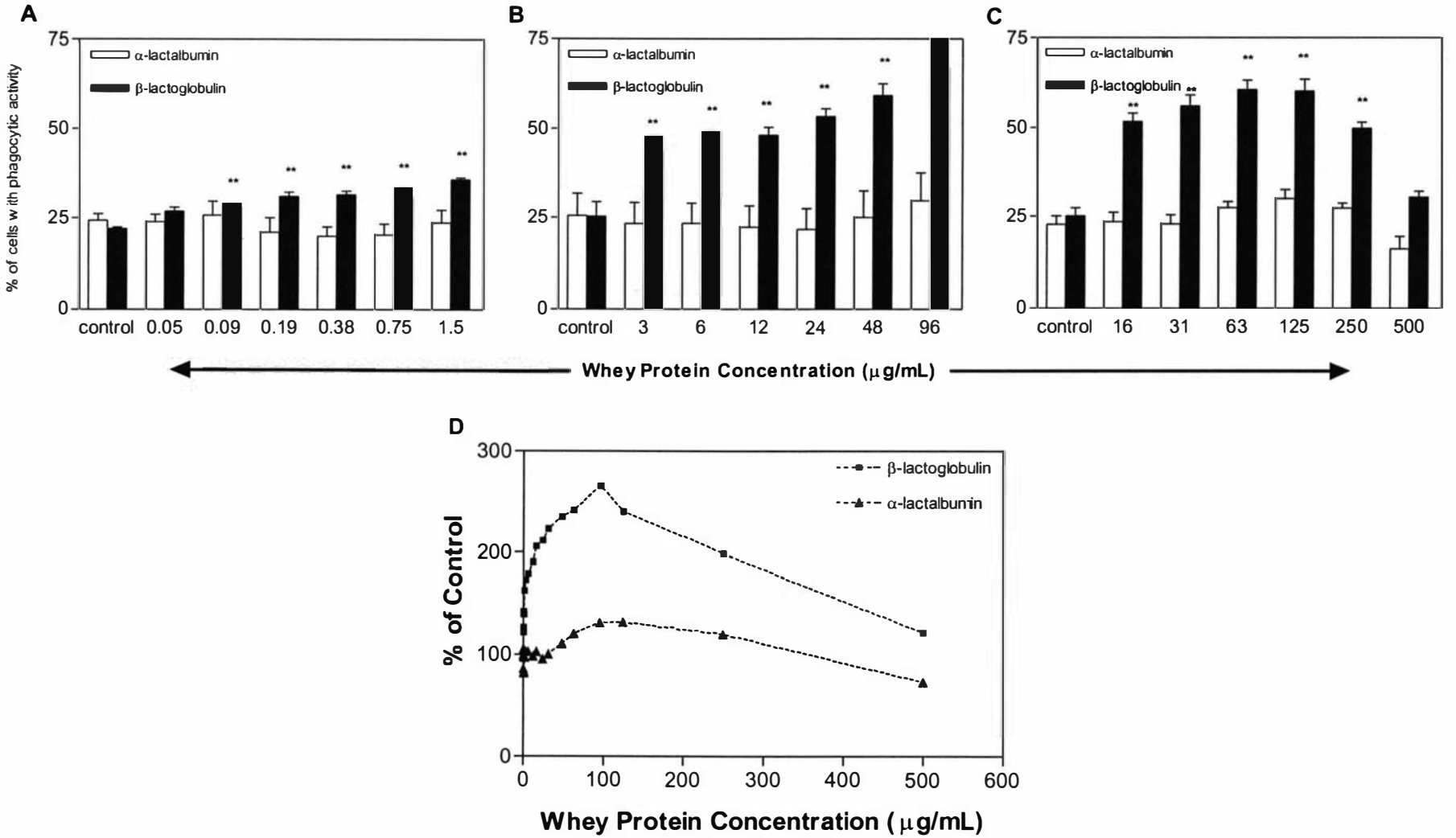

A
B
C
D
α-lactalbumin
β-lactoglobulin
% of cells with phagocytic activity
control
0.05
0.09
0.19
0.38
0.75
1.5
3
6
12
24
48
96
16
31
63
125
250
500
Whey Protein Concentration (μg/mL)
% of Control
Whey Protein Concentration (μg/mL)

FIGURE LEGEND

Figure 4.1 β-Lactoglobulin (NZDB), but not α-lactalbumin (NZDB), enhances the ability of murine peritoneal cells to phagocytose *E. coli*. Murine peritoneal cells pooled from 12 BALB/c mice were pre-incubated with 0.05 - 1.5 μg/mL **(A)**, 3 - 96 μg/mL **(B)**, or 16 - 500 μg/mL **(C)** of β-lactoglobulin or α-lactalbumin for 2 hours. Cells were then incubated with FITC-labelled *E. coli* and the level of phagocytosis determined using flow cytometry. Results are expressed as mean percentage of cells with phagocytic capacity ± SEM (n = 8 mean results obtained from independent experiments). * $P < 0.05$, ** $P < 0.01$. Figure 4.1 **D** summarises the phagocytosis of FITC-labelled *E. coli* by murine peritoneal cells after incubation with β-lactoglobulin or α-lactalbumin (0.05 - 500 μg/mL). Results are represented as a percentage of the control (no added protein, 0 μg/mL).

4.3.3 The effect of β-lactoglobulin (NZDB) on the ability of a human monocytic cell line (THP-1) to phagocytose *E. coli*

The β-lactoglobulin (NZDB) also exerted a dose-dependent stimulatory effect on the ability of human monocytes to phagocytose FITC-labelled *E. coli.* At concentrations ranging between 0.5 - 1.5 μg/mL, β-lactoglobulin had no significant effect on the ability of human monocytes to phagocytose FITC-labelled *E. coli* (Figure 4.2 A). However, at concentrations between 3 to 96 μg/mL the percentage of cells with the ability to phagocytose FITC-labelled *E. coli* increased 100 – 350% compared to the control ($P < 0.01$) (Figure 4.2 B). This positive effect was strongest at concentrations of β-lactoglobulin ranging between 16 - 125 μg/mL with the phagocytic capacity of human monocytes increasing by 100 - 400% compared to the control ($P < 0.01$) with maximum enhancement occurring at 96 μg/mL (Figure 4.2 B). At concentrations greater than or equal to 250 μg/mL, β-lactoglobulin increased the phagocytic ability of human monocytes (Figure 4.2 C), however this increase was not significant, thus at these higher concentrations the level of enhancement was less than that observed at the lower concentrations. The phagocytosis of FITC-labelled *E. coli* by human THP-1 cells after incubation with β-lactoglobulin (0.05 - 500 μg/mL) is summarised in Figure 4.2 D.

4.3.4 The effect of α-lactalbumin (NZDB) on the phagocytic capacity of a human monocytic cell line (THP-1) to phagocytose *E. coli*

α-Lactalbumin (NZDB), at the lower concentration range of 0.05 - 1.5 μg/mL, had no significant effect on the percentage of cells with the ability to phagocytose FITC-labelled *E. coli* (Figure 4.2 A). Furthermore, no significant effect was observed at a concentration range of 3 - 12 μg/mL (Figure 4.2 B). However, at concentrations in the range of 24 - 96 μg/mL α-lactalbumin significantly reduced phagocytic capacity by 20 - 40% ($P < 0.05$) compared to the control (Figure 4.2 B). A decrease in the proportion of cells with phagocytic ability was observed at α-lactalbumin concentrations ranging between 16 to 500 μg/mL ($P < 0.01$) (Figure 4.2 C). The phagocytosis of FITC-labelled *E. coli* by human THP-1 monocytes after incubation with α-lactalbumin (0.05 - 500 μg/mL) is summarised in Figure 4.2 D.

FIGURE 4.2

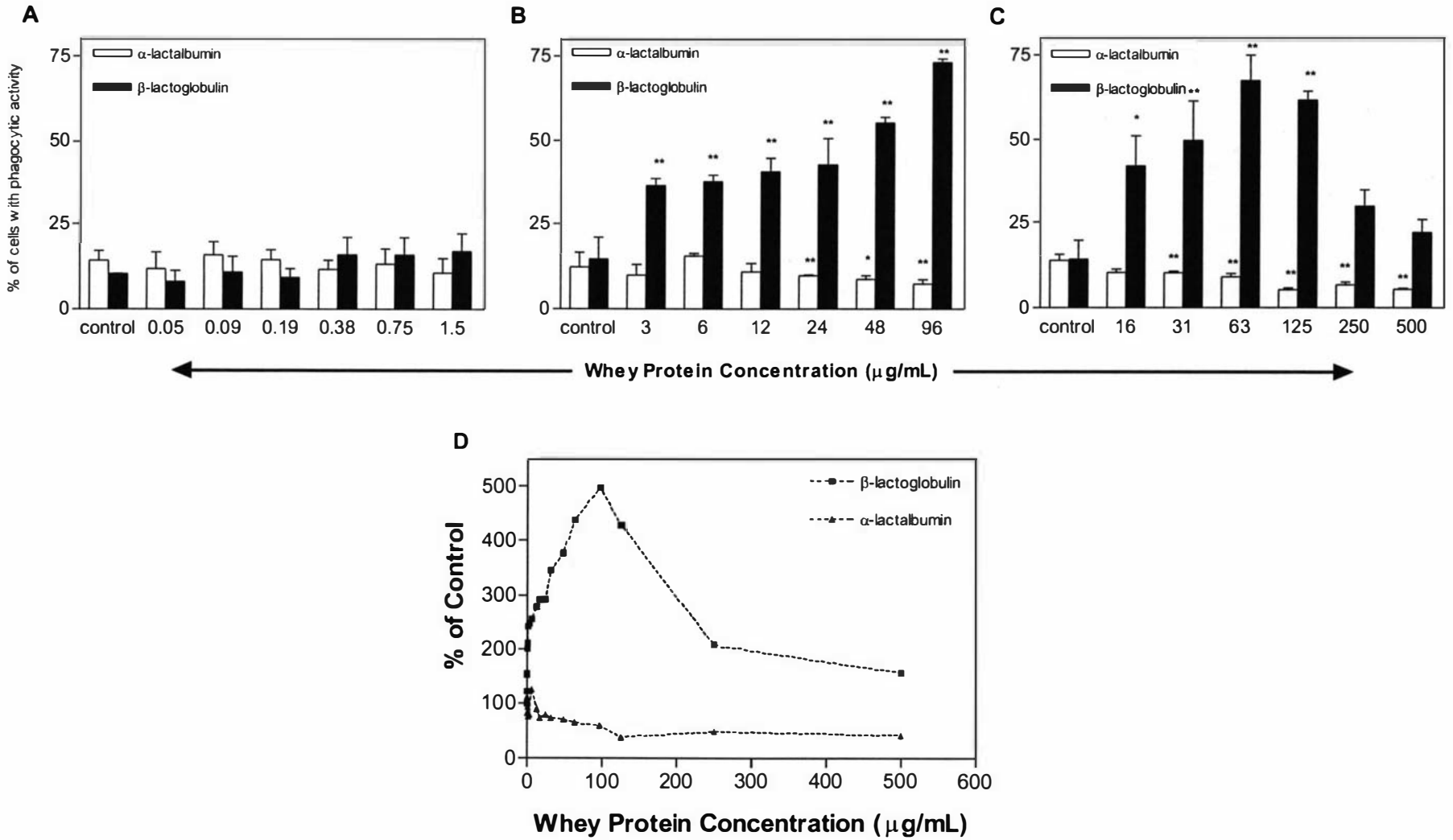

A
B
C
α-lactalbumin
β-lactoglobulin
% of cells with phagocytic activity
75
50
25
0
control
0.05
0.09
0.19
0.38
0.75
1.5
3
6
12
24
48
96
16
31
63
125
250
500
Whey Protein Concentration (μg/mL)
D
% of Control
500
400
300
200
100
0
600
Whey Protein Concentration (μg/mL)

FIGURE LEGEND

Figure 4.2 β-Lactoglobulin (NZDB), but not α-lactalbumin (NZDB), enhances the ability of a human monocytic cell line (THP-1) to phagocytose *E. coli*. Human monocytic cells were pre-incubated with 0.05 - 1.5 μg/mL **(A)**, 3 - 96 μg/mL (**B**), or 16 - 500 μg/mL (**C**) of β-lactoglobulin or α-lactalbumin for 2 hours. Human monocytic cells (THP-1) were then incubated with FITC-labelled *E. coli* and the level of phagocytosis determined using flow cytometry. Results are expressed as mean percentage of cells with phagocytic capacity ± SEM (n = 8 mean results obtained from independent experiments). * $P < 0.05$, ** $P < 0.01$. Figure 4.2 **D** summarises the phagocytosis of FITC-labelled *E. coli* by human monocytic cells (THP-1) after incubation with β-lactoglobulin or α-lactalbumin (0.05 - 500 μg/mL). Results are represented as a percentage of the control (no added protein, 0 μg/mL).

4.3.5 The effect of β-lactoglobulin (NZDB) or α-lactalbumin (NZDB) on the phagocytosis of latex beads by murine peritoneal cells and a human monocytic cell line (THP-1)

To investigate the effect of bovine whey proteins on the ability of murine peritoneal cells and human monocytic cells (THP-1) to phagocytose different particles, the phagocytosis of unopsonised latex beads was also measured. β-Lactoglobulin (NZDB) and α-lactalbumin (NZDB) did not enhance the ability of murine peritoneal cells to phagocytose fluorescent latex beads at the concentrations evaluated (Table 4.1). Similarly, these whey proteins had no significant effect on the ability of human monocytes to phagocytose latex beads (Table 4.1). Notably, the level of phagocytosis of unopsonised latex beads was significantly lower than that observed for *E. coli*. For example the base line (control) percentage of phagocytosis of the unopsonised latex beads by murine peritoneal cells or human THP-1 monocytes was no greater than 6.5% or 1.7% respectively, while the base line (control) percentage of phagocytosis of *E. coli* by murine peritoneal macrophages or human THP-1 monocytes ranged from 20 - 30% or 10 - 20% respectively.

TABLE 4.1

At the concentrations evaluated, β-lactoglobulin (NZDB) and α-lactalbumin (NZDB) have no significant effect on the phagocytosis of latex beads by murine peritoneal and THP-1 cells[1].

Protein Concentration (μg/mL)	Whey protein	
	β-Lactoglobulin	α-Lactalbumin
	Murine Peritoneal Cells	
Control	6.5 ± 0.2	5.9 ± 1.5
0.05	7.0 ± 1.3	5.8 ± 1.1
0.09	7.9 ± 1.9	7.2 ± 1.8
0.19	6.0 ± 1.1	6.4 ± 1.4
0.38	6.9 ± 0.5	8.2 ± 1.6
0.75	7.0 ± 0.8	6.4 ± 1.6
1.5	7.7 ± 0.7	7.5 ± 2.8
Significance[2]	NS	NS
	Human Monocytic Cell Line (THP-1)	
Control	1.7 ± 0.3	1.6 ± 0.3
0.05	1.5 ± 0.3	1.5 ± 0.3
0.09	1.4 ± 0.3	1.4 ± 0.3
0.19	1.7 ± 0.4	1.7 ± 0.4
0.38	1.5 ± 0.4	1.5 ± 0.3
0.75	1.4 ± 0.3	1.4 ± 0.3
1.5	1.4 ± 0.3	1.4 ± 0.3
Significance[2]	NS	NS

[1] Percentage of cells with phagocytic capacity expressed as mean ± SEM (n = 8 mean results obtained from independent experiments)
[2] ANOVA main effect of protein concentrations (NS = not significant)

4.3.6 The effect of β-lactoglobulin (ICN) and α-lactalbumin (ICN) on the phagocytic capacity of murine peritoneal cells

After incubation with 0 - 1.5 μg/mL of β-lactoglobulin (ICN) or α-lactalbumin (ICN) the percentage of cells with phagocytic ability was not significantly different from the controls (no added protein, 0 μg/mL) (Figure 4.3 A). No significant enhancement of the ability of murine peritoneal cells to phagocytose FITC-labelled *E. coli* was observed after incubation with 0 - 96 μg/mL of ICN β-lactoglobulin (Figure 4.3 B). At the highest concentration tested (96 μg/mL) α-lactalbumin (ICN) significantly decreased the percentage of cells with phagocytic ability compared to the control ($P < 0.01$) (Figure 4.3 B). This reduction may have been due to factors such as toxicity of the α-lactalbumin at this high concentration, hence cell viability (trypan blue) or metabolism (MTT reduction) assays are recommended for future experiments.

4.3.7 The effect of β-lactoglobulin (ICN) and α-lactalbumin (ICN) on the phagocytic capacity of the human monocytic cell line THP-1

Similar to murine peritoneal cells, incubation with 0 - 1.5 μg/mL of β-lactoglobulin (ICN) or α-lactalbumin (ICN) had no significant effect on the percentage of the human monocytic cells (THP-1) with phagocytic ability compared to controls (no added protein, 0 μg/mL) (Figure 4.3 C). No significant enhancement of the ability of human monocytes to phagocytose FITC-labelled *E. coli* was observed after incubation with 0 - 96 μg/mL of β-lactoglobulin (ICN) (Figure 4.3 D). Co-incubation with 48 or 96 μg/mL of α-lactalbumin (ICN) significantly reduced (by 40%) the percentage of cells with phagocytic ability (Figure 4.3 D). Again, the reduction of phagocytic activity at these higher concentrations may be an artefact caused by the toxicity of β-lactoglobulin at these higher concentrations and cell viability and metabolism assays are recommended for future experiments.

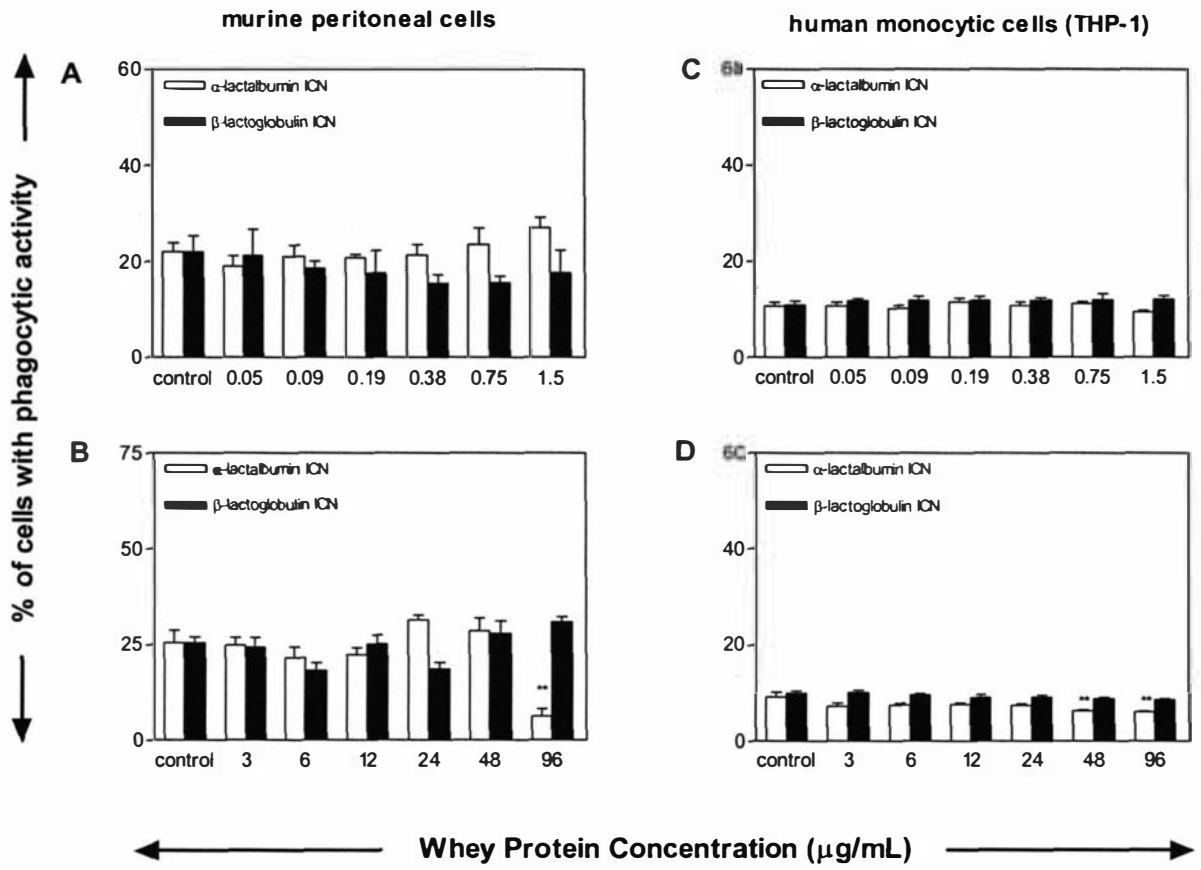


Figure 4.3 The effect of β-lactoglobulin (ICN) and α-lactalbumin (ICN) on the ability of murine peritoneal and the human monocytic cell line THP-1 to phagocytose *E. coli*. Murine peritoneal cells pooled from 12 BALB/c mice were incubated with 0.05 - 1.5 μg/mL **(A)** or 3 - 96 μg/mL **(B)** of lactoglobulin (ICN) or α-lactalbumin (ICN). Human monocytic cells (THP-1) were also incubated with 0.05 - 1.5 μg/mL **(C)** or 3 - 96 μg/mL **(D)** of β-lactoglobulin (ICN) or α-lactalbumin (ICN) for 2 hours. Cells were then incubated with FITC-labelled *E. coli* and the level of phagocytosis determined using flow cytometry. Results are expressed as mean percentage of cells with phagocytic capacity ± SEM (n = 8 mean results obtained from independent experiments). ** $P < 0.01$.

4.3.8 The effect of lactoferrin (ICN) on the ability of murine peritoneal cells to phagocytose *E. coli*

At the lower concentrations evaluated (0.01 - 0.31 μg/mL) lactoferrin (ICN) had no significant effect on the percentage of cells with the ability to phagocytose FITC-labelled *E. coli* (Figure 4.4 A). However, when incubated with 2.5 - 20 μg/mL lactoferrin, the ability of murine peritoneal cells to phagocytose FITC-labelled *E. coli* was 45 to 63% greater than the control (no added protein, 0 μg/mL) ($P < 0.01$). This enhancement occurred in a dose-dependent manner, plateauxing at the two highest concentrations tested (10 and 20 μg/mL) (Figure 4.4 B). Concentrations of lactoferrin greater than 20 μg/mL were not tested in this study due to limited supplies of lactoferrin (ICN).

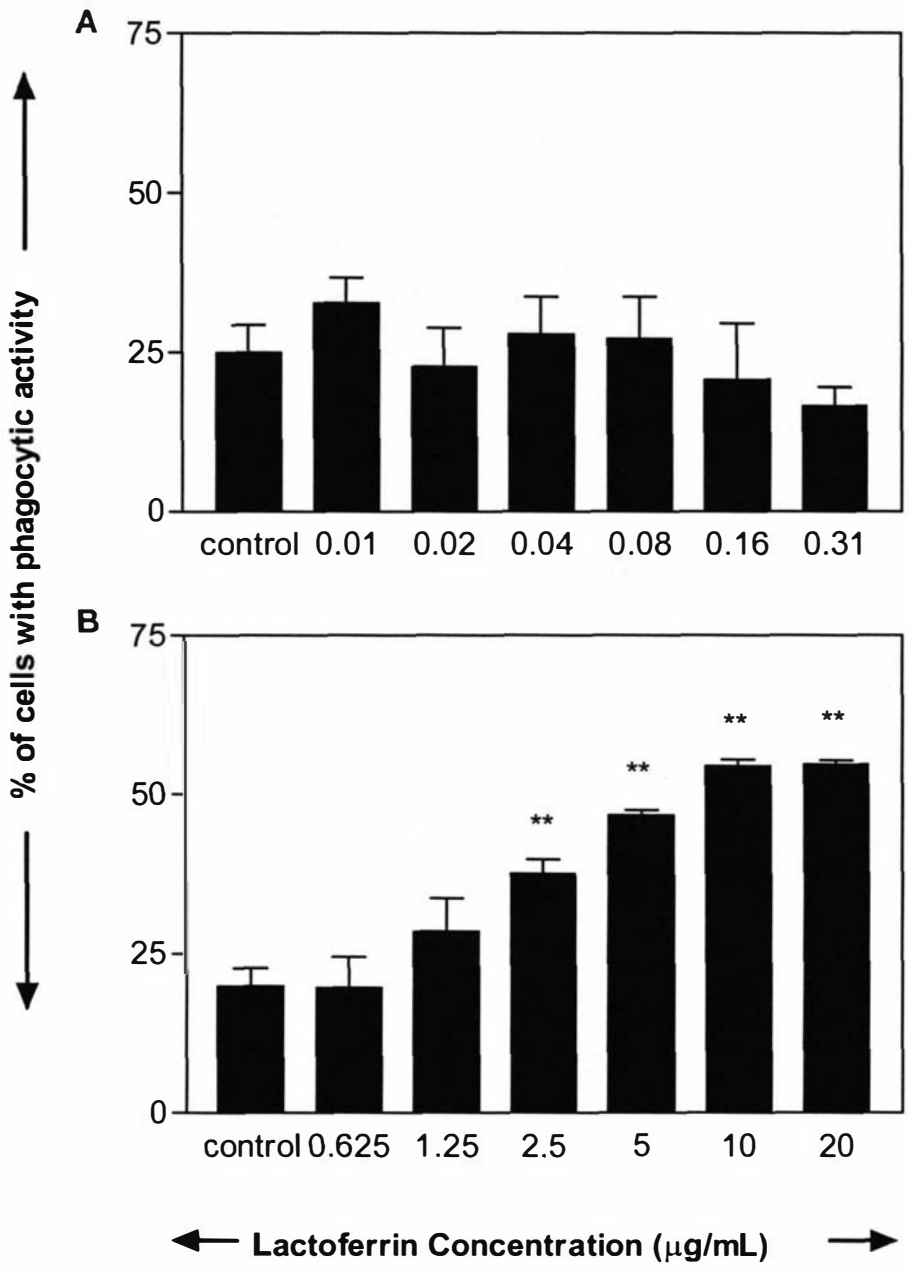


Figure 4.4 Lactoferrin (ICN) enhances the ability of murine peritoneal cells to phagocytose FITC-labelled *E. coli*. Murine peritoneal cells pooled from 12 BALB/c mice were pre-incubated with 0.01 - 0.31 μg/mL **(A)** or 0.625 - 20 μg/mL **(B)** lactoferrin for 2 hours. Cells were then incubated with FITC-labelled *E. coli* and the level of phagocytosis determined using flow cytometry. Results are expressed as mean percentage of cells with phagocytic capacity ± SEM (n = 8 mean results from independent experiments). ** $P < 0.01$.

4.4 DISCUSSION

The results of this study demonstrate that the whey protein β-lactoglobulin is able to increase the percentage of cells with phagocytic activity *in vitro*. This stimulation may be due to an enhancement of the rate of phagocytosis and/or an increase in the number of FITC-labelled *E. coli* phagocytosed by the murine peritoneal macrophages or human monocytic THP-1 cells. Bovine milk contains a number of potentially biologically active substances such as cytokines (Ogra & Ogra, 1978; Hanson *et al.*, 1985; Hoffman-Goetz *et al.*, 1985) and complement (reviewed by Korhonen *et al.*, 2000), raising the possibility that one or more of these substances could have contaminated the β-lactoglobulin. However, (as mentioned previously in Chapter 3, Section 3.4) studies by Wong *et al.* (1998) have demonstrated that the immune enhancing properties (murine spleen cell IgM production and proliferation) of β-lactoglobulin were not due to the presence of interleukin-2, interleukin-4, interleukin-10, interferon-γ or tumour necrosis factor-α. In addition, treatment of β-lactoglobulin with buffer at pH 8 significantly reduced (but did not eliminate) the activity (Wong *et al.*, 1998). This finding supports the possibility that the immunostimulating activity was due to the β-lactoglobulin *per se*, because β-lactoglobulin is denatured at slightly alkaline pH (Waissbluth & Grieger, 1974).

In the present study the phagocytic activity of murine peritoneal macrophages and human THP-1 cells was enhanced after a short pre-incubation time of 2 hours with β-lactoglobulin. Many of the amino acids present in β-lactoglobulin itself have also been demonstrated to possess the ability to enhance phagocytic function after relatively short pre-incubation times. β-Lactoglobulin contains high levels of aspartic acid, glutamic acid and lysine (Morr & Ha, 1993). Pre-incubation with glutamic and aspartic acids for 15 minutes has been shown to enhance the phagocytosis of *Staphylococcus* by murine peritoneal macrophages *in vitro* (Belokrylov *et al.*, 1992). Pre-incubation for 30 minutes with proline and arginine (also present in β-lactoglobulin) has been shown to stimulate the phagocytic activity of guinea pig peritoneal polymorphonuclear neutrophil phagocytes (Stueden *et al.*, 1983). Therefore, the ability of β-lactoglobulin to enhance the phagocytic capacity of murine peritoneal cells and human THP-1 cells may be due, in part, to its amino acid composition. In addition, β-lactoglobulin is glutamine rich. Laboratory data and clinical data suggest that glutamine may play an essential role in

immunologic responses particularly during certain inflammatory conditions such as infection and injury (reviewed by Wilmore & Shabert, 1998). A decrease in the levels of glutamine has not only been demonstrated to reduce the rate of proliferation of human lymphocytes but also decrease the rate of phagocytosis (of ^{125}I-labelled zymosan) by murine peritoneal macrophages when included in culture for 38 hours (Parry-Billings *et al.*, 1990). Hence, further study investigating the effect of pre-incubating monocytes and macrophages with β-lactoglobulin for longer periods of time (i.e. 12 - 72 hours) in cell culture is warranted. The enhancement of phagocytic activity by immunomodulating peptides has been postulated to occur via the modulation of Fc and/or C_3 receptors present on the surface of monocytic and macrophagic cells (Gattegno *et al.*, 1988). However, the mechanisms by which whey proteins may modulate phagocytic function, particularly after short incubation times *in vitro,* are not completely defined. The identification and isolation of the putative receptor molecules present on the surface of phagocytes may increase understanding of the molecular details of the modulation of phagocytic function by whey proteins such as β-lactoglobulin. Further study on the binding characteristics of whey proteins on the surface of monocytes and macrophages may also be advantageous in elucidating the mechanisms by which the observed stimulations occur.

There is also a notable relationship between the immune system and the opioid peptides (β-lactorphin, residues 102 - 105 (Yoshikawa *et al.*, 1986)) present in β-lactoglobulin. Opioid receptors for endorphins have been found on human phagocytic leucocytes and T lymphocytes (Meisel & Schlimme, 1990). Endogenous opioid peptides in β-lactoglobulin may have endorphin-like capacity with respect to the development of cellular immunity, and can consequently enhance both phagocytic capacity and T-cell function. β-Lactoglobulin is also a member of the lipocalin protein super family classified as transport proteins, some of which (such as α_1-microglobulin) are known to have immunomodulatory properties (Wong *et al.*, 1998). Trans-retinoic acid (carried by β-lactoglobulin (Guimont *et al.*, 1997)) has also been postulated to enhance mitogen-stimulated human propria-derived lymphocyte proliferation by affecting accessory cells such as macrophages (Elitsur *et al.*, 1997).

Opioid peptide-like sequences have also been found in α-lactalbumin (residues 50 - 53). This peptide (named α-lactorphin) has been shown to have opioid agonist activity (Yoshikawa *et al.*, 1986). However, α-lactalbumin contains lower amounts of the amino acids that have been demonstrated to possess immune-enhancing properties than β-lactoglobulin (Eigel *et al.*, 1984). The α-lactalbumin investigated in this study did not enhance the phagocytic ability of murine peritoneal macrophages (Figure 4.1) or human THP-1 cells (Figure 4.2). In fact, at concentrations greater than 12 μg/mL, α-lactalbumin significantly reduced the phagocytic capacity of human monocytic cells but not murine peritoneal cells (Figure 4.2). Whether or not this reduction in phagocytic capacity was due to an inhibitory and/or toxic action of α-lactoglobulin remains to be clarified by cell viability and/or metabolism assays. In contrast to these results a tripeptide (Gly-Leu-Phe obtained from casein digests) present in both human and bovine α-lactalbumins (Migliore-Samour *et al.*, 1992), has been shown to enhance the phagocytosis of red blood cells by mouse macrophages and human monocytic/macrophage cells *in vitro* (Berthou *et al.*, 1987; Gattegno *et al.*, 1988). This capacity was thought to be correlated to the presence of specific binding sites on the human phagocytic cells (Jaziri *et al.*, 1992). Furthermore, the inclusion of α-lactalbumin (prepared from bovine Cheddar cheese whey) in culture for 12 hours has been found to enhance interleukin-1β production by ovine bronchoaveolar lavage macrophages (Wong *et al.*, 1997b).

The varying immunomodulatory effects of α-lactalbumin observed in this study (and in Chapter 3) compared to other studies (Berthou *et al.*, 1987; Gattegno *et al.*, 1988; Wong *et al.*, 1997b) may not only be attributed to variations in the immunological methods employed, but also variations in the preparation or initial source of the whey protein components tested. For example, the commercially available whey proteins investigated in this study did not stimulate phagocytic capacity of murine peritoneal cells or human monocytes (Figure 4.3). The whey proteins purchased from ICN were produced from different whey sources compared to the whey proteins produced by the NZDB. Widely differing processing conditions can result in products with varying compositions, degrees of protein denaturation and aggregation, and different physicochemical functional properties which may affect their immune-enhancing capabilities (Morr & Ha, 1993). Consequently, some of the immune-enhancing proteins may have been

absent in the commercial whey protein preparations tested in this study or industrial processing may have destroyed their biological activity. It cannot be completely dismissed that other immunomodulatory components or contaminants may have been present in the whey protein preparations tested hence further investigation is warranted to determine the presence of such components.

In this study, the incubation time with FITC-labelled *E. coli* or latex beads was restricted to 20-30 minutes (see section 4.2.3) to avoid a maximal uptake of particles by all of the phagocytes pre-incubated with different concentrations of the various whey proteins. Hence, the percentage of cells with phagocytic activity ranged from 5.8% to 70% for murine peritoneal macrophages and 1.4% to 75% for human monocytic THP-1 cells. At the lower concentrations investigated in this study (0.05 - 1.5 μg/mL), the type and size of the particle being phagocytosed appeared to influence the degree of immunomodulation attributable to β-lactoglobulin. No enhancement of the phagocytosis of the inert latex beads by murine peritoneal cells and human monocytes after incubation with 0.05 – 1.5 μg/mL of β-lactoglobulin or α-lactalbumin was observed (Table 4.1). The level of phagocytosis of the unopsonised fluorescent latex beads was considerably less than the level of phagocytosis of the FITC-labelled *E. coli* by the murine macrophages and human monocytes. This is expected as phagocytes bear several different receptors that recognise microbial components and induce phagocytosis (Janeway *et al.*, 1999). The larger diameter of the latex beads (1 μm) compared to the smaller *E. coli* particles may have also contributed to the reduction in the level of phagocytosis. Additionally, the enhancement of the phagocytosis of FITC-labelled *E. coli*, but not latex beads, by murine macrophages and human monocytes pre-incubated with β-lactoglobulin suggests that β-lactoglobulin may have bound to the surface of FITC-labelled *E. coli*, facilitating phagocytosis in a manner similar to opsonisation. Indeed, Lima & Kierszenbaum (1987) postulated that lactoferrin bound via cell surface receptors to both murine peritoneal monocytes and *Trypanosoma cruzi* (*T. cruzi*) may have created a molecular bridge facilitating parasitic uptake in a manner similar to opsonisation. However, lactoferrin was also found to stimulate latex bead (lacking surface receptors for lactoferrin) uptake, suggesting that, whether or not molecular bridging by lactoferrin was involved, the possible opsonisation of *T. cruzi* by lactoferrin was not necessary for murine peritoneal macrophages to display enhanced phagocytic activity.

Lactoferrin is well known to play a role in iron metabolism as well as host defence via antimicrobial and antibacterial activities (reviewed by Reiter, 1985; Vorland, 1999) and to possess immune-modulating potential *in vitro* (discussed in Chapter 1 and Chapter 3). Shinoda *et al.* (1996) reported that both bovine lactoferrin and lactoferricin (a peptide from lactoferrin) could stimulate the release of neutrophil-activating chemokine interleukin-8 from human polymorphonuclear leucocytes. When bovine lactoferrin was depleted of lactoferricin no stimulatory effect was found (Miyauchi *et al.*, 1997). Additionally, bovine lactoferricin has been found to suppress interleukin-6 production by human monocytic cells in responses to LPS stimulation (Mattsby-Baltzer *et al.*, 1996). Lima and Kierszenbaum (1985) have previously demonstrated an increase in the uptake, intracellular destruction and association of intracellular forms of a protozoan (*T. cruzi*) with murine peritoneal macrophages or human blood monocytes after pre-incubation with human lactoferrin for lengths of time ranging from 30 minutes to 2 hours *in vitro*. Concentrations of human lactoferrin enhancing the interaction of the phagocytic cells with *T. cruzi* ranged from 0.1 to 10 μg/mL, however, the present study is the first to show that bovine (rather than human) lactoferrin can also significantly enhance the phagocytic capability of murine peritoneal macrophages, confirming and extending the immunomodulatory potential of bovine lactoferrin to include phagocytosis-stimulating capacity *in vitro*. In this study, bovine lactoferrin significantly increased the ability of murine peritoneal cells to phagocytose *E. coli* by up to 60% (Figure 4.4) at concentrations ranging between 2.5 to 20 μg/mL. These levels of lactoferrin compare with normal human plasma concentrations of 1.5 ± 1.8 μg/mL, and pathologic conditions varying between 4 and 28 μg/mL in cases of burn injury, 12 - 22 μg/mL in cases of chronic myeloid leukaemia in relapse and 5 - 12 μg/mL in hypersplenism (Lima & Kierszenbaum, 1985). Concentrations of lactoferrin greater than 20 μg/mL were not tested in the present study however Lima and Kierszenbaum (1985) found that higher concentrations of human lactoferrin (50 μg/mL) did not increase the uptake and intracellular destruction of *T. cruzi* by human blood monocytes or murine peritoneal macrophages *in vitro*. It was postulated that this lack of enhancement may be due to the saturation of lactoferrin receptors on the surface of the phagocytic cells (Lima & Kierszenbaum, 1985), thus concentrations greater than 20 μg/mL may have become supra-optimal.

In vitro, lactoferrin has been shown bind to human macrophages and monocytes by receptor like binding within a time course of 5 - 80 minutes at a magnitude 90% greater than the binding to lymphocytes and neutrophils (Birgens *et al.*, 1984). Britigan *et al.* (1991) confirmed that both monocytes and macrophages have the ability to assimilate large quantities of milk lactoferrin from the microenvironment over a short period of incubation, mediated by lactoferrin specific receptors on the cell surface. However, the functional importance of lactoferrin uptake by mononuclear phagocytes remains a subject of continued controversy (Broxmeyer *et al.*, 1978; Lima & Kierszenbaum, 1985). Findings by Britigan *et al.* (1991) have suggested that the binding of apo-lactoferrin could maintain optimal phagocyte function by complementing the role of cellular anti-oxidant enzymes and other factors in protecting phagocytes from auto-oxidative injury.

The results of this chapter and the previous chapter have demonstrated that certain whey proteins have the ability to enhance several different aspects of immune function, namely the proliferation of lymphocytes and the phagocytic capacity of macrophages and monocytes *in vitro*. *In vitro* studies can provide a valuable means of screening and of possibly predicting immunomodulating properties *in vivo*. These results suggest that particular whey proteins may also have the ability to enhance immune function *in vivo*. This notion is supported by Belokrylov *et al.* (1992) who found that the positive influence of several natural peptides on phagocytic capacity takes place both *in vitro* and *in vivo*. Bovine milk whey protein, especially its basic protein fraction (termed milk basic protein) has been shown in both *in vitro* and *in* vivo studies to promote bone formation and suppress bone resorption (Takada *et al.*, 1996; Aoe *et al.*, 2001; Toba *et al.*, 2001) and a synthetic casein peptide has been found to stimulate phagocytosis of SRBC by murine and human macrophages when included in culture for 20 hours and also been active *in vivo* by exerting a protective effect against *Klebsiella pneumoniae* infection in mice (Parker *et al.*, 1984; Migliore-Samour *et al.*, 1989). Additionally, Otani *et al.* (1995) reported that a κ-casein glycopeptide prepared from whey protein concentrate inhibited the mitogen-induced proliferation of murine spleen lymphocytes *in vitro*, but found mitogen induced proliferative responses of spleen lymphocytes fed a CGP-added diet increased significantly compared with mice fed a control diet (ovalbumin alone) (Monnai *et al.*, 1998). Therefore, the immunomodulatory effects of milk proteins on immune functions such as phagocytic function and lymphocyte

proliferation can vary *in vitro* and *in vivo*. Consequently, further research into the effects of our whey proteins on immune function *in vivo* is warranted to fully elucidate the immune-enhancing properties of these proteins.

CHAPTER 5

The Effect of Dietary α-Lactalbumin, β-Lactoglobulin or Lactoferrin on Humoral Immune Responses

5.1 INTRODUCTION

Whey proteins are well-known for their high nutritional value and versatile functional properties in food products (de Wit, 1998). During recent decades, interest has grown in the immunomodulatory capabilities of whey proteins. Many *in vitro* studies, including those reported here in Chapters 3 and 4, have provided evidence that the major protein components of whey (β-lactoglobulin and α-lactalbumin) and the minor protein component lactoferrin, possess immunobiological properties when co-cultured directly with leucocytes (Chapter 3, Chapter 4, Ogra & Ogra, 1978; Newby *et al.*, 1982; Juto, 1985; Stoeck *et al.*, 1989; Mincheva-Nilsson *et al.*, 1990; Barta *et al.*, 1991; Politis *et al.*, 1991). Whey protein components have also been found not only to provide a rich and balanced source of amino acids (Regester *et al.*, 1996), but in a number of instances, also have specific physiological actions *in vivo*. Spleen cells of mice primed by intravenous inoculation with SRBC and fed 28 g of whey lactalbumin hydrolysate/100 g diet exerted an almost 5 fold increase in IgM measured by the plaque-forming cell responses to SRBC (Bounous & Kongshavn, 1982) compared to mice fed a casein diet. Bounous & Kongshavn (1985) further identified the proteins in the whey portion of milk as key immunoenhancing factors by demonstrating that mice immunised with a T-independent antigen (trinitrophenylated ficoll) had an increased number of plaque-forming cells per spleen when fed bovine whey proteins (20 g/100 g lactalbumin) compared with those mice on standard mouse chow (control) diets. The humoral antibody response to immunisation with SRBC was found to be even greater. Conversely, cellular immune functions such as mitogen responsiveness and delayed type hypersensitivity did not appear to be affected by dietary whey proteins, thus the effects of whey proteins were postulated to primarily influence B-cell function and activity (Bounous & Kongshavn, 1985). The oral administration of bovine lactoferrin has also been found to induce both intestinal and peripheral specific antibody responses in mice (Debabbi *et al.*, 1998) and increase antigen specific proliferation of Peyer's patches and spleen cells (Tome & Debabbi, 1998).

The immunomodulatory properties of dietary bovine whey proteins have therefore been well documented, however new membrane separation and chromatographic techniques have made it possible to fractionate and enrich various components of whey more effectively than before (Korhonen *et al.*, 1998). This provides new opportunities for

dairy industries to develop and screen for valuable whey protein-based immune-enhancing products, with a view to exploit their possible industrial and pharmaceutical applications. Earlier *in vitro* studies demonstrated that previously untested whey protein isolates (manufactured by the NZDB) have powerful mitogenic activity on murine splenic lymphocytes (Chapter 3) and phagocytosis-stimulating activity on murine peritoneal macrophages and human monocytic THP-1 cells (Chapter 4). Stimulation of these two immune functions suggests that the whey protein isolates may also possess strong immune-enhancing properties *in vivo*. Such findings have prompted further investigation of the immune-enhancing properties of these whey proteins following dietary delivery to mice with the aim to determine the immunomodulatory capabilities of these whey proteins *in vivo*. Therefore, the objective of this study was to evaluate the immunomodulatory effects of dietary α-lactalbumin, β-lactoglobulin and lactoferrin in mice using a panel of *ex vivo* and *in vitro* immuno-assays. Because several previous studies (Bounous & Amer, 1988; Bounous *et al.*, 1989a; Parker & Goodrum, 1990; Wong & Watson, 1995; Debabbi *et al.*, 1998) have reported an enhancement of humoral immunity by dietary whey proteins, the effect of dietary whey proteins on humoral immune responses to several parentally-administered and orally administered vaccines was evaluated in this study. Two important indices of *ex vivo* lymphoid and non-lymphoid cell function, lymphocyte proliferation and phagocytic ability, were also assessed.

5.2 MATERIALS AND METHODS

5.2.1 Mice and diets

Male 6-7-week-old BALB/c mice were housed individually at 22 ± 1°C under a light-dark photoperiod and offered feed and water *ad libitum.* Control animals were fed a ground milk-free mouse chow diet (pellet diet) comprising 18% w/w total protein of dry weight diet (Sharps, Lower Hutt, New Zealand) as described in Section 2.2. Test animals were fed a complete diet (Table 2.1) containing 18% w/w protein of a β-lactoglobulin-enriched whey protein isolate (PT3886, NZDB, Wellington, New Zealand) as the sole protein source, a α-lactalbumin-enriched isolate (PT6378, NZDB, Wellington, New Zealand) as the sole protein source, or a ground milk-free pellet diet containing 1% w/w lactoferrin (Tatua Biologics, Morrinsville, New Zealand). Dietary compositions are shown in Table 5.1. Diets containing 18% - 20% w/w protein have been shown to be nutritionally adequate and sustain normal growth in mice (Bounous & Kongshavn, 1982; Parker & Goodrum, 1990). The typical microbiological estimates of the whey proteins evaluated in this study are given in Chapter 3, Table 3.2 (β-lactoglobulin (NZDB) and α-lactalbumin (NZDB)) and Table 5.2 (lactoferrin (Tatua)).

Table 5.1

Composition of Diets

	Pellet (control)	β-lactoglobulin	α-lactalbumin	Lactoferrin
Protein	Meat meal 6% Lucerne meal 5% Fish meal 7% Soybean meal 1.5%	PT3886 19.15% (β-lac 85% protein)	PT6378 20.69% (α-lac 69% protein)	Lactoferrin 1.03% Pellet diet 18%
Total Protein Content	**18g/100g dry weight**	**18g/100g dry weight**	**18g/100g dry weight**	**19g/100g dry weight**
Total Fat Content	**7g/100g dry weight**	**8g/100g dry weight**	**8g/100g dry weight**	**8g/100g dry weight**

TABLE 5.2

Typical microbiological estimates for the lactoferrin evaluated in this study

Whey Protein Isolates	Standard plate count (non-specific bacteria)	Coliforms	Yeasts & moulds	*S. aureus*	*Salmonella*
Lactoferrin (Tatua)	<1/g max	Neg/0.1 g	50/g max	Neg/0.1 g	Neg/5 g

Note:
1. All counts calculated from number of colony forming units
2. Negative (Neg)
3. *Staphylococcus aureus (S. aureus)*
4 Tatua product details obtained from Tatua Biologics, Morrinsville, New Zealand

5.2.2 Experimental design and immunisations

Mice (8 mice per diet/time on diet) were fed control or test diets for a total of 0, 2, 4 or 6 weeks (Figure 5.1). The mice were immunised by subcutaneous injection (25 μL/mouse) of absorbed diphtheria toxoid and tetanus toxoid vaccine (ADT) (CSL, Victoria, Australia) and inactivated influenza vaccine (Fluvax) (CSL). Mice also received oral inoculations of cholera toxin (10 μg/mouse) (Sigma, St Louis, MO, USA), ovalbumin (1 mg/mouse) (CSL, Victoria, Australia) in 25μL 0.5 M NaH_2CO_3 (pH 8.2), and live attenuated polio vaccine (6 μl/mouse) (SmithKline Beecham Biologicals, Mt Wellington, Auckland). All mice received subcutaneous and oral vaccinations at week 0 of dietary treatment (1° immunisation) and were given booster immunisations (2° immunisation) at week 4 of dietary treatment (Figure 5.1). At the end of 0, 2, 4 or 6 weeks of dietary treatment mice were euthanased as described in Section 2.2 and immunological parameters of mice from test and control diets were assessed (Figure 5.1).

5.2.3 The assessment of systemic and mucosal antibody responses to various vaccines of mice fed dietary whey proteins

An ELISA was used to determine systemic serum antibody responses to flu vaccine (Flu), tetanus toxin (TT) and diphtheria (Dip); and gut mucosal antibody responses to cholera toxin (CT), ovalbumin (OV) and polio, as described in Section 2.9 and antibody units (units/mL) assigned as described in Section 2.9.2.

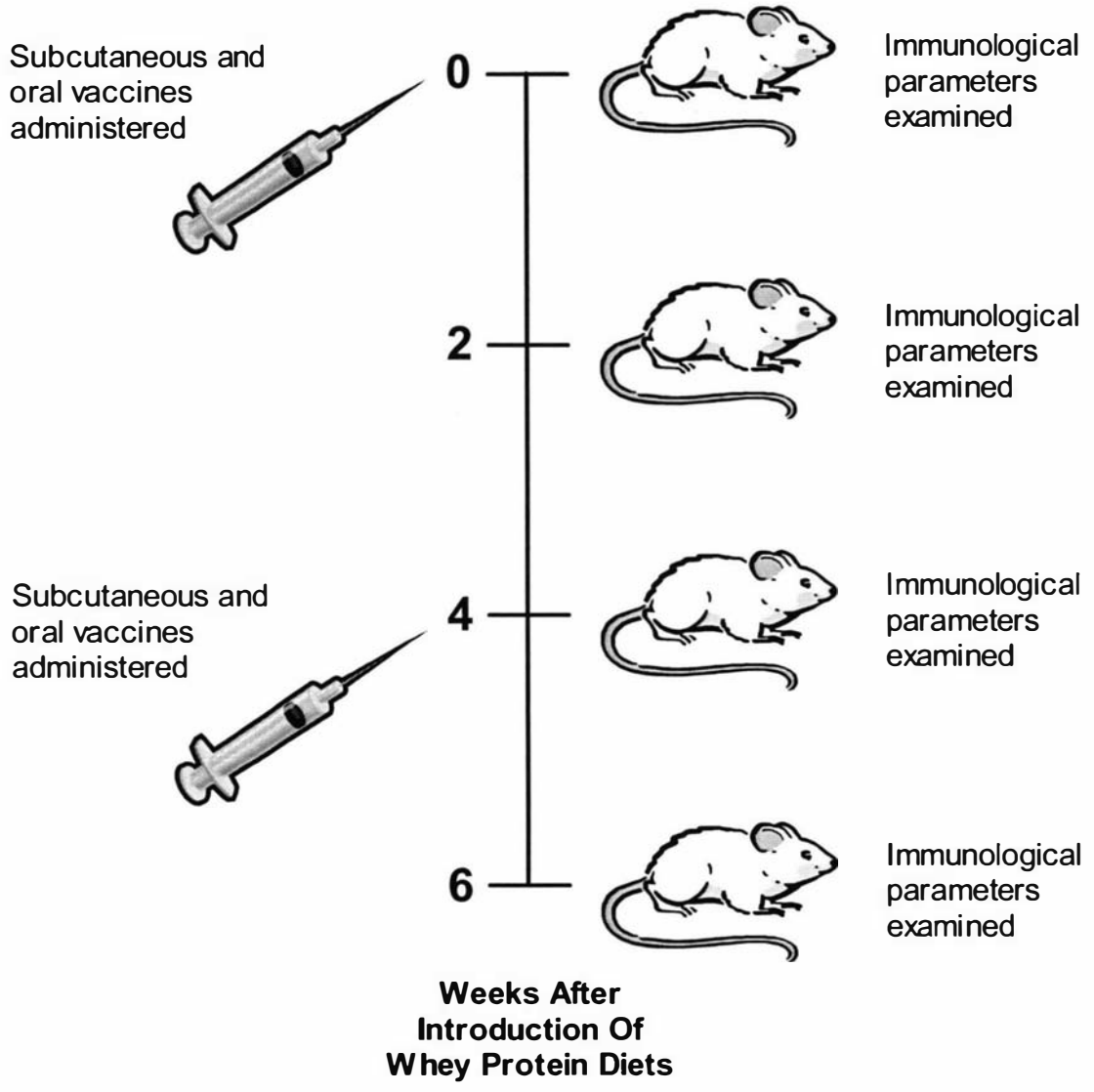


Figure 5.1 Experimental design and immunisation schedule. Schematic representation of the animal feeding trial designed to assess the impact of dietary whey proteins on several humoral and cellular parameters of immune function. At the end of week 0, 2, 4 or 6 immunological parameters were examined. For each test diet, control mice were fed milk free pellets for 0, 2, 4, or 6 weeks. Each group contained 8 mice.

5.2.4 Lymphocyte proliferation assay to assess the effect of dietary whey proteins on the mitogen-induced proliferative potential of splenic lymphocytes

Spleens were removed aseptically from the mice and weighed prior to the preparation of single cell suspensions as described in Section 2.3. Splenic lymphocytes were enumerated using a Becton Dickinson FACSCalibur flow cytometer. *In vitro* proliferative responses of splenic lymphocytes to mitogens were determined using an enzyme-based colourimetric cell proliferation kit (Boehringer Mannheim, Mannheim, Germany) as previously described in Section 2.4 with the following modification. One hundred thousand (10^5) cells were cultured in the presence or absence of the following mitogens: 2.5 μg/mL Con A; 5 μg/mL LPS; or 13.3 μg/mL PHA.

5.2.5 Phagocytosis assay to assess the effect of dietary whey proteins on phagocytic activity

To assess the phagocytic activity of cells derived from mice fed β-lactoglobulin, α-lactalbumin, lactoferrin (1% w/w) or the control (pellet) diets, peritoneal macrophages were prepared as described in Section 2.6. To assess the phagocytic ability of peripheral blood cells, blood was obtained as described in Section 2.5 and 2.7. Phagocytic ability of murine peritoneal cells and peripheral blood cells was assessed as previously described in Section 2.8 with the following modification. Peritoneal cells or peripheral blood cells were incubated separately with 20 μl of FITC-labelled *E. coli* for 20 minutes prior to flow cytometric analysis of phagocytic activity. Optimal incubation times were determined in preliminary experiments.

5.2.6 Statistical analysis

Statistical differences between the control and test diet groups were determined at each time point using ANOVA and Dunnett's multiple comparison post-hoc test (GraphPad Prism®, USA, 1999). *P* values less than 0.05 were considered significant.

5.3 RESULTS

5.3.1 The mucosal antibody response to orally-administered antigens of mice fed α-lactalbumin, β-lactoglobulin or lactoferrin

The effect of dietary α-lactalbumin, β-lactoglobulin or lactoferrin on the mucosal antibody responses to three different orally-administered antigens was evaluated. After the primary immunisation, dietary α-lactalbumin or β-lactoglobulin had no effect on the mucosal antibody response to cholera toxin (bacterial toxoid) (Figure 5.2 A), ovalbumin (protein) (Figure 5.2 B) or polio (attenuated virion) (Figure 5.2 C). However, 2 weeks after the secondary immunisation, dietary α-lactalbumin and β-lactoglobulin significantly increased (by up to 3 fold) mucosal antibody responses to CT, OV and polio compared to mice fed milk free pellets ($P < 0.05$ or $P < 0.01$). Similarly, dietary lactoferrin (1% w/w) had no effect on mucosal antibody responses to CT, OV and polio after the primary immunisation. However, 2 weeks after the secondary immunisation, mice treated with dietary lactoferrin exhibited significantly greater (2 fold) mucosal immune responses to orally administered CT, OV and polio compared to control mice (Figure 5.2 A, B, C).

5.3.2 The systemic antibody response to parenterally-administered antigens of mice fed α-lactalbumin, β-lactoglobulin or lactoferrin

The effect of dietary α-lactalbumin, β-lactoglobulin or lactoferrin on the systemic antibody response to 3 different parenterally-administered antigens was evaluated. Dietary α-lactalbumin, β-lactoglobulin or lactoferrin (1% w/w) had no significant effect on systemic antibody responses to Flu vaccine (attenuated virion) after the primary immunisation compared to controls (Figure 5.3 A). However, 2 weeks after the secondary immunisation all the dietary whey proteins evaluated in this study enhanced the secondary systemic immune responses to the flu vaccine 3 fold compared to the control mice ($P < 0.05$) (Figure 5.3 A). Similarly, dietary α-lactalbumin, β-lactoglobulin and lactoferrin had no significant effect on systemic antibody responses to the bacterial toxoid vaccines, tetanus toxoid (Figure 5.3 B) and diphtheria (Figure 5.3 C) following the primary immunisation. However, 2 weeks after secondary immunisation dietary α-lactalbumin, β-lactoglobulin and lactoferrin significantly enhanced systemic antibody immune responses to TT and Dip by more than 2 fold compared to the control mice ($P < 0.05$, $P < 0.001$) (Figure 5.3 B, C).

FIGURE 5.2

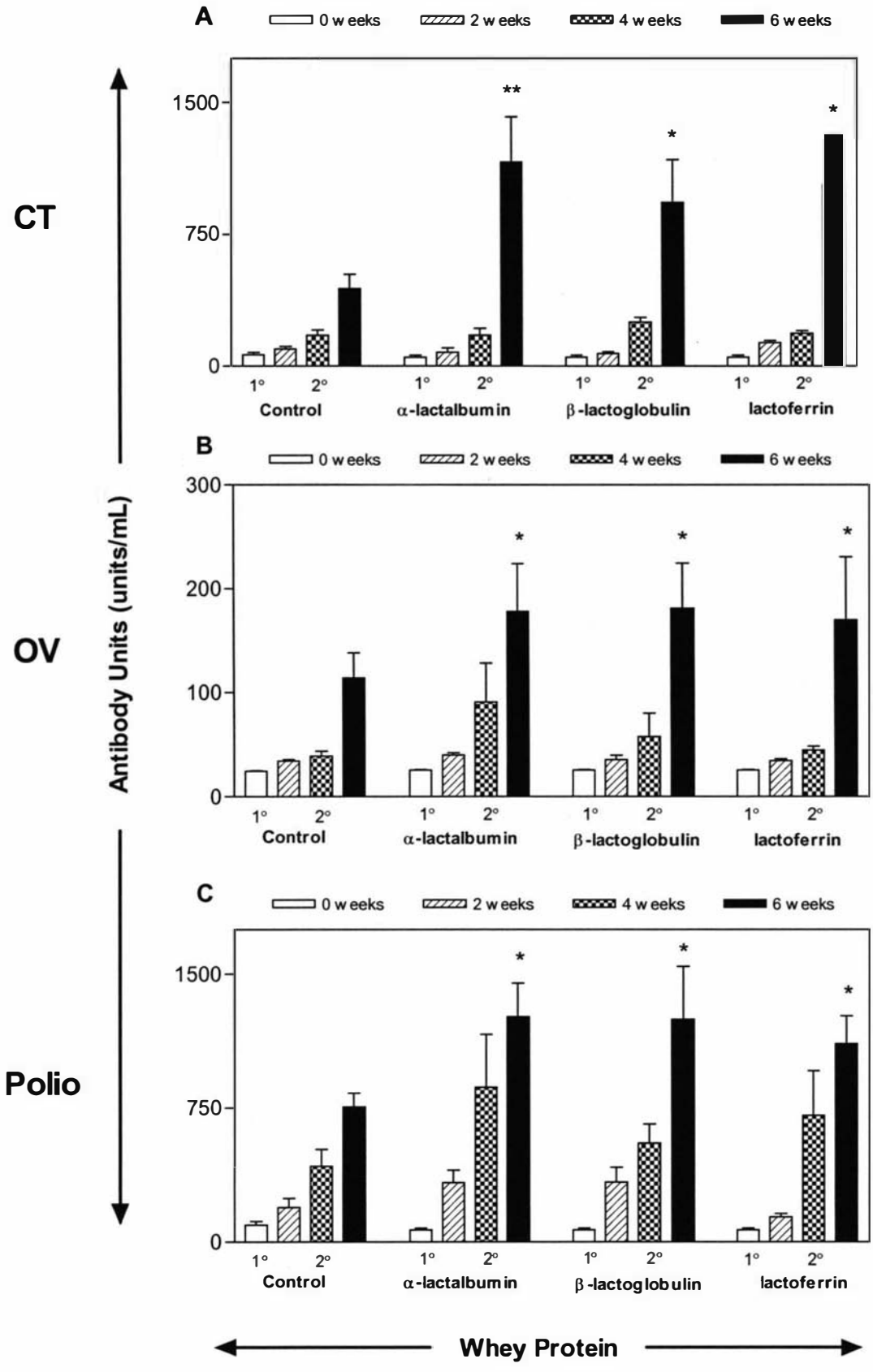
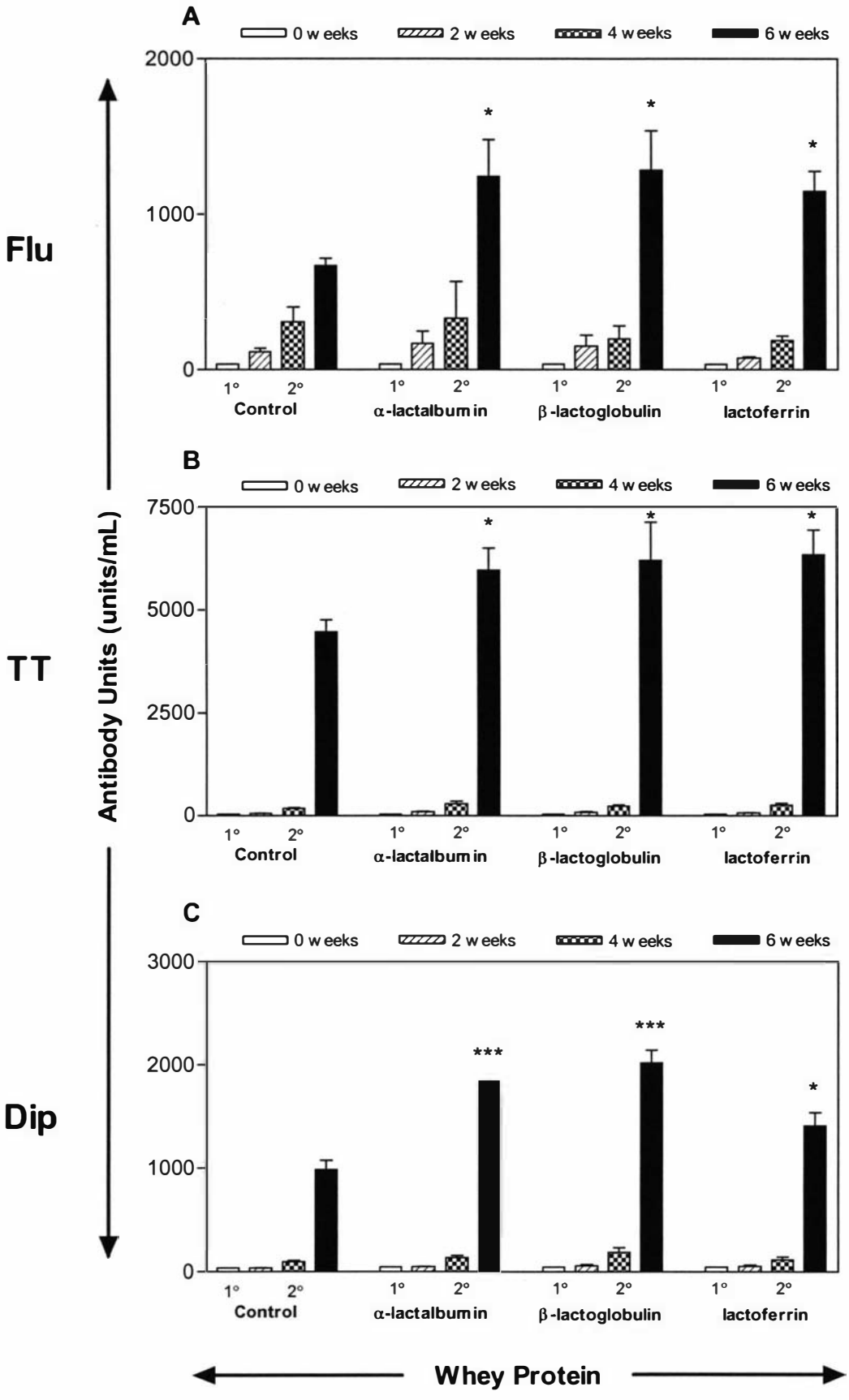

A
0 weeks
2 weeks
4 weeks
6 weeks
1500
750
0
**
*
*
1° 2°
Control
α-lactalbumin
β-lactoglobulin
lactoferrin
CT
B
300
200
100
0
OV
C
Polio
Antibody Units (units/mL)
Whey Protein

FIGURE LEGENDS

Figure 5.2 Dietary α-lactalbumin, β-lactoglobulin and lactoferrin enhances the mucosal immune response to orally-administered CT, OV and polio by up to 3 fold compared to the controls. Immunisations with CT (**A**), OV (**B**) or polio (**C**) were administered at the beginning of week 0 and week 4. Antibody responses were assessed using an enzyme-linked immunosorbent assay (ELISA) after 0, 2, 4 or 6 weeks on the dietary regime. The results are expressed as the mean ± SEM (n = 8) antibody units (units/mL). ** $P < 0.01$, * $P < 0.05$.

Figure 5.3 Dietary α-lactalbumin, β-lactoglobulin and lactoferrin enhances the systemic antibody response to subcutaneously administered Flu, TT and Dip by up to 3 fold compared to the controls. Immunisations with Flu (**A**), TT (**B**) or Dip (**C**) were administered at the beginning of week 0 and week 4. Antibody responses were assessed using an enzyme-linked immunosorbent assay (ELISA) after 0, 2, 4 or 6 weeks on the dietary regime. The results are expressed as the mean ± SEM (n = 8) antibody units (units/mL). *** $P < 0.001$, * $P < 0.05$.

FIGURE 5.3


A
0 weeks
2 weeks
4 weeks
6 weeks
2000
1000
0
Flu
1° 2°
Control
α-lactalbumin
β-lactoglobulin
lactoferrin
B
7500
5000
2500
0
TT
C
3000
2000
1000
0
Dip
Antibody Units (units/mL)
Whey Protein

5.3.3 The effect of dietary whey proteins on mitogen-induced lymphocyte proliferation

To evaluate the effect of dietary α-lactalbumin, β-lactoglobulin and lactoferrin on lymphocyte function, mitogen-induced splenic lymphocyte proliferation was measured. Dietary α-lactalbumin, β-lactoglobulin and lactoferrin had no significant effect on the Con A (T cell mitogen), LPS (B cell mitogen) and PHA (T cell mitogen)-induced lymphocyte proliferation compared to control mice fed pellets over 6 weeks of dietary treatment (Figure 5.4).

5.3.4 The effect of dietary whey proteins on the phagocytic activity of murine peritoneal cells and peripheral blood cells

To evaluate the effect of dietary α-lactalbumin, β-lactoglobulin and lactoferrin on phagocytic function, the phagocytic ability of both peritoneal cells and peripheral blood cells was measured. Dietary α-lactalbumin, β-lactoglobulin and lactoferrin had no significant effect on the phagocytic activity of peritoneal cells or peripheral blood cells compared to control mice fed pellets over 6 weeks of dietary treatment (Figure 5.5).

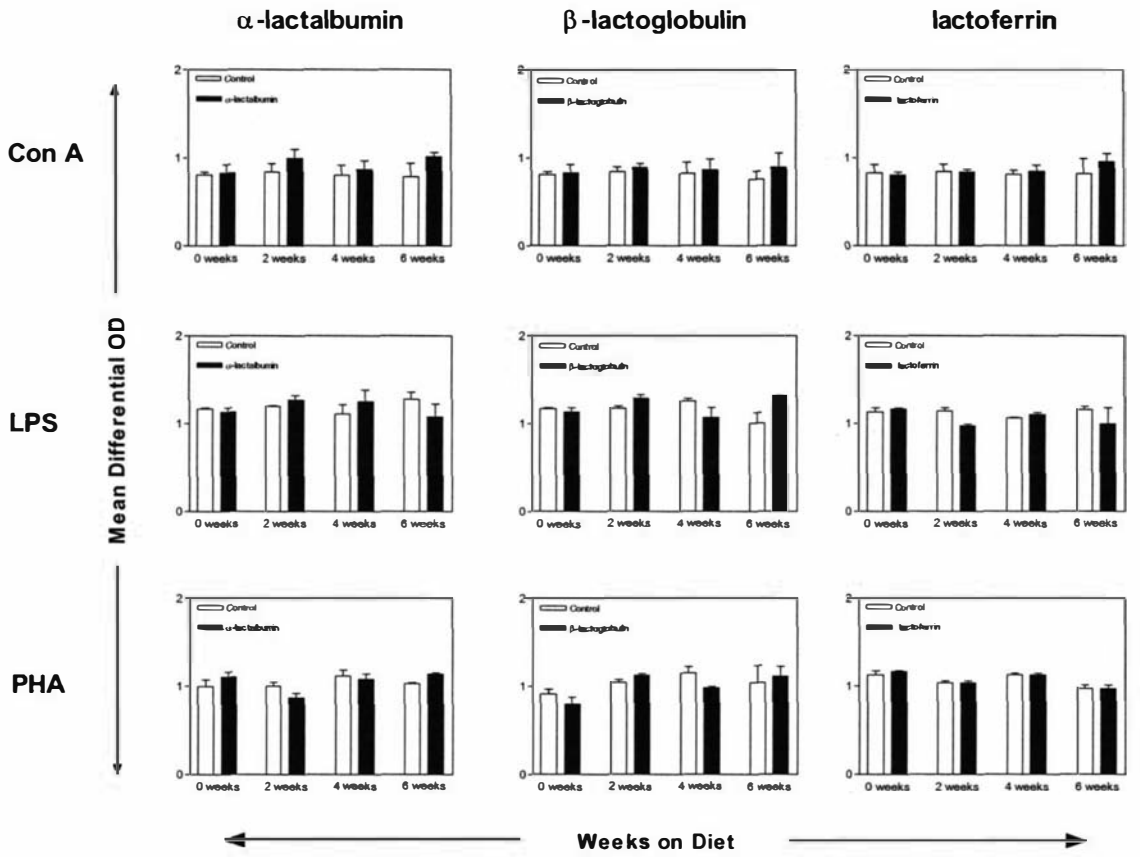


Figure 5.4 Dietary α-lactalbumin, β-lactoglobulin and lactoferrin had no significant effect on Con A, LPS or PHA-induced lymphocyte proliferation. Mice were fed α-lactalbumin, β-lactoglobulin, lactoferrin or pellets for 0, 2, 4 or 6 weeks. At the end of each feeding regime, the proliferative responses of murine spleen cells to Con A, LPS or PHA were quantified using a colourimetric assay based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation. Each value represents the mean differential OD ($OD_{MITOGEN}$ - $OD_{NO\ MITOGEN}$) ± SEM (n = 8).

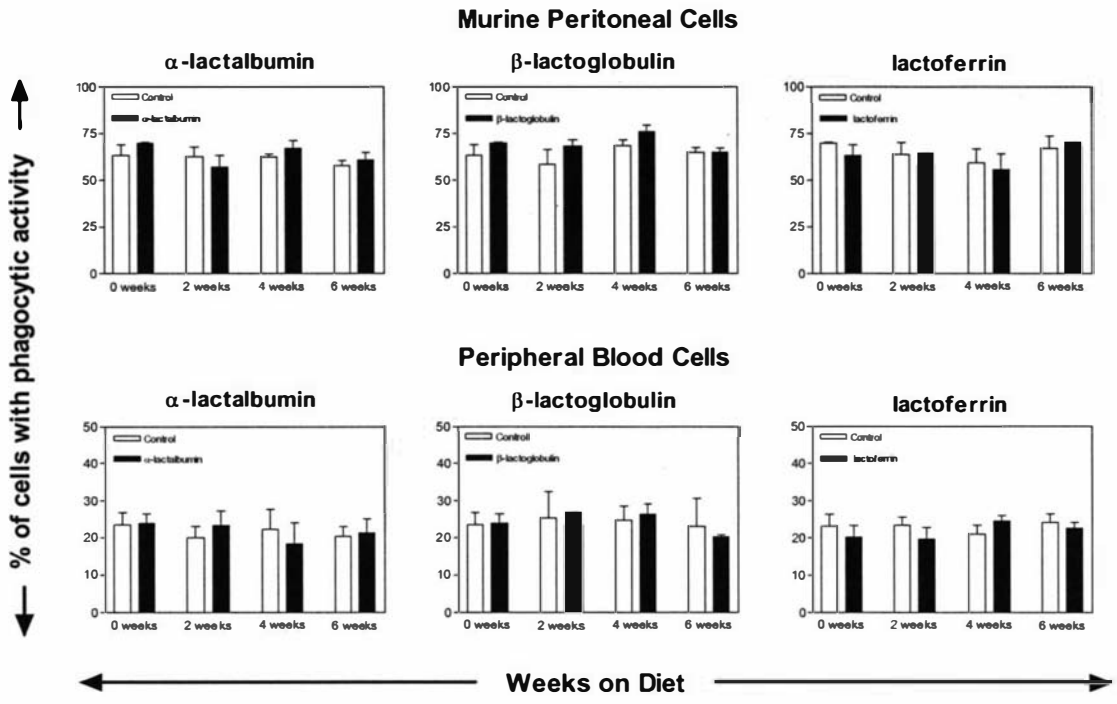


Figure 5.5 Dietary α-lactalbumin, β-lactoglobulin and lactoferrin had no significant effect on the phagocytic activity of murine peritoneal or peripheral blood cells. Mice were fed α-lactalbumin, β-lactoglobulin, lactoferrin or pellets for 0, 2, 4 or 6 weeks. At the end of each feeding regime, the percentage of peritoneal cells or peripheral blood cells with the ability to phagocytose FITC-labelled *E. coli* was determined using a FACSCalibur flow cytometer. Each value represents the mean percentage of macrophages with phagocytic ability ± SEM (n = 8). (Note: Due to differences in the ratio of phagocytes to target cells (FITC-labelled *E. coli*) in the *ex vivo* phagocytosis assay implemented in this chapter, the base line (control) phagocytic activity was recorded at approximately 60% while the *in vitro* phagocytic assay implemented in Chapter 4, Section 4.3 recorded a base line (control) phagocytic activity of 30%. In both chapters the phagocytic activity of the test groups were evaluated in comparison to their respective control groups.)

5.3.5 Murine splenic weight over 6 weeks of dietary treatment

To evaluate the effect of α-lactalbumin, β-lactoglobulin and lactoferrin on splenic weight, the spleen weight of mice fed dietary whey proteins for 0, 2, 4 or 6 weeks was recorded and compared to the control animals. Dietary α-lactalbumin, β-lactoglobulin and lactoferrin had no significant effect on the spleen weight of mice fed for 0, 2, 4 or 6 weeks compared to control mice fed pellets (Table 5.3).

TABLE 5.3

Dietary whey protein has no significant effect on spleen weight

Weeks on Diet	Diet					
	α-lactalbumin	Control	β-lactoglobulin	Control	Lactoferrin	Control
	Spleen Weight (grams)[1]					
0	0.098 ± .004	0.092 ± .004	0.095 ± .004	0.923 ± .004	0.089 ± .002	0.086 ± .003
2	0.106 ± .004	0.099 ± .004	0.099 ± .006	0.107 ± .003	0.090 ± .004	0.087 ± .005
4	0.108 ± .004	0.102 ± .004	0.107 ± .006	0.114 ± .005	0.098 ± .006	0.083 ± .007
6	0.113 ± .003	0.108 ± .002	0.114 ± .003	0.111 ± .003	0.105 ± .004	0.094 ± .005

[1] Results represented as mean ± SEM (n = 8)

5.4 DISCUSSION

Previous studies have shown that dietary whole whey protein concentrates (Wong & Watson, 1995) or hydrolysate-rich whey protein isolate preparations (such as lactalbumin) have immunomodulatory properties in mice (Bounous *et al.*, 1981; Bounous *et al.*, 1989a; Parker & Goodrum, 1990). However, the results of this chapter are the first to demonstrate conclusively that purified whey protein isolates can affect immune function when incorporated into a diet. In this study dietary bovine α-lactalbumin, β-lactoglobulin and lactoferrin were found to enhance specific humoral antibody responses against systemically and orally administered antigens *in vivo*.

The results of this chapter demonstrate that the consumption and subsequent digestion of whey proteins can markedly enhance specific humoral antibody responses beyond a basal immune response *in vivo*. This enhancement may be due, in part, to the fact that α-lactalbumin and β-lactoglobulin are readily digestible and offer a supply of essential amino acids (Vorland, 1999). This notion is supported by Bounous & Kongshavn (1989) who found that the plasma amino acid profile of mice fed a whey protein diet essentially conforms to the amino acid profile of the ingested protein. Hence, changes in the amino acid plasma profile in the body (murine), resulting from the digestion of whey proteins, may promote a more effective humoral response. In addition, protein transport studies conducted by Takada *et al.* (1996) found that the inner solution of an everted gut-sac (made from rat small intestine) incubated in a solution of intact whey protein (prepared from WPC by dialysis), pepsin-digested whey protein or pepsin/pancreatin-digested whey protein stimulated the proliferation (^{3}H-thymidine incorporation) of an osteoblastic cell line *in vitro*. This finding suggests that the immunomodulatory components in the whey protein can permeate or be absorbed by the intestines (Takada *et al.*, 1996). The enhancement of both the mucosal and systemic immune responses in the present study by dietary whey proteins suggests that these whey protein components are not only readily incorporated into the physiological system of the mouse but may also modulate the localised gut-associated lymphoid tissue (GALT) immune response.

The results of this chapter concur with previous reports that dietary protein type can influence humoral immune responsiveness. Lactalbumin (a denatured bovine milk

protein, ICN), has been shown by Parker & Goodrum (1990) to have significant immune-enhancing properties in mice when compared to dietary casein and soy. After immunisation with sheep erythrocytes, the number of IgM plaque-forming cells was found to be 1.5 times greater in mice fed lactalbumin compared to mice fed casein (Parker & Goodrum, 1990). Increases in the production of B lymphocytes or the processes leading to the activation of B cells (such as an increase in lymphokines secreted by helper T cells) may have contributed to the observed enhanced humoral immune responses of whey protein-fed mice. Bounous *et al.* (1985) investigated the possibility that lactalbumin was either directly affecting B-lymphocyte development in the bone marrow or that lactalbumin was affecting B-lymphocyte proliferation *in situ* in lymphoid tissue prior to immunisation. However, results showed that there was no change in B-cell ontogeny in lactalbumin-fed mice (Bounous *et al.*, 1985) suggesting that the effect on B cells may be in the events of antigen processing and presentation, or cytokine production, or immunoglobulin secretion. Although the underlying cause and mechanism of immune-enhancement by dietary whey proteins remains to be elucidated, there are considerable differences in the relative concentration of some of the amino acids found in whey protein components compared to casein or soy proteins (Bounous & Amer, 1988; Parker & Goodrum, 1990). It is possible that any one amino acid at the concentration found in whey proteins components may be responsible for the immunoenhancing effects seen. Alternatively the ratio of essential amino acids or the metabolic products of whey protein component degradation could be responsible for observed changes in the immune systems of whey protein-fed mice. A study by Bounous *et al.* (1989a) determined that the cysteine levels in lactalbumin contribute to the immune enhancing effect of lactalbumin by providing this precursor for the synthesis of glutathione. Glutathione plays an important role in the stability of cell membranes and in its reduced state protects cells from the effects of oxidative damage by radiation and oxygen radicals (Meister & Anderson, 1983). Glutathione is also critical to the functional state or activation of many cells including T and B lymphocytes (Noelle & Lawrence, 1981; Fidelus & Tsan, 1987). The anti-oxidant property of glutathione makes it an important factor in the optimal immune function of both macrophages and lymphocytes. However, cysteine alone may not be totally responsible for the observed immune-enhancement. While the present study did not find a significant effect of dietary whey protein on splenic weight (a major lymphomyeloid organ) or *ex vivo* T-cell function (Con A or PHA stimulated proliferation), Parker &

Goodrum (1990) did find an effect of dietary whey protein on T cells by observing an increase in the thymic weight in mice fed lactalbumin compared to mice fed casein. This may contribute to the mechanism of an enhanced immune response in lactalbumin-fed mice by increasing the number of T lymphocytes able to respond to antigen. Humoral immune enhancement by dietary whey proteins may also be due, in part, to a significant increase in macrophage secretory products such as lysozyme and complement C3 (important in the initial activation of the B lymphocytes (Klaus & Humphrey, 1986)) secreted by peritoneal macrophages in lactalbumin fed mice compared to casein fed mice after 2 weeks (Parker & Goodrum, 1990). Further investigation into the possible causes and mechanisms of the immune-enhancing activity of dietary bovine whey proteins is required.

Debabbi *et al.* (1998) have previously shown that repeated oral-administration of lactoferrin (1 mg/g body weight per day) can enhance the biosynthesis of lactoferrin-specific A and G isotype antibodies in both intestinal secretions and serum of mice compared to controls. Total immunoglobulins were also higher in the intestinal fluid of mice given lactoferrin (orally or intramuscularly) compared to controls (Debabbi *et al.*, 1998). The present study has shown that whole dietary lactoferrin (1% w/w) can significantly enhance specific mucosal and systemic antibody responses (whole Ig) to orally and parenterally-administered vaccines respectively. These findings are in agreement with Miyauchi *et al.* (1997) who found the level of specific IgA (measured by ELISA) against orally-administered cholera toxin in the bile and intestinal contents of mice fed liquid diets containing a bovine lactoferrin pepsin hydrolysate (1% w/volume) was greater than those of mice fed control diets (amino acid mixture, casein hydrolysate). Furthermore, human lactoferrin at concentrations ranging from 1-10 μg/mL has been found to stimulate (up to 5 times) the humoral immune response (number of plaque-forming cells) to SRBC when injected (intraperitoneally) into mice 3 hours before immunisation (Zimecki *et al.*, 1991). Lactotransferrin-treated thymocytes, given intravenously into mice, have also been shown to enhance the immune response to SRBC to the same extent as interleukin-1 and induce *in vitro* $CD4/CD8^-$ thymocytes to mature into the $CD4^+$ helper phenotype (Zimecki *et al.*, 1991) suggesting that lactotransferrin stimulates the immune response by a process which involves the promotion of T-cell differentiation. These findings are in disagreement with the *in vitro* data of Duncan & McArthur (1981) who showed that iron-saturated and native human

lactoferrin (10^{-10} to 10^{-6} M) significantly decreased the number of direct (IgM) plaque-forming cells to a T-dependent antigen (sheep erythrocytes) and a T-independent antigen (trinitrophenolated *Brucella abortus*) when included in culture with murine splenic cells. The exposure of isolated macrophage-rich but not lymphocyte-rich populations to lactoferrin resulted in a significant decrease in the anti-sheep erythrocyte response (Duncan & McArthur, 1981). Thus in the *in vitro* system of Duncan & McArthur (1981), lactoferrin (which is synthesised and released by polymorphonuclear leucocytes and present in inflammatory lesions) may play a role in modulating the antibody response through an effect on the macrophage cell fraction rather than on lymphocytes directly.

Previous studies have reported that bovine lactoferrin can stimulate *in vitro* murine lymphocyte proliferation and immunoglobulin production (Hashizume *et al.*, 1983; Debabbi *et al.*, 1998). Administered orally (1 mg/g body weight per day), bovine milk lactoferrin was found to significantly increase the mitogen-induced proliferation of Peyer's patch and spleen cells (Debabbi *et al.*, 1998) compared to control mice. These findings suggest that lactoferrin may interact with B and/or T cells in mucosal inductive sites (eg. Peyer's patches) and possibly in systemic lymphoid tissue (eg. spleen). Actor *et al.* (2002) demonstrated that subcutaneous immunisation with lactoferrin enhanced (up to 17 fold) delayed type hypersensitivity responses to SRBC in a dose dependent manner, with maximum enhancement occurring at 50 mg/dose and 250 mg/dose. In the same study, murine macrophages (peritoneal and J774A.1 cell line) stimulated with lactoferrin showed an increase in tumour necrosis factor-α protein production and an up-regulation of interleukin-12 and interleukin–15 mRNA. Levels of chemokines also increased in a dose-dependent manner. Taken together these results suggested that lactoferrin (as an adjuvant) may stimulate immune cells to generate a local environment likely to push immune responses towards the development and maintenance of cell-mediated immunity (Actor *et al.*, 2002). To further determine the effect of the whey proteins on lymphoid and non-lymphoid function, spleen lymphocyte proliferation and phagocytic activity were investigated in this chapter. However, no *ex vivo* changes in the lymphocyte proliferative potential (Figure 5.4) or phagocytic activity of peritoneal and peripheral blood cells (Figure 5.5) were detected in mice after dietary treatment with these whey proteins. This result is consistent with Bounous *et al.* (1989a) who also found that dietary protein type had no significant effect on lymphocyte-mediated

immune responses such as splenic cell proliferative response to mitogens (PHA and Con A) and DTH reactions. However, the α-lactalbumin, β-lactoglobulin and lactoferrin were found here to have stimulating effects on lymphocyte proliferative potential and phagocytic ability *in vitro* (Chapter 3 and 4). Such findings support the hypothesis that the immunomodulatory effect of whey proteins on specific immune functions can differ between *in vitro* and *in vivo* studies. The differences observed in the effects of the whey proteins on lymphocyte and phagocytic function *in vitro* and *in vivo* may be due to several reasons which are also discussed in Chapter 9 (General Discussion). For example, the complex interaction of all the components of the immune system in an *in vivo* model may alter any direct effects of the whey proteins on isolated immune functions such as cell proliferation and phagocytic activity achieved in *in vitro* studies. Additionally, the end point concentration of whey proteins present in the plasma of animals after digestion may have differed from the concentrations used in the *in vitro* studies (Chapter 3 and Chapter 4). Future studies evaluating the levels and types of the amino acids present in the plasma of mice which have ingested and digested whey proteins compared to control mice are necessary.

No difference in the spleen weights (Table 5.3) of mice fed whey protein-based diets were observed compared to control mice suggesting that dietary whey protein components were nutritionally adequate and had no gross clinical effects on this organ. This finding is comparable with Parker & Goodrum (1990) who found no change in spleen weight between groups of mice fed lactalbumin, casein or soy proteins. Furthermore, preliminary observational evidence from this study indicated no obvious differences in the physical appearance of mice fed whey protein diets compared to control animals. This was expected as previous studies by Bounous & Kongshavn (1982) and Parker & Goodrum (1990) have shown that diets comprising of whey protein components (18 - 20% w/w) were nutritionally adequate and able to provide the required daily support of essential amino acids for normal growth. However, longer-term feeding safety studies investigating the nutritional effects of the whey protein diets in mice using parameters such as total body weight, serum protein, peripheral white blood counts and liver enzyme production as quantitative indicators of normal growth and health are required.

The results presented in this chapter provide clear indications and opportunities for the dairy industry in the processing and marketing of α-lactalbumin, β-lactoglobulin and lactoferrin as valuable ingredients, and provide a rational basis for the development of functional foods based on whey proteins. The immune-enhancing potential of individual whey protein isolates on immune functions (such as T-lymphocyte responsiveness) have been found to be reduced when combined fractions (such as lactoferrin and lactoperoxidase) or a whey protein concentrate (consisting of all whey protein components) were tested *in vitro* (Wong *et al.*, 1997b). This finding suggests that the individual whey protein components may counteract each other with or without influences of other trace factors. However, several other studies have demonstrated that diets containing whey protein concentrate have immunostimulatory (reviewed in Chapter 1) and anticarcinogenic effects in mice and rats (Bounous *et al.*, 1988c; McIntosh *et al.*, 1998). Therefore further investigation into the immunomodulatory properties of dietary whey proteins combined in a whey protein concentrate is warranted.

CHAPTER 6

Influence Of Dietary Whey Protein Concentrate On Mucosal Antibody Responses

Published as: Enhancement Of Mucosal Antibody Responses By Dietary Whey Protein Concentrate. Low PPL, Rutherfurd KJ, Cross ML & Gill HS (2001) *Food and Agricultural Immunology* 13, 255-264.

6.1 INTRODUCTION

During the past decade there has been increased interest in finding new economic uses for whey (Wong *et al.*, 1996c). Among the potential uses, whey proteins have recently been highlighted for their utilisation as functional food ingredients (McIntosh *et al.*, 1998), since several studies have demonstrated a physiological impact of dietary whey consumption (Chapter 5, Bounous *et al.*, 1991; Bounous & Gold, 1991; Kennedy *et al.*, 1995; Smithers *et al.*, 1996; McIntosh *et al.*, 1998). The immune system, in particular, has been shown to be modulated by dietary WPC consumption in both animal models and human studies (Bounous & Amer, 1988; Bounous *et al.*, 1988a; Bounous *et al.*, 1988b; Bounous & Kongshavn, 1989; Wong & Watson, 1995).

Whey protein concentrate comprises major proteins, for example, β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins (Eigel *et al.*, 1984), as well as minor proteins such as lactoferrin and lactoperoxidase. Many of these proteins have been found to possess a variety of bio-active properties including modulation of the immune system (Stoeck *et al.*, 1989; Mincheva-Nilsson *et al.*, 1990; Barta *et al.*, 1991; Wong *et al.*, 1996c; Wong *et al.*, 1997c; Wong *et al.*, 1997b; Monnai *et al.*, 1998). Furthermore, minor changes in dietary amino acid profile, due to consumption of different whey protein mixtures, have been found to modify the immune response without having a significant impact on nutritional status (Bounous & Kongshavn, 1978), suggesting that different WPC preparations can have different effects on the immune system.

Bounous *et al.* (1988a) showed that an α-lactalbumin-based WPC enhanced murine immune responses, including splenic antibody production against parenterally-administered antigen (SRBC). Dietary consumption of immunomodulating WPC has also been found to promote enhanced protection against gastrointestinal tract microbial pathogens (Bounous & Kongshavn, 1989) and tumour development (Smithers *et al.*, 1996; McIntosh *et al.*, 1998) in some animal model systems, reflecting a potential end-point benefit for immunoenhancing activity. Other potential uses for immunostimulatory WPCs include their use as dietary immunopotentiators to enhance key immune functions that are important to health, for example boosting responses to vaccination.

A further study (Wong & Watson, 1995) has provided additional evidence that dietary WPC can enhance antibody responses to systemically-administered antigen in mice, possibly via a mechanism of enhanced lymphocyte activation and cellular function (Bounous & Kongshavn, 1985; Bounous & Kongshavn, 1989; Wong *et al.*, 1996c). Thus, the common paradigm appears to be that some WPCs can potentiate immune activity, in particular B-cell production of antibodies in response to systemic immunisation (Bounous *et al.*, 1985; Wong & Watson, 1995). However, it is currently unclear whether a dietary WPC preparation could enhance local (i.e. gut mucosal) antibody responses, especially in the case of antigens which are administered via the oral route. This is an important consideration for the potential development of immunopotentiating functional foods based on WPCs, since several human vaccines are administered orally.

Cholera toxin, one of the most potent mucosal immuno-adjuvants identified to date, has been shown to enhance the antigenicity of relatively poor mucosal immunogens, such as the influenza virus (Mestecky *et al.*, 1994), when co-delivered orally (Elson & Dertzbaugh, 1994). A combination of cholera toxin and a protein based T-dependent soluble antigen, such as ovalbumin, is therefore a good model to study mucosal immuno-responses (Elson & Dertzbaugh, 1994). The objective of this study was to first determine whether dietary WPC could modulate mucosal antibody responses to cholera toxin and ovalbumin co-administered orally to mice. Secondly, to determine whether altered patterns of mucosal immuno-responses by WPC could be reflected by systemic indices of lymphocyte activation, such as phenotype expression in blood cells and spleen cell proliferative responses. The immunomodulatory potential of a cheese-derived WPC (ALACEN 472, containing glycomacropeptide) will be evaluated for the first time in this study. While the main focus of this study was to investigate the effect of this dietary WPC on mucosal antibody responses, as an adjunct to this, the underlying patterns of lymphocyte responses (proliferative response to mitogens) were also measured and circulating lymphocyte populations characterised (phenotyping). Future studies (Chapter 7 and 8) will examine the effect of dietary whey protein concentrates on both systemic and mucosal immune responses.

6.2 MATERIALS AND METHODS

6.2.1 Mice and diets

Male 6-7-week-old BALB/c mice were housed in pairs at 22 ± 1°C under a 12 hr light-dark photo-period and offered feed and water *ad libitum*. Control animals were fed a standard mouse pellet diet (Diet 86, Sharps, Lower Hutt, New Zealand) comprising 18% w/w total protein of the dry weight diet. Test animals were fed a diet containing 20% w/w protein of a WPC (ALACEN 472, New Zealand Dairy Board, Wellington, New Zealand) as the sole protein source. Dietary compositions are shown in Table 6.1. ALACEN 472 is a free flowing, soluble 80% milk protein concentrate, produced via spray-drying of ultra-filtered rennet whey under commercial conditions (M. Pritchard, NZDRI, *pers. comm.*). The microbiological estimates for the whey protein concentrate evaluated in this study are given in Table 6.2.

TABLE 6.1

Composition of Test Diets

	Mouse Chow Diet		**WPC Diet**
Protein	Meat meal Lucerne meal Fish meal Milk powder	6.0% 5.0% 7.0% 1.5%	ALACEN 472 25%
Total Protein Content	**18 g/100 g dry weight**		**20 g/100 g dry weight**
Total Fat Content	**7 g/100 g dry weight**		**7 g/100 g dry weight**

TABLE 6.2

Typical microbiological estimates for the whey protein concentrate evaluated in this study

Whey Protein Concentrate	Standard plate count (non-specific bacteria)	Coliforms	Yeasts & moulds	*S. aureus*	*Salmonella*
ALACEN 472	<30000/g	Neg/g	<50/g	Neg/g	Neg/750 g

Note:
1. All counts calculated from number of colony forming units
2. Negative (Neg)
3. *Staphylococcus aureus (S. aureus)*
4. ALACEN 472 product details obtained from NZDB, Wellington, New Zealand

6.2.2 Experimental designs

Two experiments were conducted. In the first experiment the effect of WPC on mucosal antibody responses was investigated. To elicit antibody responses against cholera toxin and ovalbumin, mice were given a total of 3 oral immunisations comprising cholera toxin (CT) (10 μg/mouse; Sigma, St Louis, MO, USA) and ovalbumin (OV) (1 mg/mouse; CSL, Parkville, Victoria, Australia) in 25 μL 0.5 M NaH_2CO_3 pH 8.2, with 1 week between the first and second doses, and 2 weeks between the second and third doses. A total of thirty-six mice were fed a diet containing WPC (WPC diet; Table 6.1) (18 mice per group) or mouse chow (18 mice per group) for a total of 84 days (12 weeks). To induce a primary immune response, immunisations commenced on day 0, 28 or 56 of the 84-day dietary regime. A schematic representation of the immunisation schedule is shown in Table 6.3. Intestinal washings were obtained from euthanased mice to assess humoral immune response as described in Section 6.2.3.

In the second experiment the effect of WPC on systemic lymphocyte parameters was investigated. A total of ninety-six mice were fed a diet containing WPC (WPC diet) (12 mice per group) or mouse chow (control diet) (12 mice per group) for periods of 28, 56 or 84 days. Blood samples and spleens were obtained after humanely killing the mice following the completion of each feeding regime as described in Section 6.2.4 and Section 6.2.5.

TABLE 6.3

Schematic representation of 3 dose (1°, 2° and 3°) oral immunisation schedule commencing on day 0, 28 or 56 of 84-day dietary regime

	Days After Introduction Of WPC Diet											
Group	**0**	**7**	**14**	**28**	**35**	**42**	**49**	**56**	**63**	**70**	**77**	**84**
0	↑ 1°	↑ 2°		↑ 3°								
28				↑ 1°	↑ 2°		↑ 3°					
56								↑ 1°	↑ 2°		↑3°	

6.2.3 Antibody responses to assess mucosal immune responses to CT and OV

Intestinal fluid samples were first clarified by centrifugation and an ELISA was used to determine mucosal immune responses to CT and OV, as described in Section 2.9 and antibody units (units/mL) assigned as described in Section 2.9.2.

6.2.4 Preparation of immune cell suspensions

Mice were euthanased as described in Section 2.2 and blood collected as described in Section 2.5. Spleens were removed aseptically from mice and single cell suspensions prepared as described in Section 2.3.

6.2.5 Lymphocyte proliferation assay to assess *in vitro* proliferation responses

In vitro proliferation responses of splenic lymphocytes to mitogens were determined using an enzyme-based colourimetric cell proliferation kit (Boehringer Mannheim, Mannheim, Germany) as previously described in Section 2.4.

6.2.6 Analysis of blood leucocyte subsets

Flow cytometric analysis was used for monitoring the expression of $CD4^+$ (helper T cells), $CD25^+$ (activated T cells) and $CD40^+$ (accessory cells and B cells) antigens on blood leucocytes as described in Section 2.10.

6.2.7 Statistical analysis

Significant differences between the experimental and control groups were determined using ANOVA and Tukey's post-hoc test (SAS ver. 6.12, Statistical Analysis Systems Institute Incorporated, Cary, NC, USA). Values of $P < 0.05$ were considered significant.

6.3 RESULTS

6.3.1 The effect of dietary WPC on mucosal antibody responses to cholera toxin and ovalbumin

The effect of feeding WPC on specific mucosal antibody responses to CT and OV is shown in Figure 6.1. WPC-fed mice exhibited significantly higher mucosal antibody responses to both cholera toxin and ovalbumin than chow-fed mice, post-immunisation ($P < 0.05$) (Figure 6.1). Enhanced antibody responses among WPC-fed mice were observed regardless of the time point of the first immunisation, i.e. at the beginning (day 0), during the early stage (day 28) or in the middle (day 56) of the 86 day dietary regime.

6.3.2 The effect of dietary WPC on lymphocyte proliferation responses and phenotype expression

To evaluate the effect of feeding WPC on systemic lymphocyte parameters, proliferative responses of spleen cells to the T cell mitogen Con A, the B cell mitogen LPS and the T cell-dependent B cell mitogen PWM were measured; and blood lymphocyte phenotype expression was determined. There were no significant differences in the spleen cell proliferative responses to mitogens between mice fed WPC or mouse chow, and no significant differences in mitogen responses were seen over different lengths of feeding regime ($P > 0.05$) (Figure 6.2). Similarly, no significant differences in the percentage of lymphocyte populations labelling positively for CD4, CD25 or CD40 were observed between mice fed WPC or mouse chow ($P > 0.05$) (Figure 6.2).

FIGURE 6.1

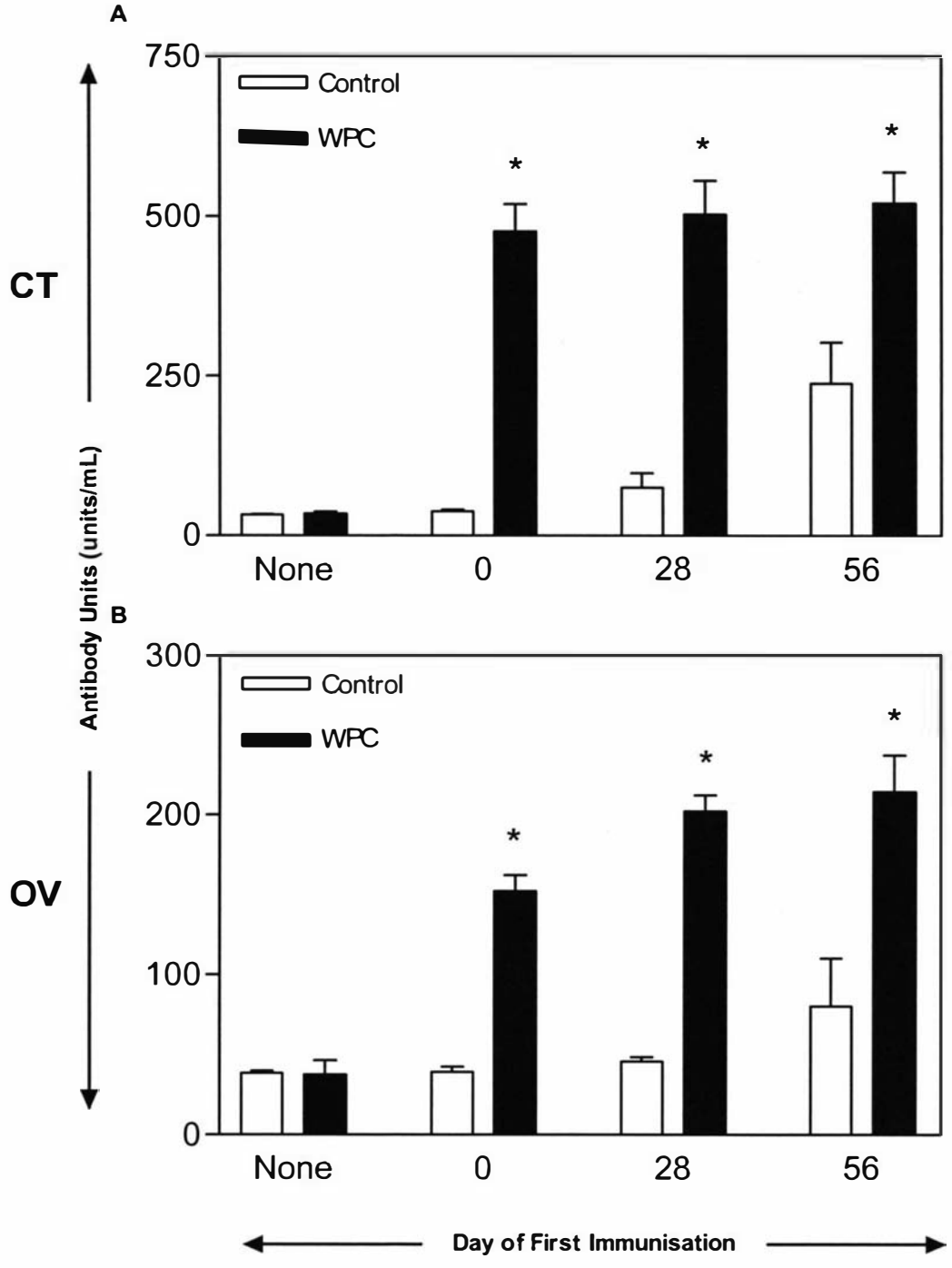

A
750
500
250
0
Control
WPC
*
*
*
CT
None
0
28
56
Antibody Units (units/mL)
B
300
200
100
0
Control
WPC
*
*
*
OV
None
0
28
56
Day of First Immunisation

FIGURE LEGENDS

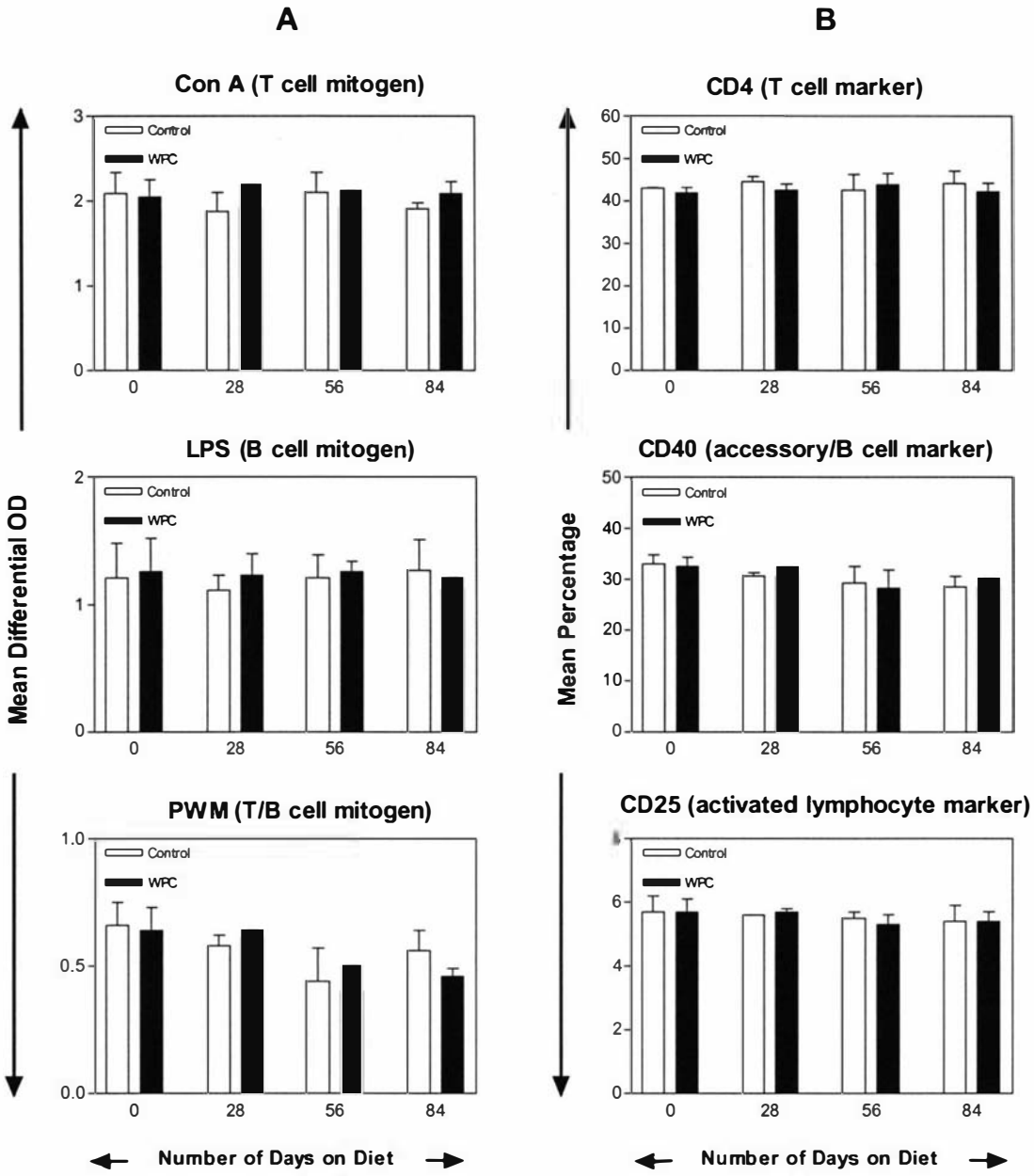
Figure 6.1 Feeding WPC for 84 days enhanced gut mucosal antibody responses to oral immunisation with cholera toxin (A) and ovalbumin (B) compared to controls. Three oral immunisations with cholera toxin and ovalbumin were administered with 1 week between the first and second doses and 2 weeks between the second and third doses, commencing on day 0, 28, or 58 of the dietary regime. Antibody responses were assessed using an enzyme-linked immunosorbent assay (ELISA) on day 84 of the dietary regime. $*P < 0.05$ between WPC-fed and control (chow-fed) mice.

Figure 6.2 Dietary WPC had no significant effect on the proliferative responses of murine spleen cells to T and B cell mitogens (A), or on murine blood lymphocyte subsets (B) compared to controls. Mice were fed chow- or WPC-based diets for periods of 28, 56 or 84 days. For each length of feeding regime, the proliferative responses of murine spleen cells to mitogens Con A, PWM or LPS were quantified using a colourimetric assay based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation. Each value represents the mean differential OD ($OD_{MITOGEN} - OD_{NO\ MITOGEN}$) ± SEM. Murine blood lymphocyte subsets were determined by labelling whole blood samples with fluorochrome-conjugated monoclonal antibodies specific for murine CD4 (T cell marker), CD25 (activated lymphocyte marker) or CD40 (accessory cell/B cell marker). The percentage of positively labelled lymphocytes was measured by flow cytometry. Each value represents the mean percentage ± SEM. (n=12) There were no significant differences in lymphocyte proliferative responses or percentage of positively-labelled leucocyte sub-sets between chow- and WPC-fed mice ($P > 0.05$).

Addendum to Fig 6.1

Samples were obtained from intestinal washings as described in Section 6.2.3. The results are expressed as the mean ± SEM (n=18) antibody units (units/mL).

FIGURE 6.2


A
B
Con A (T cell mitogen)
CD4 (T cell marker)
LPS (B cell mitogen)
CD40 (accessory/B cell marker)
PWM (T/B cell mitogen)
CD25 (activated lymphocyte marker)
Control
WPC
Mean Differential OD
Mean Percentage
0
28
56
84
Number of Days on Diet

6.3.3 The effect of long term feeding of dietary WPC on live body weight increase and spleen weight

To monitor the nutritional effects of the long term consumption of dietary WPC, live body weight increase and spleen weights of mice were measured every 14 days (2 weeks) for 84 days (12 weeks). The weight increase (final weight - initial weight) of mice fed dietary WPC was not statistically different from mice fed pellets (control) at every time point measured in this study (Figure 6.3 A). Similarly, dietary WPC had no significant effect on the spleen weight of mice compared to control animals over 84 days (12 weeks) of dietary treatment (Figure 6.3 B). Observational evidence indicated no obvious differences in the physical appearance of mice fed whey protein diets compared to control animals.

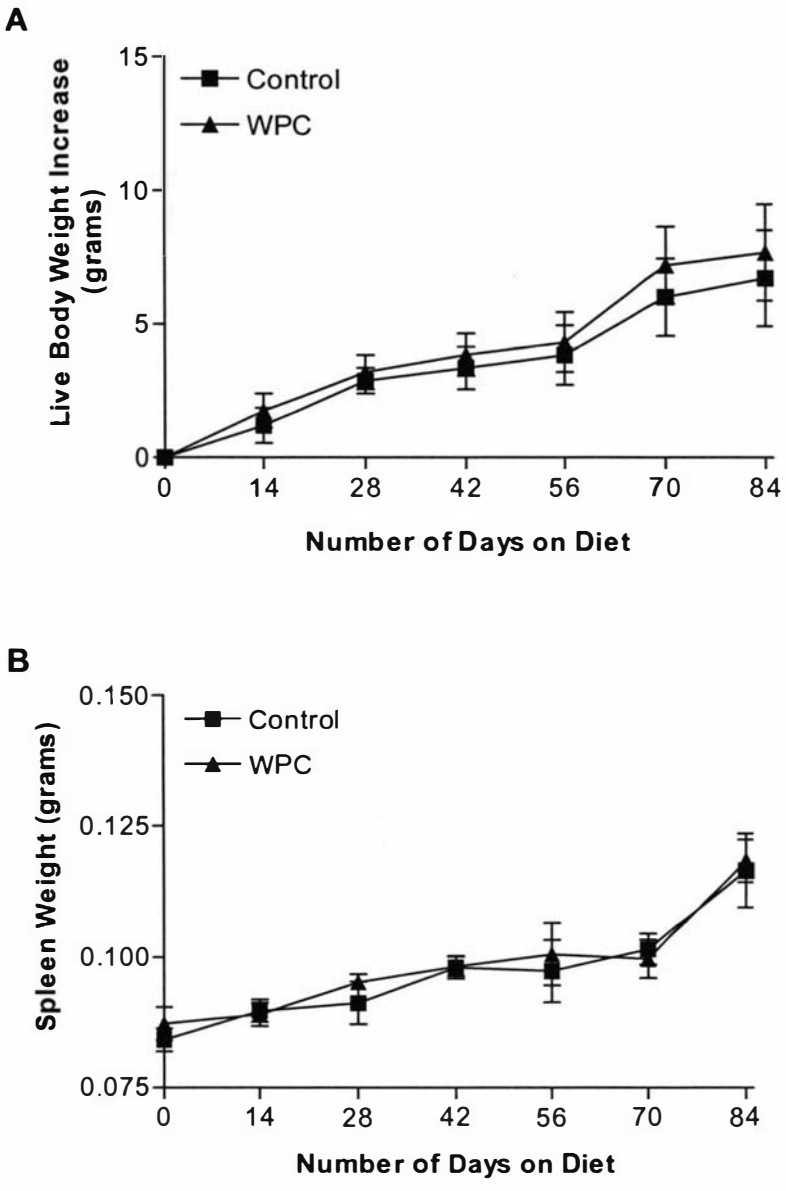


Figure 6.3 Dietary WPC has no significant effect on live body weight increase (A) or spleen weight (B) compared to control animals. The increase in live body weight (final weight - initial weight) (**A**) and spleen weight (**B**) of mice fed dietary WPC was measured every 14 days (2 weeks) for 84 days (12 weeks) and compared to animals fed a pellet diet (control). Results are expressed as mean ± SEM (n = 12).

6.4 DISCUSSION

Bounous & Kongshavn (1982) and Wong & Watson (1995) have previously shown that dietary WPC can enhance serum antibody responses to systemically-administered antigens in mice. The present study is the first to show that dietary WPC can significantly enhance gut mucosal antibody responses to orally-administered antigens. Furthermore, the WPC utilised here (ALACEN 472) was able to enhance mucosal antibody responses to orally-administered CT and OV in healthy mice, irrespective of when immunisations commenced throughout a 12-week feeding regime (Figure 6.1). Previously, dietary supplementation using amino acids or immunoregulatory strains of lactic acid bacteria have been shown to be an effective strategy for enhancing gut mucosal antibody responses to orally-delivered antigens (Kobayashi *et al.*, 1998; Takahashi *et al.*, 1998). However, the results presented in this chapter are the first to describe an ability of milk whey protein to enhance these types of responses. In contrast to these results, Monnai *et al.* (1998) demonstrated that orally-fed milk CGP could suppress, rather than enhance, circulating IgG responses to orally-administered ovalbumin, although the authors did not investigate the effect of CGP on mucosal responses in their study.

The results of this chapter are in agreement with previous reports that dietary whey protein concentrate can significantly enhance humoral immunity in mice. Dietary WPC has been shown by Bounous & Kongshavn (1982) to enhance antibody responses to a systemically administered T-dependent antigen (SRBC), and to increase protection against salmonellosis (Bounous *et al.*, 1981). Specific humoral immune responses to SRBC among mice fed whey proteins were found to be almost five times higher than those of mice fed casein (Bounous *et al.*, 1983a). Furthermore, ingestion of bovine milk whey proteins by mice consistently enhanced secondary humoral antibody responses following systemic immunisation with ovalbumin, when compared with other protein sources such as soybean protein isolate and ovine colostral whey proteins (Wong & Watson, 1995). The results of this chapter have shown that ALACEN 472 can increase murine gut mucosal antibody responses to orally administered antigens by 2 - 5 fold, indicating that this WPC has potent immunoenhancing capabilities on the mucosal immune system. Whole immunoglobulin antibody responses were measured in this study, therefore it remains unclear whether these enhanced responses were due

primarily to a stimulation of IgA and/or IgG antibody production. In previous studies on the immunoenhancing effects of dietary WPC, increased plaque-forming (IgM/IgG) cell-responses to SRBC were observed after 2 weeks and persisted for at least 8 weeks after dietary treatment (Bounous & Amer, 1988). In this study enhanced mucosal immunity was observed while feeding WPC for a total of 84 days (12 weeks). This long term feeding demonstrates that the immunoenhancing effects of dietary WPC is not short lived and may be expressed as long as the protein is ingested. Furthermore, throughout this long term study no significant differences in physical appearance, live body weight gain and splenic weight were observed between mice fed WPC and control mice (Figure 6.3). Such results indicate that a WPC based diet is not only immune enhancing but also nutritionally adequate and able to provide the daily support of amino acids required for normal murine growth.

The results of this chapter demonstrate that a dietary WPC preparation can enhance local (gut mucosal) antibody responses following antigen administration via the oral route, therefore, to determine if this was due to a systemic immunoenhancing effect of WPC on lymphocyte function, several lymphocyte parameters were investigated. However, no changes in lymphocyte proliferative potential or major phenotype expression were detected in mice fed WPC compared to mice fed chow. The absence of significant variations in immunoactive leucocyte sub populations (CD4, CD25, CD40) implies that, in this case, dietary WPC did not significantly alter the cellular composition or activation status of the major immune cell populations that contribute to humoral immunity (Figure 6.2). In common with this result, previous studies have also reported no significant effect of WPC on cell-mediated immune responses, including murine splenic T-cell mitogenesis (Bounous *et al.*, 1981; Bounous & Kongshavn, 1982; Bounous *et al.*, 1983a; Bounous *et al.*, 1983b; Bounous & Kongshavn, 1985; Bounous *et al.*, 1988a). In contrast, however, Wong & Watson (1995) demonstrated that mice fed a WPC-supplemented diet for 6 weeks had enhanced proliferative responses to the T cell mitogen Con A. These results re-enforce the suggestion that different WPC preparations can enhance the immune system in different ways. As proposed by Wong & Watson (1997), whey is a complex mixture of immunoregulatory components. Consequently, different WPC preparations are likely to have subtly different immunomodulatory effects, and thus the thorough characterisation of WPCs is warranted in research on immuno-active foodstuffs.

Bounous *et al.* (1985) have suggested that the effects of altered dietary protein type on humoral immune responsiveness is due to changes either in the functional responsiveness of the B lymphocytes themselves or in the processes leading to their activation and differentiation in the peripheral lymphoid tissues. In this study, ALACEN 472 enhanced gut antibody responses without measurable effects on the systemic lymphocyte parameters that are relevant to antibody production (i.e. lymphocyte proliferative potential, increased helper T-cell and B-cell proportions, or activation). Therefore, the possible mode of action of this WPC may be on the GALT system primarily, and the measurement of systemic events may thus not have detected any compartmentalised effects of dietary WPC on the GALT. Alternatively, it is possible that this WPC may primarily affect the afferent arm of the acquired immune response, such as antigen uptake and processing, rather than efferent responses. Further investigation of the effects of dietary ALACEN 472 on localised GALT immune responses is required.

Previous studies have shown that WPCs can enhance immunity and consequently increase protection against tumour development/progression, in both clinical studies (Bounous *et al.*, 1991; Kennedy *et al.*, 1995) and animal models (Papenburg *et al.*, 1990; Smithers *et al.*, 1996; McIntosh *et al.*, 1998), and can promote enhanced resistance to infectious pathogen challenge (Bounous *et al.*, 1981). The WPC used here enhanced mucosal antibody responses to orally-administered antigens, thus suggesting that it may be of use in human health as a dietary adjuvant or immunopotentiator to increase responses to other orally-administered vaccines such as poliomyelitis virus (Mestecky *et al.*, 1994). Consequently, dietary WPC may be beneficial in those groups who respond poorly to oral vaccines, such as the elderly and malnourished infants. Whey proteins contain high levels of constituent cysteine and glutamylcysteine groups which are important in the synthesis of glutathione, a natural antioxidant with advantageous physiological effects (Regester *et al.*, 1996). Supplementation with dietary WPC may therefore be beneficial in a dietary formulation to optimise/enhance immunocompetence and health. Clearly there is already a strong case for the use of whey proteins in nutritional (de Wit, 1998) and functional foods (Sadler, 1992; Regester *et al.*, 1996; Smithers *et al.*, 1996; Hilliam, 1998; Korhonen *et al.*, 1998; McIntosh *et al.*, 1998). The production of whey proteins for human and animal consumption may provide an economic way to utilise whey and take dual advantage of its nutritional and

immunoregulatory properties. Further studies on the immunoregulatory properties of whey proteins, including the effect of whey protein concentrate on systemic antibody responses, will no doubt be invaluable in revealing the full potential of bovine whey proteins.

CHAPTER 7

Short-term effects of feeding a novel WPC and a novel WPC enriched with lactoferrin on immune responses in mice

7.1 INTRODUCTION

In recent decades, interest has grown in improving the biological efficacy of whey proteins in infant formulae and in dietetic and health foods beyond its nutritional value (Regester *et al.*, 1996; McIntosh *et al.*, 1998). For example, Immunocal® (Immunotec Research, USA), a powdered non-denatured whey protein dietary supplement, is currently available claiming to benefit body processes such as immune function, liver function, wound healing, creation of lean muscle mass and cancer treatment by providing glutathione precursors (Kennedy *et al.*, 1995; Bounous, 2000). Hence, the use of bovine whey protein in such dietary supplements and infant formulae, not only as a nutritional source, but also as functional food (especially to enhance immune function) is currently strong and is further strengthened by the findings of previous Chapters presented in this thesis.

Most commercial infant formula preparations contain about 1.5% w/w protein formulated from bovine milk, that itself contains about 3% protein w/w (Janas *et al.*, 1985). However, human breast milk contains only about 1% w/w protein (Jenness, 1979). The differences between human and bovine milks exist not only in the quantity of protein but also in the composition. For example, whey proteins comprise the largest fraction in human milk whereas the largest fraction in bovine milk is casein which contains specific proteins that differ in amino acid content from whey proteins (Hansen, 1974; de Wit, 1998). As the whey-predominant nature of human milk has become apparent, attention has turned to the development of formulae supplemented with bovine whey proteins in an attempt to mimic the composition of human milk (Heine *et al.*, 1991). Balmer *et al.* (1989) showed that the use of a whey-predominant formula, rather than a casein-predominant formula, induced a faecal flora generally closer to that of breast-fed babies. Furthermore, the whey component of human milk contains more lactoferrin than is found in cow's milk (Hansen, 1974). Bovine whey protein formulae manufactured for human consumption can therefore be supplemented with exogenous lactoferrin (de Wit, 1998). Little is currently known about the immunomodulatory effects resulting from the addition of lactoferrin to a dietary whey protein based formula, however, in addition to possessing bactericidal properties and immunoregulating iron binding properties (Reiter *et al.*, 1975; Reiter, 1985; De Sousa *et al.*, 1988; Lonnerdal, 1996), lactoferrin alone is known to be a unique polyfunctional

protein that influences cell proliferation (Chapter 3) and differentiation, regulates granulopoiesis and DNA synthesis in some cells (Hutchens *et al.*, 1991; He & Furmanski, 1995), activates the non-specific immune response by stimulating phagocytosis (Chapter 4) and enhances specific antibody responses *in vivo* (Chapter 5). Furthermore, lactoferrin has been found to improve the immune competence of cells from patients with systemic inflammatory response syndrome (Adamik *et al.*, 1998).

An important aspect of any potential immune-enhancing dietary component is its ability to augment existing defence mechanisms. For example, the specific antibody responses that are produced following vaccination have been shown elsewhere to be enhanced by such varied dietary additives as micronutrient vitamins and minerals (Chandra, 1992) and probiotic micro-organisms (Isolauri *et al.*, 1995). At an experimental level, bovine whey proteins have also been shown to augment specific antibody responses in animal studies. Dietary treatment with WPC has been shown to increase antibody production in mice following both parenteral (Wong & Watson, 1995) and oral immunisation (Low *et al.*, 2001) using T-dependent antigens. Further, individual proteins isolated from bovine whey are known to impact on specific immunoresponses; Debabbi *et al.* (1998) reported that mice fed the minor whey protein lactoferrin exhibited increased antigen-specific lymphocyte responses in both gastrointestinal tract and systemic lymphoid tissues. There is thus the strong possibility that bovine whey proteins could offer promise as dietary additives specifically to augment antigen-primed humoral antibody responses in humans. However, for such a regime to have practical value, it must be proven effective with known vaccine antigens, and must be shown to impart significant influence within the relatively short-time span of a vaccination regime (which typically employs a sequential prime-and-boost approach to establish high antibody titres (Roitt, 1996)). Therefore, the present study has sought to determine the impact of WPC on shorter-term antibody responses, generated in mice following experimental immunisation with a range of commonly-used T-dependent vaccine antigens. Based on the existing knowledge that lactoferrin can also impact on murine immunoresponses, this study additionally determined the effect of supplementing WPC with lactoferrin on gut mucosal and systemic antibody production.

7.2 MATERIALS AND METHODS

7.2.1 Mice and diets

Male 6-7-week-old BALB/c mice were housed in pairs at 22 ± 1°C under a 12 hr light-dark photo-period and offered feed and water *ad libitum*. Control animals were fed a ground milk-free mouse pellet diet (Diet 86, Sharps, Lower Hutt, New Zealand) comprising 18% w/w total protein of the dry weight diet. Test animals were fed a diet containing 20% w/w protein of a WPC (ALACEN 472, NZDB, Wellington, New Zealand) as the sole protein source as described in Chapter 6, Section 6.2.1 or a diet containing 20% w/w protein of a WPC enriched with 1% w/w lactoferrin (LF-enriched WPC) (Tatua Biologics, Morrinsville, New Zealand) as the sole protein source. Dietary compositions are shown in Table 7.1. Microbiological estimates of the whey proteins evaluated in this study are listed in Chapter 6, Table 6.2 for WPC (NZDB); and Chapter 5, Table 5.2 for lactoferrin (Tatua Biologics).

TABLE 7.1

Composition of Diets

	Pellet (control)	WPC	LF-enriched WPC
Protein	Meat meal 6.0% Lucerne meal 5.0% Fish meal 7.0% Soybean meal 1.5%	ALACEN 472 25%	Lactoferrin 1.03% ALACEN 472 24%
Total Protein Content	**18g/100g dry weight**	**20g/100g dry weight**	**20g/100g dry weight**
Total Fat Content	**7g/100g dry weight**	**7g/100g dry weight**	**7g/100g dry weight**

7.2.2 Experimental design and immunisation schedule

A total of 96 mice (8 mice per group) were fed control diets or test diets for a total of 0, 2, 4 or 6 weeks and immunised as described in Chapter 5, Section 5.2.2 (Figure 7.1).

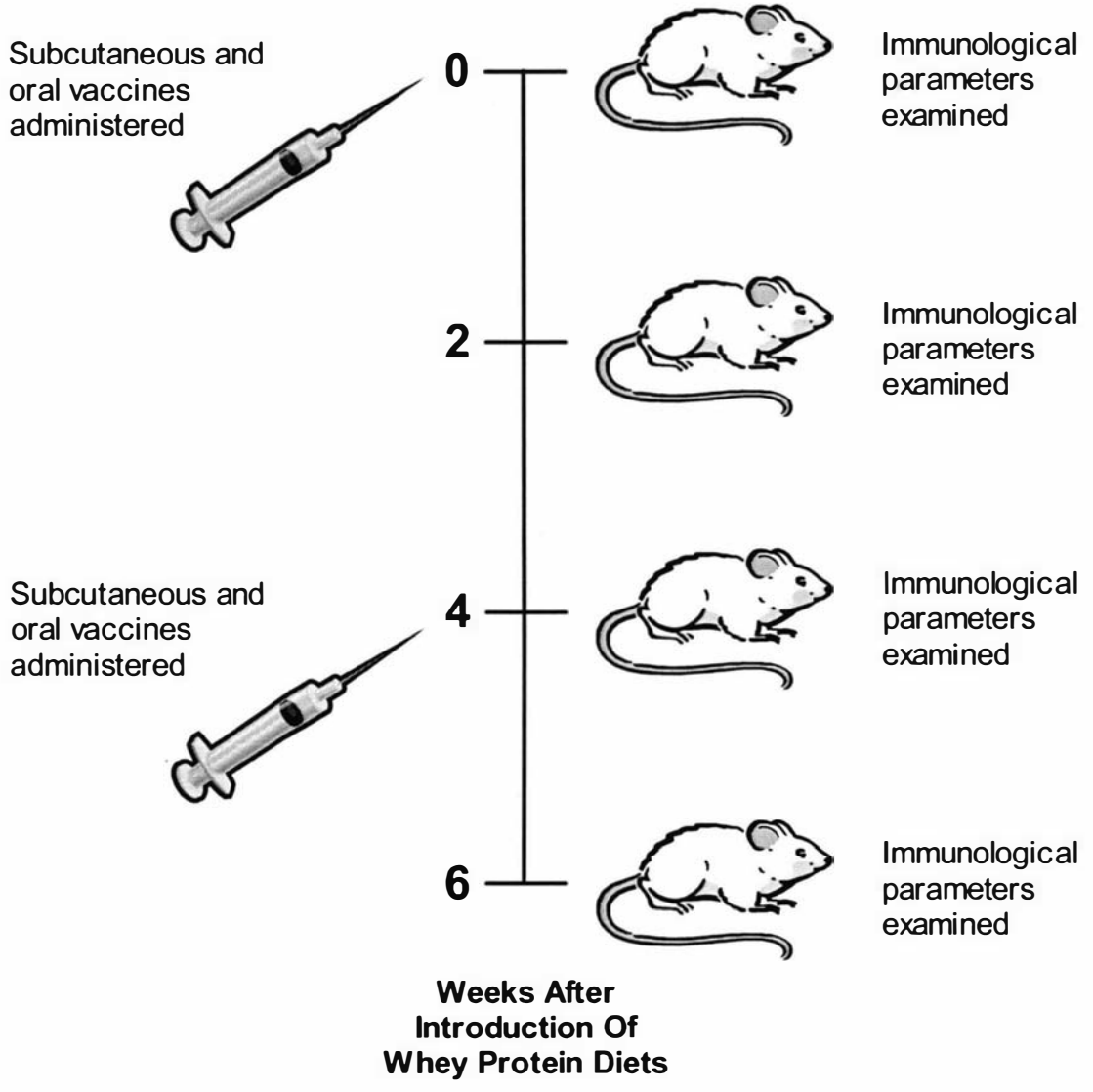


Figure 7.1 Experimental design and immunisation schedule. Schematic representation of the animal feeding trial designed to assess the impact of dietary whey proteins on several humoral and cellular parameters of immune function. At the end of week 0, 2, 4 or 6 immunological parameters were examined. For each test diet, control mice were fed milk free pellets for 0, 2, 4, or 6 weeks. Each group contained 8 mice.

7.2.3 Antibody response, lymphocyte proliferation and phagocytosis assays

An ELISA was used to assess antibody responses to the subcutaneous and orally-administered antigens as described in Section 2.9 and antibody units (units/mL) assigned as described in Section 2.9.2. The effect of dietary WPC or LF-enriched WPC on mitogen induced lymphocyte proliferative potential was performed as described in Section 2.4. The effect of dietary treatment with WPC or LF-enriched WPC compared to controls on the phagocytic ability of murine peritoneal cells and peripheral blood cells was assessed as described in Section 2.8.

7.2.4 Statistical analysis

Statistical differences between the control and test diet groups were determined at each time point using ANOVA and Dunnett's multiple comparison post-hoc test (GraphPad Prism®, USA, 1999). *P* values less than 0.05 were considered significant.

7.3 RESULTS

7.3.1 The effect of dietary WPC and WPC enriched with lactoferrin on mucosal antibody responses to orally-administered CT, OV and polio

To evaluate the short term effect of dietary WPC and dietary WPC enriched with lactoferrin (1% w/w) on mucosal immune responses to CT, OV and polio vaccines, mucosal antibody responses were measured every two weeks for 6 weeks following commencement of the immunisation schedule. Dietary WPC and WPC enriched with lactoferrin (1% w/w) enhanced mucosal antibody responses to all three orally-administered antigens used in this study compared to control animals. Two weeks and 4 weeks after primary immunisation, dietary WPC and dietary WPC enriched with lactoferrin (1% w/w) significantly enhanced mucosal antibody responses to CT, OV and polio compared to control animals ($P < 0.05$) (Figure 7.2 A, B, C). Furthermore, 2 weeks after secondary immunisation antibody responses to CT, OV and polio were 2 to 3 times greater in mice fed WPC and WPC enriched with lactoferrin compared to control mice ($P < 0.05$, $P < 0.001$, $P < 0.01$ respectively) (Figure 7.2 A, B, C). No significant differences in the primary and secondary mucosal antibody responses to orally-administered CT, OV and polio were observed between mice fed WPC alone or WPC enriched with lactoferrin.

FIGURE 7.2

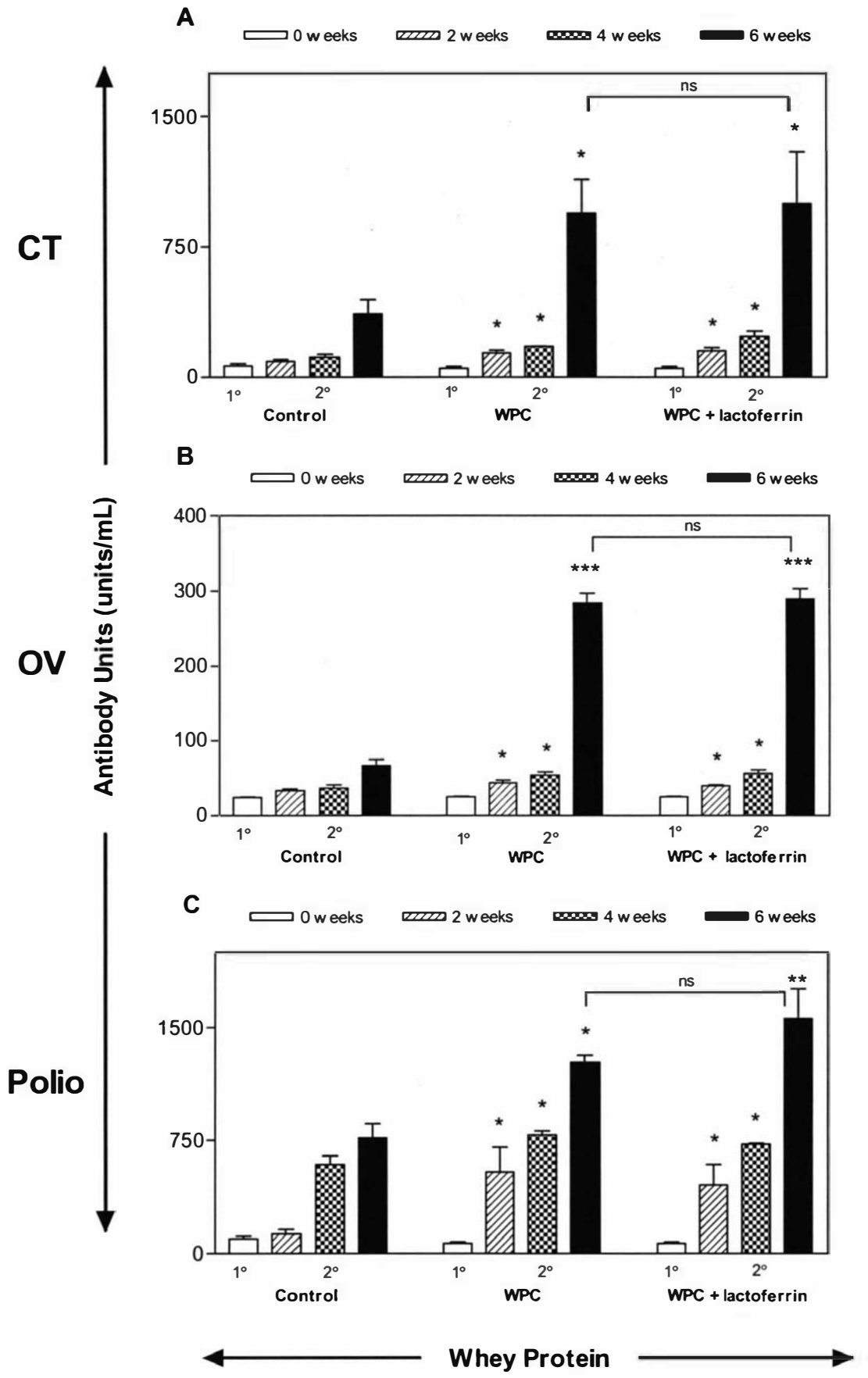

A
0 weeks
2 weeks
4 weeks
6 weeks
ns
1500
750
0
CT
1°
2°
Control
WPC
WPC + lactoferrin
B
400
300
200
100
0
OV
Antibody Units (units/mL)
C
Polio
Whey Protein

FIGURE LEGEND

Figure 7.2 Dietary WPC and LF-enriched WPC enhances primary and secondary mucosal antibody responses to orally-administered CT, OV and polio by up to 3 fold compared to the controls. Immunisations with CT (**A**), OV (**B**) or polio (**C**) were administered at beginning of week 0 and week 4. Mucosal antibody responses were assessed using an enzyme-linked immunosorbent assay (ELISA) after 0, 2, 4 or 6 weeks on the dietary regime. The results are expressed as the mean ± SEM (n = 8) antibody units (units/mL). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to the control at the same time point (ns = not significant).

7.3.2 The effect of dietary WPC and WPC enriched with lactoferrin on systemic antibody responses to subcutaneously administered Flu, TT and Dip

To evaluate the short-term effects of dietary WPC and dietary WPC enriched with lactoferrin (1% w/w) on systemic immune responses to Flu, TT and Dip vaccines, systemic antibody responses were measured every two weeks for 6 weeks following commencement of the immunisation schedule. Dietary WPC and WPC enriched with lactoferrin (1% w/w) enhanced systemic antibody responses to all three systemically-administered antigens used in this study in a similar manner, compared to control animals. Two weeks and 4 weeks after primary immunisation, dietary WPC and WPC enriched with lactoferrin (1% w/w) had no significant effect on primary antibody responses to Flu, TT or Dip. However, 2 weeks after secondary immunisation, systemic antibody responses to Flu, TT and Dip were up to 2 times greater in mice fed WPC and LF-enriched WPC compared to control mice ($P < 0.05$) (Figure 7.3, A, B, C). No significant differences in the primary and secondary systemic antibody responses to systemically administered Flu, TT or Dip were observed between mice fed WPC alone or WPC enriched with lactoferrin.

FIGURE 7.3

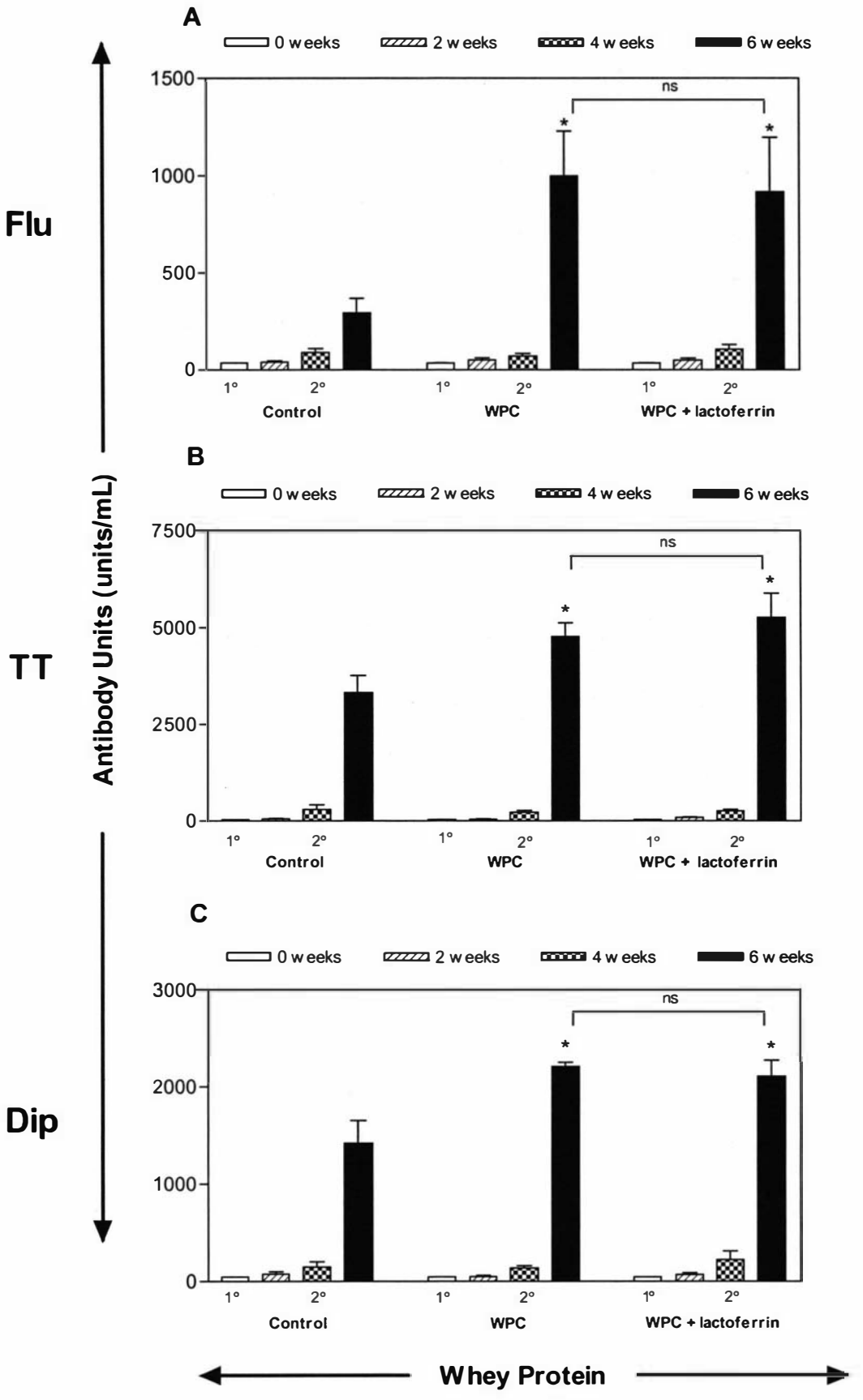

A
0 weeks
2 weeks
4 weeks
6 weeks
1500
1000
500
0
ns
*
*
1°
2°
1°
2°
1°
2°
Control
WPC
WPC + lactoferrin
Flu
B
0 weeks
2 weeks
4 weeks
6 weeks
7500
5000
2500
0
ns
*
*
1°
2°
1°
2°
1°
2°
Control
WPC
WPC + lactoferrin
TT
Antibody Units (units/mL)
C
0 weeks
2 weeks
4 weeks
6 weeks
3000
2000
1000
0
ns
*
*
1°
2°
1°
2°
1°
2°
Control
WPC
WPC + lactoferrin
Dip
Whey Protein

FIGURE LEGEND

Figure 7.3 Dietary WPC and LF-enriched WPC enhances the systemic antibody responses to subcutaneously-administered Flu, TT and Dip by up to 2 fold compared to the controls. Immunisations with Flu (**A**), TT (**B**) or Dip (**C**) were administered at beginning of week 0 and week 4. Systemic antibody responses were assessed using an enzyme-linked immunosorbent assay (ELISA) after 0, 2, 4 or 6 weeks on the dietary regime. The results are expressed as the mean ± SEM (n = 8) antibody units (units/mL). * $P < 0.05$ compared to the control at the same time point (ns = not significant).

7.3.3 The effect of dietary WPC alone and dietary LF-enriched WPC on the proliferative potential of splenic lymphocytes induced by Con A, LPS and PHA

To evaluate the short term effect of dietary LF-enriched WPC on lymphocyte function, mitogen-induced proliferation was measured every 2 weeks for 6 weeks. Dietary LF-enriched WPC increased Con A (T cell mitogen)-induced lymphocyte proliferation by approximately 50%, 2 weeks after commencement of dietary treatment compared to dietary WPC and controls (Figure 7.4 A). LPS (B cell mitogen)-induced lymphocyte proliferation was at least 50% greater in mice fed LF-enriched WPC compared to mice fed WPC alone or pellets (control) 2, 4, and 6 weeks after commencement of dietary treatment (Figure 7.4 B). Two weeks after dietary treatment commenced, PHA (T cell mitogen)-induced lymphocyte proliferation was 50% greater in mice fed LF-enriched WPC compared to mice fed WPC alone or pellets (control) (Figure 7.4 C).

FIGURE 7.4

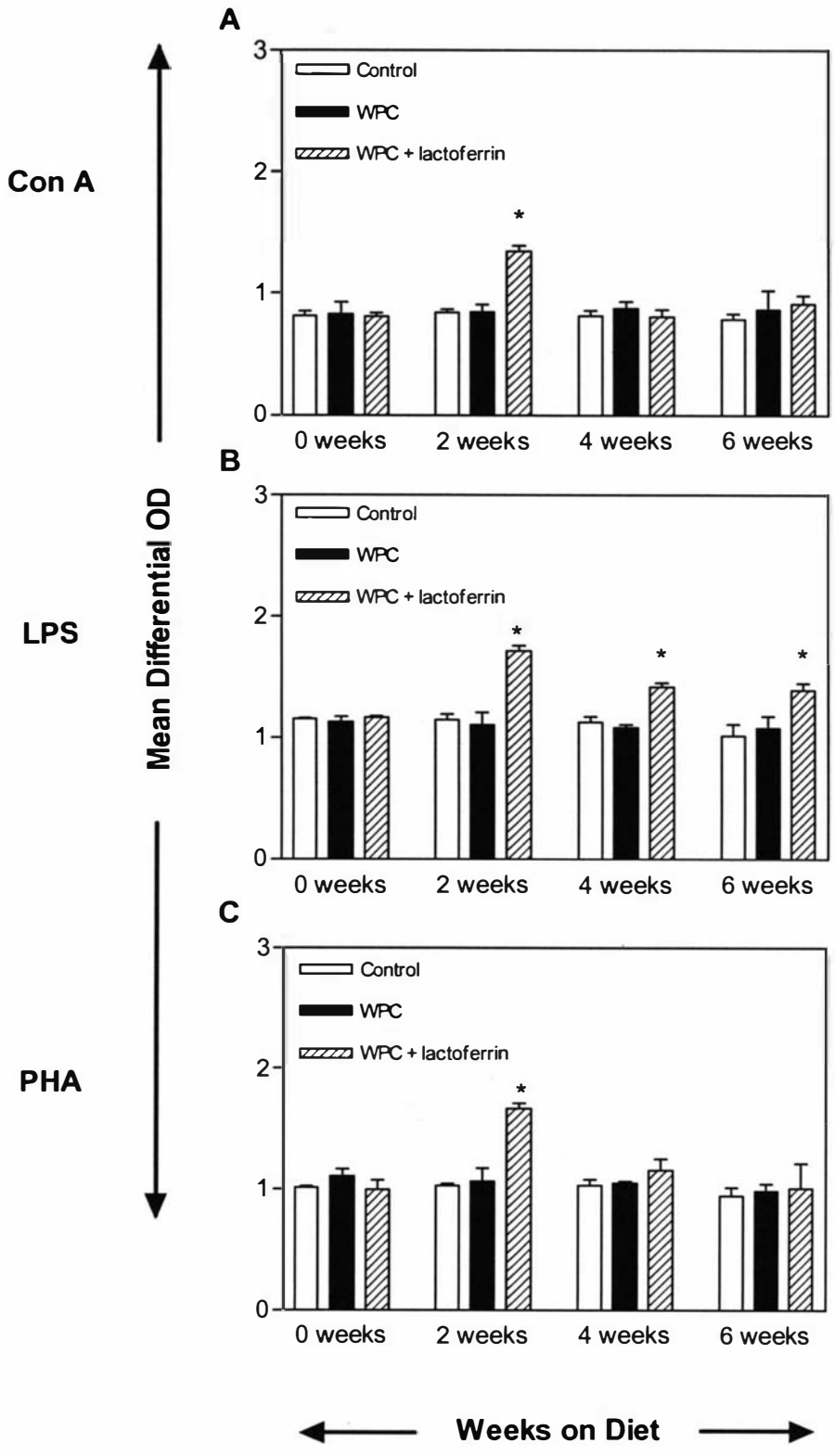

A
B
C
Control
WPC
WPC + lactoferrin
Con A
LPS
PHA
Mean Differential OD
0
1
2
3
*
0 weeks
2 weeks
4 weeks
6 weeks
Weeks on Diet

FIGURE LEGEND

Figure 7.4 Dietary LF-enriched WPC (but not WPC alone) affects Con A, LPS and PHA-induced lymphocyte proliferation. Mice were fed WPC, LF-enriched WPC or pellets for 0, 2, 4 or 6 weeks. At the end of each feeding regime, the proliferative responses of murine spleen cells to Con A (**A**), LPS (**B**) or PHA (**C**) were quantified using a colourimetric assay based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation. Each value represents the mean differential OD ($OD_{MITOGEN}$ - $OD_{NO\ MITOGEN}$) ± SEM (n = 8). * $P < 0.05$ compared to the control at the same time point.

7.3.4 The effect of dietary WPC alone and dietary LF-enriched WPC on the phagocytic activity of murine peritoneal cells

To evaluate the effect of dietary LF-enriched WPC on phagocytic function, the phagocytic ability of both peritoneal and peripheral blood cells was measured. After 6 weeks of dietary treatment, the phagocytic ability of peritoneal cells from mice fed LF-enriched WPC was significantly greater than mice fed WPC alone or pellets (control) (Figure 7.5 A). However, no significant differences in the phagocytic ability of murine peripheral blood cells was observed between mice fed LF-enriched WPC, WPC alone or pellets (control) after 2, 4 and 6 weeks of dietary treatment (Figure 7.5 B).

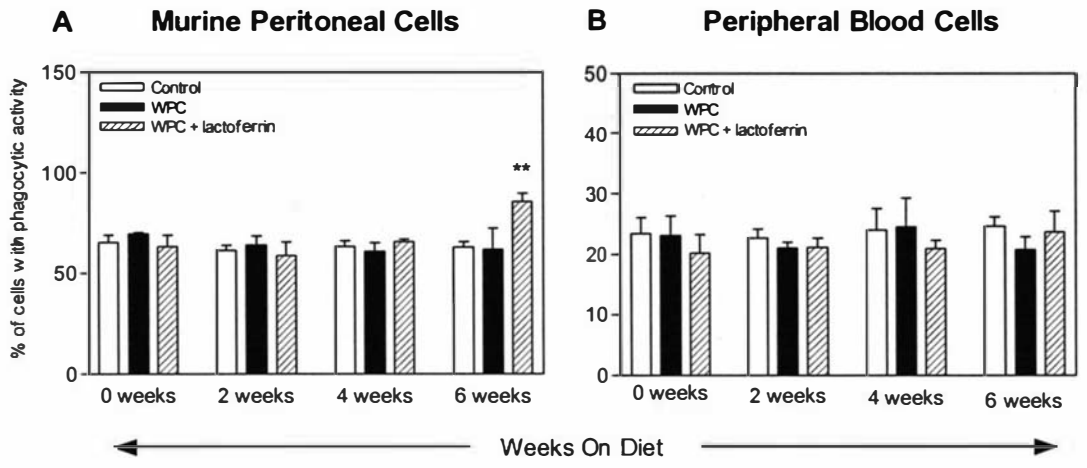


Figure 7.5 Effect of dietary WPC or LF-enriched on the phagocytic activity of murine peritoneal or peripheral blood cells. Mice were fed WPC, LF-enriched WPC or pellets for 0, 2, 4 or 6 weeks. At the end of each feeding regime, the percentage of peritoneal cells (**A**) or peripheral blood cells (**B**) with the ability to phagocytose FITC-labelled *E. coli* was determined using a FACSCalibur flow cytometer. Each value represents the mean percentage of macrophages with phagocytic ability ± SEM (n = 8). ** $P < 0.01$ compared to the control at the same time point.

7.4 DISCUSSION

Previous reports (Chapter 6, Wong & Watson, 1995) have indicated that whole WPC preparations can effectively augment antigen-specific antibody responses in mice, following long-term feeding periods (i.e. over 8 weeks) in response to model T-dependent antigens (such as ovalbumin). The present study has shown that WPC feeding for as little as 2 weeks was able to significantly increase intestinal tract antibody responses to orally-administered antigens, in response to primary immunisation. Both WPC and LF-enriched WPC preparations were effective in this regard, and it was noteworthy that secondary intestinal tract responses (following boost immunisation) were also elevated among WPC and LF-enriched WPC fed mice. These results indicate an immunopotentiating effect of WPC, within a relatively short time-span, is effective on the localised humoral response in the gastrointestinal tract. Quite possibly, this reflects a direct effect of whey proteins on lymphoid foci within the gut environment, and it has been reported elsewhere that bovine whey proteins are able to directly stimulate immunocompetent cells in the gut (Kuhara *et al.*, 2001). It is presently unclear whether this localised immune enhancement reflects increased cellular biosynthesis of immunoglobulin by existing lymphoid cells, and/or an increase in lymphocyte populations leading to elevated antibody production in the gut tissues. While this remains to be determined experimentally, previous research using other dietary supplements (e.g. probiotics) as antibody-enhancing agents has provided clear proof of the latter (Perdigon *et al.*, 1999), with significant increases in gut-dwelling lymphocyte populations as a result of dietary modification.

In the present study, both WPC and LF-enriched WPC dietary treatments were shown to increase serum antibody responses following systemic immunisation of mice with commonly-used T-dependent vaccine antigens (influenza vaccine, tetanus toxoid and diphtheria toxin). Interestingly, consistent enhancement of secondary antibody production (i.e. following booster immunisation) was observed in whey protein-fed mice, while neither WPC nor LF-enriched WPC appeared to affect the primary systemic response. These results are in agreement with a previous study on the immunoenhancing effects of WPC (Wong & Watson, 1995), where whey-fed mice were shown to mount increased secondary (but not primary) antigen-specific serum IgG responses following systemic immunisation with ovalbumin. Other studies have reported increased systemic-

level humoral immunoresponses in mice fed bovine whey protein hydrolysates (Bounous *et al.*, 1981; 1983a), thus it appears that dietary whey proteins are able to up-regulate circulating antibody levels, although this is most noticeable during the anamnestic response to recall antigen administration (i.e. renewed rapid production of an antibody on the second (or subsequent) encounter with the same antigen, immunologic memory). Whole Ig antibody responses were measured in this study therefore it remains unclear whether enhanced humoral immune responses were primarily due to stimulation of IgA and/or IgG antibody responses. Bounous *et al.* (1985) observed an enhancement of IgM/early IgG isotype antibody production by measuring plaque-forming spleen cell responses to SRBC, whereas Wong & Watson (1995) observed an increase in the secondary serum IgG antibody response to systemically-administered ovalbumin. Therefore it appears that dietary WPC is able to stimulate the production of several different isotypes of antibody, however further investigation into the specific antibody enhancement by dietary WPC is warranted.

Previous chapters described the enhancement of secondary antibody responses to orally-administered antigen (CT, OV and polio) and systemically-administered antigen (Flu, TT and Dip) by individual dietary whey components (β-lactoglobulin, α-lactalbumin and lactoferrin) (Chapter 5). However, the present study demonstrates that both secondary and primary antibody responses to orally- and systemically-administered antigen can be enhanced by dietary WPC. This result implies that the humoral immune-enhancing property of WPC (a mixture of individual whey proteins and other immunomodulatory proteins and peptides) is greater than the humoral immune-enhancing potential of individual whey protein components alone. These findings are similar to Bounous & Kongshavn (1989) who found mice fed diets containing any one of the major components of whey (β-lactoglobulin, α-lactalbumin, γ-globulin or serum albumin) developed an inferior humoral immune response (measured by plaque-forming cell response to SRBC) than mice fed a whey protein mixture. This observation suggests that the immune enhancing effect of whey protein concentrate is dependent on the overall amino acid pattern resulting from the contribution of all its protein components (Bounous *et al.*, 1989a).

In a previous study of the immunomodulatory effects of WPC in mice (Chapter 6), it was demonstrated that the long term (up to 84 days) dietary treatment of mice could

enhance antibody responses to T-dependent antigens, but this occurred in the apparent absence of any concomitant up-regulation of systemic lymphocyte activity (such as cellular proliferation or the expression of surface activation markers. The present study has re-confirmed this effect over a shorter time-frame, in that mice fed WPC over a period of 2, 4 or 6 weeks produced elevated intestinal tract and serum antibody responses to heterologous antigens, however, no associated increases in the mitogen-responsiveness of splenic lymphocytes in these animals were detected. These results differ to previous studies involving hydrolysates of α-lactalbumin (a major whey protein) or WPC, which have reported increased splenic lymphocyte proliferation to T and B cell mitogens following dietary treatment of mice (Bounous & Kongshavn, 1982; Bounous *et al.*, 1983a; Wong & Watson, 1995). The reasons for these discrepancies are unclear, however it is acknowledged that the immunomodulatory capacity of WPC is dependent on its constituent protein profile (Cross & Gill, 2000) such that different preparations of WPC are likely to exert subtly different influences on the immune system. This notion is highlighted by the finding that lactoferrin, when fed alone, has no significant effect on mitogen induced murine lymphocyte proliferation (Chapter 5, Figure 5.4) or the phagocytic activity of murine peritoneal cells (Chapter 5, Figure 5.5) however when lactoferrin is fed in conjunction with WPC both lymphocyte proliferation (Figure 7.4) and the phagocytic activity (after 6 weeks of feeding) (Figure 7.5) is significantly enhanced compared to WPC alone and control diets.

Interestingly, while the humoral immune-enhancing effects of dietary LF-enriched WPC were not significantly different from dietary WPC alone (Figure 7.2 and 7.3), the cell proliferative potential of mice fed LF-enriched WPC was significantly higher than both WPC fed and control mice (Figure 7.4). Hence the addition of LF to WPC appears to have initiated an increase in splenic cell proliferation to a B cell mitogen (at all time points) and to T cell mitogens (transiently, at 2 weeks post-feeding). This finding suggests that LF-enriched WPC may target antibody-producing B cells in particular. Bounous *et al.* (1985) showed that WPC acts primarily on B cells by observing that the plaque-forming cell response of primed spleen cells against a T-independent antigen (TNP-Ficoll) was significantly greater in WPC-fed mice compared to casein- or soy-fed mice. The results of this chapter also confirm the previously-reported stimulatory effect of dietary lactoferrin on lymphocyte function (Debabbi *et al.*, 1998; Zimecki & Kruzel, 2000), and highlight the fact that simple augmentation of WPC with a minor whey

protein can produce an immunoenhancing effect that is not observed with the parent material alone. Further studies will be necessary to determine whether supplementation of WPC with other known immunostimulatory minor whey proteins (such as lactoperoxidase (Wong *et al.*, 1997b)) can initiate further increments in immune performance, without impinging on the main effect of humoral enhancement by WPC.

The results of this study support the use of WPCs in infant and adult human health as a possible dietary adjuvant to enhance antibody responses to commonly-administered vaccines. In the first few months of life the lymphoid system is underdeveloped, hence human infants have relatively low levels of immunoglobulin, especially IgA (Hanson *et al.*, 1982). Therefore an immune boosting dietary adjuvant, such as WPC containing a mixture of β-lactoglobulin, α-lactalbumin and lactoferrin, that enhances humoral immune responses to not only microbes but also vaccines commonly administered at this stage of life, may be particularly beneficial. *In vivo* investigations in human infants have shown that dietary bovine lactoferrin is relatively resistant to digestion both *in vivo* and *in vitro* (Gortler *et al.*, 1993). Furthermore, the sequence analysis of bovine lactoferrin clearly indicates that this protein exhibits a strikingly similar structural homology to human LF (Magnuson *et al.*, 1990). Thus bovine lactoferrin delivered within a dietary WPC formulation may provide a suitable substitute for human breast milk lactoferrin and confer an increased degree of immunological protection in humans (Hanson *et al.*, 1985).

In summary, this study has demonstrated that dietary WPC delivered alone or in conjunction with lactoferrin, can enhance humoral immunity in mice following immunisation with several T-dependent antigens, with enhancement of intestinal tract responses being observed as early as 2 weeks following primary immunisation. The fact that several of these antigens are in routine medical use highlights the possibility that dietary whey proteins could be used as a dietary adjunct to boost vaccination responses in humans; this would be particularly beneficial for population groups who ordinarily respond sub-optimally to immunisation, such as the young and the elderly (Gill *et al.*, 2001a). Bovine whole whey protein preparations are currently utilised in health care as therapeutic agents against certain forms of metastatic carcinoma (Kennedy *et al.*, 1995; Bounous, 2000) and He *et al.* (2001) has demonstrated that dietary bovine colostral whey can modulate humoral immune responses to attenuated *S. typhi* Ty21 (an orally-

delivered microbial vaccine) in humans suggesting further opportunities for increasing host defence responses using this form of dietary treatment. In future research to define immunoenhancing effects of dietary whey proteins, it is recommended that consideration is also given to the additional supplementation of minor whey proteins into existing WPC preparations.

CHAPTER 8

Long-term Effects of Dietary Whey Protein Concentrate on Mucosal And Systemic Immune Responses To Commonly Used Vaccines

Published as: Effect Of Dietary Whey Protein Concentrate On Primary And Secondary Antibody Responses In Immunized BALB/c Mice. Low PPL, Rutherfurd KJ, Gill HS & Cross ML (2003) *International Immunopharmacology* 3, 393-401.

8.1 INTRODUCTION

Vaccination is one of the most important contributions to public health in the past 100 years and has dramatically reduced deaths from certain infectious diseases (Roitt, 1996). Immunisation is now considered so important that most countries, particularly first world countries, have extensive public health vaccination programmes against pathogens such as poliovirus, tetanus and diphtheria (Janeway *et al.*, 1999). The boosting of humoral antibody responses to these vaccines would undoubtedly enhance the efficacy of vaccination regimes.

The use of dietary supplements to boost immunoresponses (such as humoral immune responses) has received considerable research attention, as a non-pharmaceutical means of optimising immune competence and benefiting health (Gill *et al.*, 2001a). Several dietary components, including trace minerals, anti-oxidant vitamins, nucleotides/nucleic acids, essential fatty acids and probiotic microbes have been shown to offer promise as effective oral agents for modulating cellular and humoral immune responses (Chandra, 1992; Kaila *et al.*, 1992; Schilling *et al.*, 1996; Gogos *et al.*, 1998; Gill *et al.*, 2000a; Adolfsson *et al.*, 2001; Gill *et al.*, 2001b). Relatively less attention has been paid to the use of whole protein supplements as dietary immunomodulators, although evidence has existed for several years that appropriate protein nutrition can boost immune competence (Bounous & Kongshavn, 1982).

Studies have demonstrated that concentrates of bovine whey proteins exhibit anti-carcinogenic and anti-cancer activity via their effect on increasing glutathione concentration in relevant tissues (Bounous, 2000). Elevated levels of tissue glutathione provided by a whey protein rich diet have also been implicated in the increased resistance to diseases that occur with ageing (Fidelus & Tsan, 1987; Bounous *et al.*, 1989b) and auto-immune diseases (Fidelus & Tsan, 1987). Furthermore, recent research has identified unique immunomodulatory properties of a WPC derived from cheese whey (Chapter 6 and Chapter 7). After long-term (12 weeks) WPC feeding, BALB/c mice were shown to produce markedly increased intestinal tract antibody responses following oral sensitisation to soluble T-dependent antigen (ovalbumin) co-administered with a mucosal adjuvant (cholera toxin sub-unit) (Low *et al.*, 2001). In that study, three sequential immunisations of antigen plus adjuvant were administered. In another study

(Chapter 7), the shorter-term feeding of WPC and LF-enriched WPC resulted in the enhancement of both mucosal and systemic responses to vaccines administered in single priming and boosting doses. However, it remains to be determined what effect WPC feeding might have on primary and secondary antibody responses in an experimental design where immunisations are administered according to schedules used to vaccinate humans during a long-term feeding regime. Therefore, this chapter has sought to understand the kinetics of WPC-mediated effects on the humoral immune response to orally- and parenterally-administered vaccine antigens that are currently in routine medical use by feeding WPC not for 6 weeks (Chapter 7), but for 12 weeks.

Previous studies have not investigated whether or not the pre-feeding of WPC prior to exposure to antigenic challenge can cause a greater enhancement of immune responses compared to those without prior dietary exposure to WPC. Therefore, this chapter has also sought to expand the scope of this work to investigate whether or not the pre-feeding of WPC for a long period of time (4 weeks), prior to the first immunisation, elicits a greater enhancement of humoral immune responses against vaccine antigens that are currently in routine medical use. An additional aim of this chapter was to identify and delineate the effect of feeding WPC (for 12 weeks) on immunoglobulin sub-class responses (i.e. IgA and IgG) to orally-administered vaccine antigens.

8.2 MATERIALS AND METHODS

8.2.1 Mice and diets

Male BALB/c mice were bred, raised and housed as described in Chapter 7, Section 7.2.1. Control animals were fed a milk-free pellet diet (Sharps, Lower Hutt, New Zealand) and test animals were fed a 20% w/w protein of a WPC (ALACEN 472, NZDB, Wellington, New Zealand) as a sole protein source as described in Chapter 7, Section 7.2.1.

8.2.2 Experimental designs

Experimental investigations were conducted in two parts. In part one the effect of pre-feeding WPC on humoral immune responses to several vaccines was investigated. A total of 140 mice were fed a WPC diet or a pellet diet for 4 weeks prior to the first immunisation. Dietary treatment with WPC or milk free pellets (control) continued for 0, 2, 4, 6, 8, 10 or 12 weeks after the administration of the first immunisation. Mice were given a total of 2 oral immunisations (according to the recommended immunisation schedules used to vaccinate humans) of cholera toxin (CT) and ovalbumin (OV) (as a model T–dependent soluble antigen); and polio as described in Chapter 2, Table 2.3, with 4 weeks between the first and second doses. Mice were also given 2 subcutaneous immunisations (according to the recommended immunisation schedules used to vaccinate humans) of absorbed diphtheria (Dip) and tetanus toxoid (TT) vaccine (ADT) and Flu vaccine (Flu), as described in Chapter 2, Table 2.3, with 4 weeks (Flu) or 8 weeks (Dip, TT) between the first and second doses (Figure 8.1, Part I). Blood and intestinal samples were obtained after humanely killing the mice 0, 2, 4, 6, 8, 10 or 12 weeks after the first immunisation was administered, as described in Section 2.9. Each group contained 10 mice.

In part two, the effect of no pre-treatment with dietary WPC on humoral immune response to several vaccines was investigated. Experimental design is as described above except mice received their first immunisation on the same day dietary treatment with WPC or pellets (control) began (Figure 8.1, Part II).

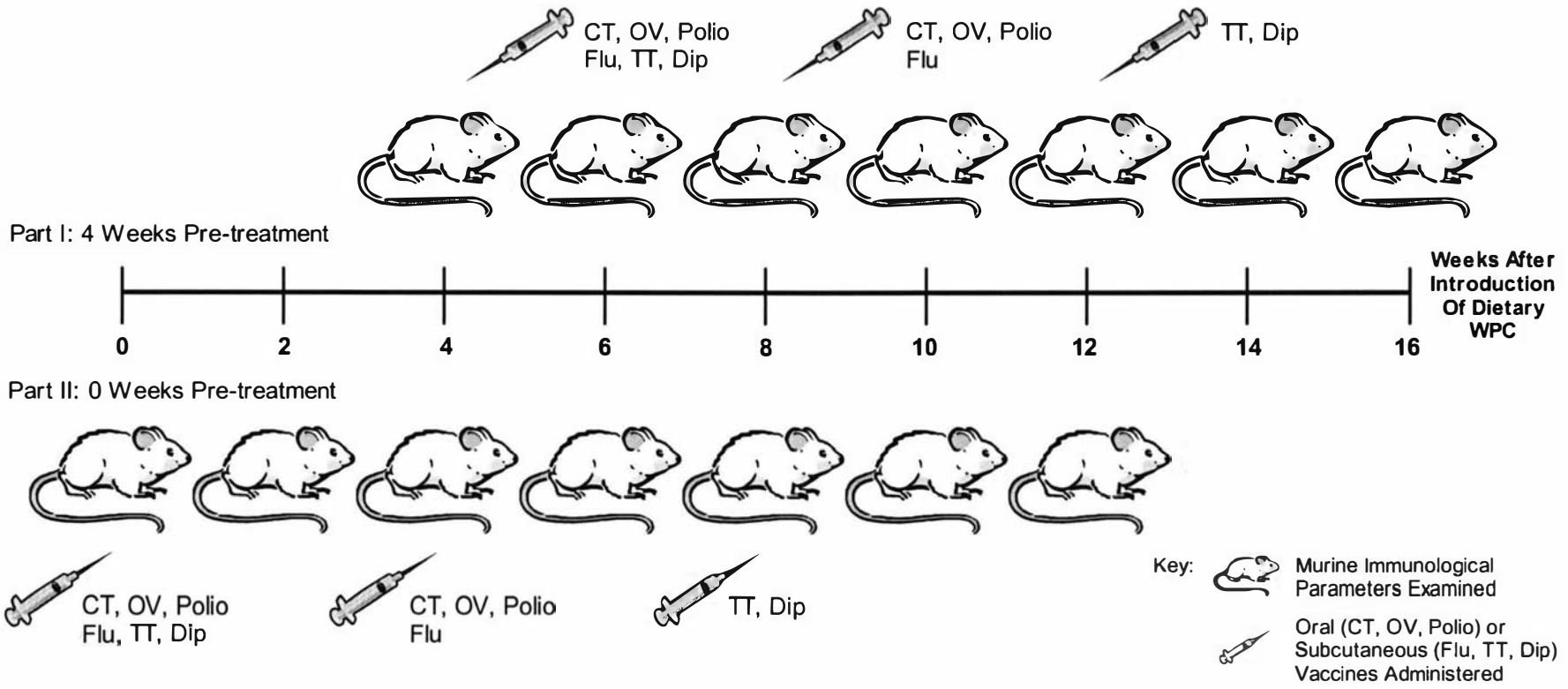


Figure 8.1 Experimental design and immunisation schedule. Schematic representation of the animal feeding trial designed to assess the impact of dietary WPC pre-treatment or no pre-treatment on humoral immune responses to several vaccines. At the end of 0, 2, 4, 6, 8, 10 or 12 weeks, humoral immune responses were examined. Control mice were fed milk-free pellets for 0, 2, 4, 6, 8, 10 or 12 weeks.

8.2.3 Antibody responses to assess the effect of WPC pre-treatment or no pre-treatment on humoral immune responses to several commonly used vaccines

An ELISA was used to determine whole Ig systemic antibody responses to flu vaccine (Flu), tetanus toxin (TT) and diphtheria (Dip) and whole Ig mucosal antibody responses to cholera toxin (CT), ovalbumin (OV) and polio, as described in Section 2.9 and antibody units (units/mL) assigned as described in Section 2.9.2. An ELISA was used to determine the IgA and IgG mucosal antibody responses to CT and OV as described in Section 2.9 with the following exception; specific antibody binding was visualised using alkaline phosphatase-conjugated rat anti-mouse IgA (Silenus) or IgG (Silenus) and antibody units assigned as described in Section 2.9.2.

8.2.6 Statistical analysis

Statistical differences between the control and test diet groups were determined at each time point using ANOVA and Dunnett's multiple comparison post-hoc test (GraphPad Prism®, USA, 1999). *P* values less than 0.05 were considered significant.

8.3 RESULTS

8.3.1 The effect of dietary WPC on mucosal immune responses to orally administered CT, OV and polio vaccines in both WPC pre-treated and non-pre-treated mice

The effect of pre-treating and not pre-treating mice with dietary WPC on mucosal antibody responses to CT, OV and polio vaccines was evaluated every two weeks for 12 weeks following commencement of the immunisation schedule. Regardless of 4 weeks pre-treatment, dietary WPC considerably enhanced (up to 2 fold) mucosal antibody responses to CT every two weeks for 12 weeks following commencement of the immunisation schedule compared to controls (Figure 8.2 A, B) ($P < 0.05$). No significant differences in the kinetics or magnitude of enhanced mucosal immune responses to CT were observed between mice pre-treated and non-pre-treated with dietary WPC. Regardless of 4 weeks pre-treatment, dietary WPC also considerably enhanced (up to 2 fold) mucosal antibody responses to OV every two weeks for 12 weeks following commencement of the immunisation compared to controls (Figure 8.2 C, D) ($P < 0.05$). No significant differences in the kinetics or magnitude of enhanced mucosal immune responses to CT were observed between mice pre-treated and mice not pre-treated with dietary WPC. Similarly, every two weeks for 12 weeks following commencement of the immunisation schedule, mucosal antibody responses to polio were significantly higher (2 fold) in mice pre-treated and non-pre-treated with dietary WPC compared to control animals (Figure 8.2 E, F) ($P < 0.05$). The level of the mucosal antibody response to polio began to decline 4 weeks after the secondary immunisation in both treated mice and control mice, however mice treated with dietary WPC continued to exhibit significantly higher mucosal antibody responses to polio compared to control mice over the period measured (Figure 8.2 E, F) ($P < 0.05$). The use of a slightly higher dose of polio vaccine may have resulted in higher levels of antibodies persisting for a longer period of time after the administration of the secondary immunisation. No significant differences in the kinetics or magnitude of enhanced mucosal antibody responses to polio were observed between WPC pre-treated and non-pre-treated mice.

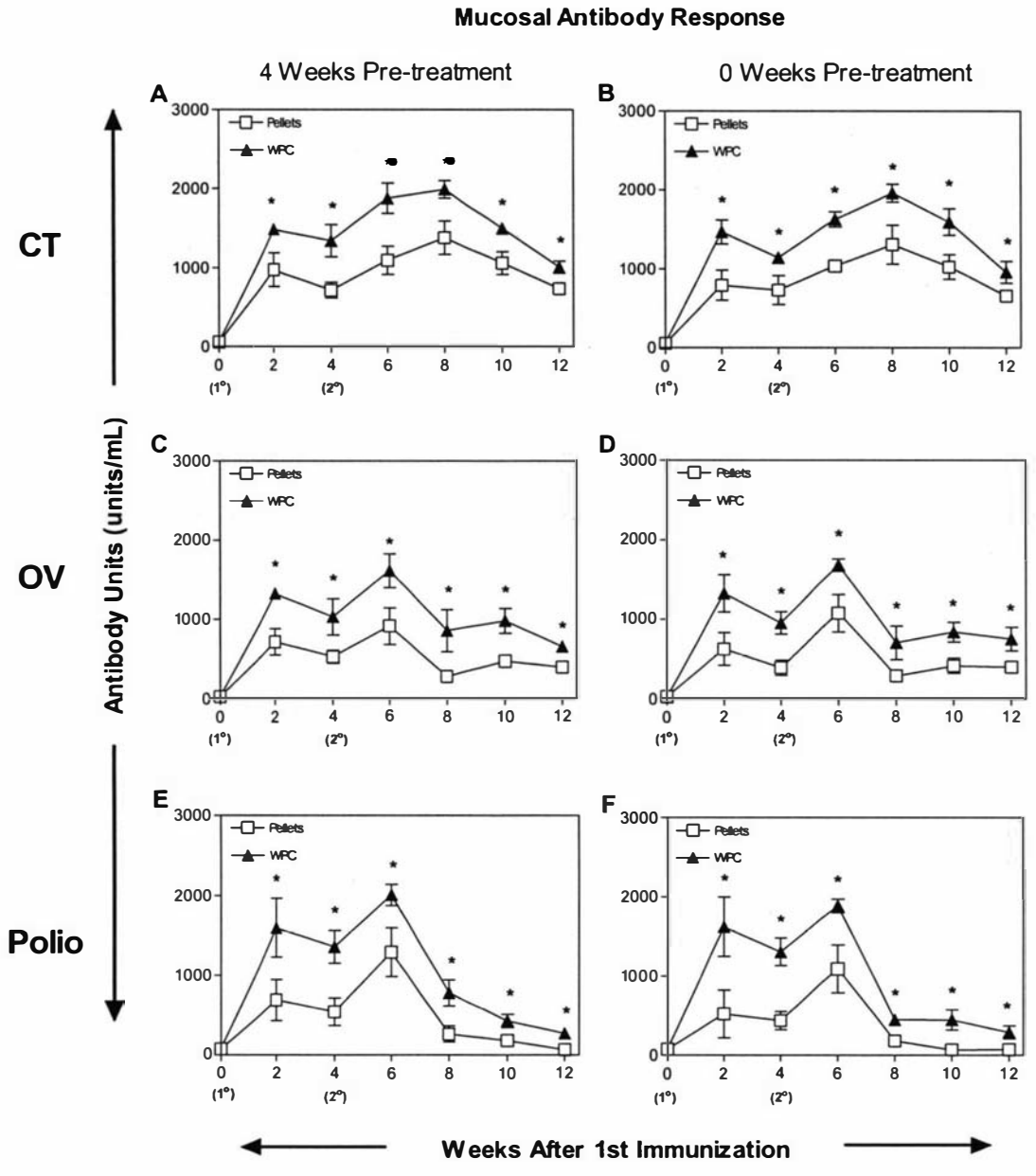


Figure 8.2 Dietary WPC enhances both primary and secondary mucosal immune responses to orally administered CT, OV and polio by up to 2 fold compared to the controls in both WPC pre-treated and non-pre-treated mice. Immunisations of CT (**A, B**), OV (**C, D**) or polio (**E, F**) were administered at the beginning of week 0 and week 4. Antibody responses were assessed using an enzyme-linked immunosorbent assay (ELISA) at 0, 2, 4, 6, 8, 10 or 12 weeks after primary (1°) immunisation. The results are expressed as the mean ± SEM (n = 10) antibody units (units/mL). * $P < 0.05$.

8.3.2 The effect of dietary WPC on systemic immune responses to subcutaneously administered Flu, TT and Dip vaccines in both WPC pre-treated and non-pre-treated mice

The effect of pre-treating and not pre-treating mice with dietary WPC on systemic antibody responses to Flu, TT and Dip vaccines was evaluated every two weeks for 12 weeks following commencement of the immunisation schedule. Regardless of 4 weeks pre-treatment, dietary WPC had no significant effect on the systemic antibody responses to Flu after primary immunisation, however two weeks after the secondary immunisation, dietary WPC began to significantly enhance (3 fold) systemic antibody responses to Flu compared to controls (Figure 8.3 A, B) ($P < 0.05$). This significant enhancement was maintained until the last time point measured in this study (12 weeks after primary immunisation) (Figure 8.3 A, B) ($P < 0.05$). No significant differences in the kinetics or magnitude of enhanced antibody response to Flu were observed between mice pre treated and mice not pre-treated with dietary WPC. Similarly, regardless of 4 weeks pre-treatment, dietary WPC did not affect systemic antibody responses to either Dip or TT after primary immunisation (Figure 8.3 C, D and Figure 8.3 E, F respectively). However, two weeks after secondary immunisation (administered 8 weeks after first immunisation) the systemic antibody responses of mice pre- and non-pre-treated with dietary WPC were significantly higher (up to 2 fold) than the control animals ($P < 0.05$). Antibody responses to Dip and TT remained significantly higher in animals treated with dietary WPC until the last time point measured in this study (12 weeks after primary immunisation) compared to control animals (Figure 8.3 C, D and Figure 8.3 E, F respectively) ($P < 0.05$).

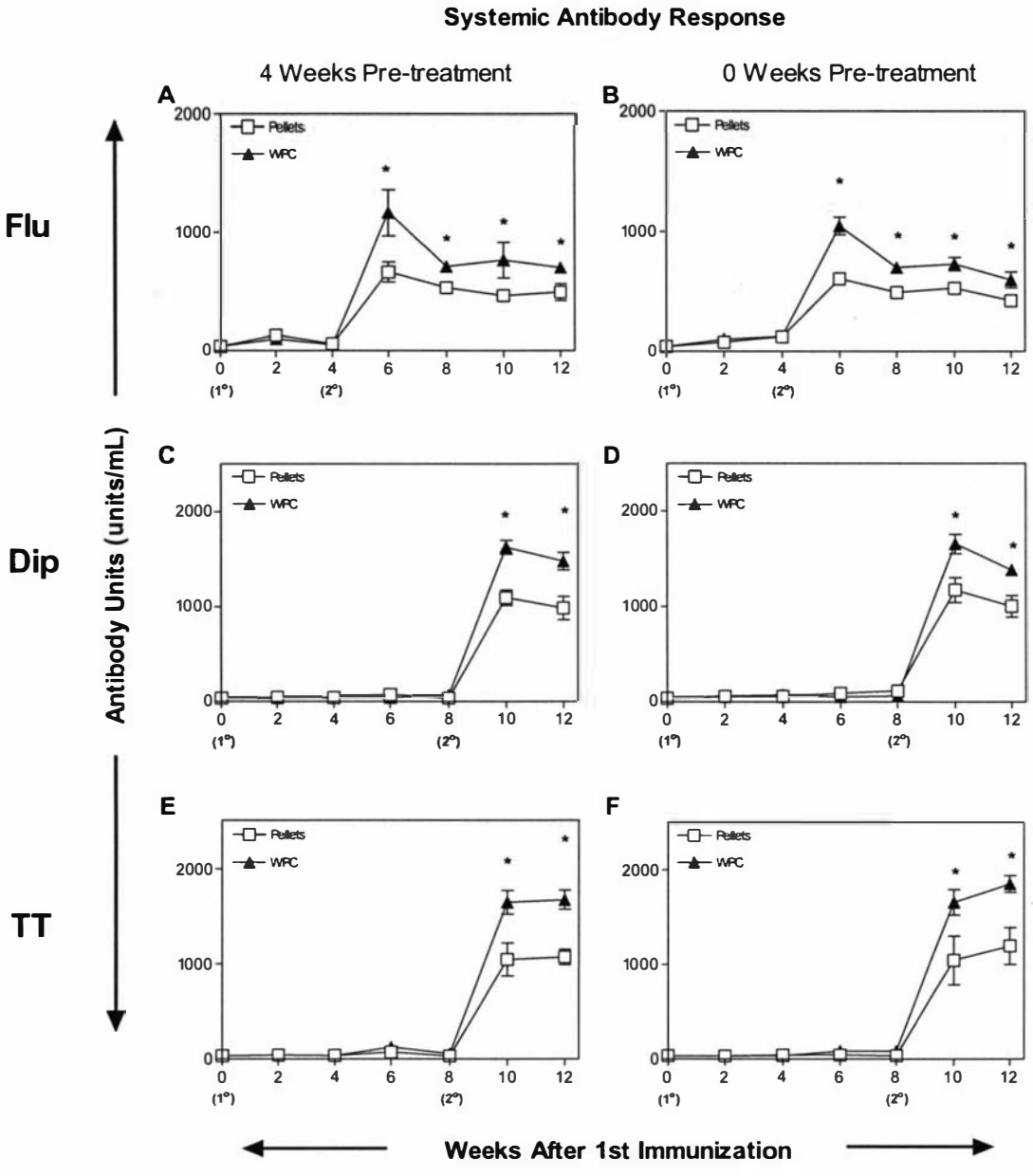


Figure 8.3 Dietary WPC enhances secondary systemic immune responses to subcutaneously administered Flu, TT and Dip by up to 3 fold compared to the controls in both WPC pre-treated and non-pre-treated mice. Immunisations with Flu (**A, B**) were administered at the beginning of week 0 and week 4. Immunisations with TT (**C, D**) or Dip (**E, F**) were administered at the beginning of week 0 and week 8. Antibody responses were assessed using an enzyme-linked immunosorbent assay (ELISA) at 0, 2, 4, 6, 8, 10 or 12 weeks after primary (1°) immunisation. The results are expressed as the mean ± SEM (n = 10) antibody units (units/mL). * $P < 0.05$.

8.3.3 The effect of dietary WPC on mucosal IgG and IgA production to orally administered CT and OV in pre-treated and non-pre-treated mice

Mucosal IgG production, in response to primary and secondary oral vaccinations of CT and OV was greater in mice pre-treated and not-pre-treated with dietary WPC at least 2 weeks after immunisations were administered, however this increase was not significant compared to control mice (Figure 8.4). Similarly, dietary WPC did not significantly increase mucosal IgA production against CT and OV in both pre-treated and non-pre-treated mice compared to controls, however mice fed WPC exhibited higher levels of IgA production especially following primary and secondary immunisations compared to pellet fed mice (Figure 8.4).

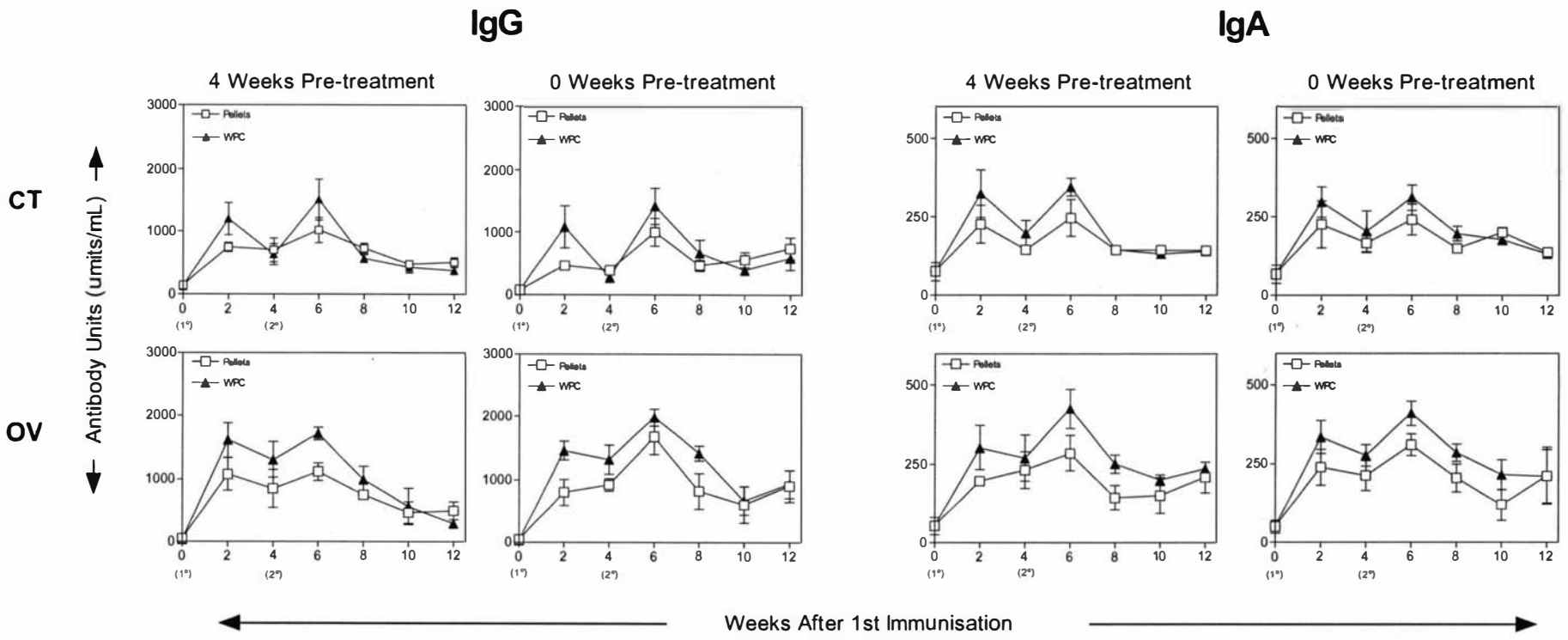


Figure 8.4 Dietary WPC does not significantly alter mucosal IgG and IgA production against orally administered CT and OV compared to the controls in both pre-treated and non-pre-treated mice. Immunisations with CT and OV were administered at beginning of week 0 and week 4. Antibody responses were assessed using an enzyme-linked immunosorbent assay (ELISA) after 0, 2, 4, 6, 8, 10 or 12 weeks on the dietary regime. The results are expressed as the mean ± SEM (n = 10) antibody units (units/mL).

8.4 DISCUSSION

Supplementation of the diet with proven oral immunopotentiating agents has been postulated as a safe and effective means of optimising immune function (Gill *et al.*, 2001a). The results of this chapter have confirmed previous reports from animal model studies that the whey fraction of bovine milk can modulate immune responses (Torre & Oliver, 1989; Wong & Watson, 1995). While the results presented here can provide no further insight into either the proposed mechanism of action or the individual components of bovine whey that might be immunologically active, these results are important since they highlight the health potential of a readily available food-quality protein source. Currently, bovine whey protein is utilised in health care as a glutathione substrate and cysteine donor for restorative therapy against certain forms of metastatic carcinoma (Bounous, 2000; Kuhara *et al.*, 2001). The nutritional qualities of WPC as a readily-digestible protein source, rich in sulphur-containing amino acids, could thus support further exploitation of this material into an immune-enhancing dietary protein supplement.

The present chapter sought to understand the kinetics of immune-enhancement conferred by WPC, by investigating its effects on systemic and intestinal tract antigen-specific antibody production. For this purpose, BALB/c mice were sensitised to several commonly-used T-dependent vaccine antigens using a single prime/single boost regime in order to emulate typical vaccination schedules. As in previous chapters (Chapters 5, 6 and 7) ovalbumin was also included with cholera toxin as an experimental soluble antigen/mucosal adjuvant model. Mice pre-treated and not pre-treated with dietary WPC exhibited considerably higher (2 fold increase) mucosal antibody responses to orally administered CT, OV and polio every 2 weeks for 12 weeks following the commencement of the immunisation schedule compared to control animals (Figure 8.2). These significantly higher levels of whole Ig antibodies to CT, OV and polio were observed for 4 weeks after primary immunisation and persisted for 8 weeks after the secondary immunisation. Furthermore, regardless of pre-treatment dietary WPC significantly enhanced secondary systemic antibody responses to Flu, TT and Dip by up to 3 fold compared to control animals (Figure 8.3). In mice pre-treated and non-pre treated with WPC significantly higher levels of whole Ig antibodies persisted for up to 8 weeks (Flu) or 4 weeks (Dip, TT) after the secondary immunisation. The longest time

point investigated in this study was 12 weeks after the primary immunisation therefore even longer term studies incorporating booster immunisations are recommended to fully elucidate the immune enhancing impact of dietary WPC throughout the entire life time of a mouse. However, from these results it can be concluded that WPC-fed mice can express elevated antibody levels in response to each of the test vaccine antigens, regardless of the route of administration. In addition, these results confirm that orally-delivered WPC can significantly increase humoral immune responses (Chapter 7, Wong & Watson, 1995), and further identify that the kinetics and magnitude of WPC-mediated immune enhancement are similar whether dietary treatment begins prior to or at the same time as commencement of an immunisation regime.

The fact that WPC appeared to impact quite rapidly on intestinal tract humoral responsiveness, with detectable influences as early as 2 weeks following primary oral immunisation, re-enforces the initial conclusion that WPC acts principally upon the GALT system (Low *et al.*, 2001). Indeed, some individual bovine whey proteins (such as lactoferrin) are known to directly activate immunoresponses in intestinal lymphatic tissues following oral delivery (Debabbi *et al.*, 1998; Kuhara *et al.*, 2001), while other non-whey milk components (such as caseinophosphopeptide) have been shown to modulate intestinal tract antibody production (Monnai *et al.*, 1998; Otani *et al.*, 2000). Thus, although previous reports have demonstrated that feeding WPC to mice can significantly enhance systemic-level cellular and humoral immunoresponses (Wong & Watson, 1995; Ford *et al.*, 2001), the present study (coupled with previous observations (Chapter 6 and 7)) provides the first clear evidence that WPC can also up-regulate immune responses in the intestinal tract, in a relatively short time-frame, when used as a whole protein dietary supplement.

Interestingly, in both the present Chapter and Chapter 7, consistent enhancement of both primary and secondary intestinal tract responses to orally-administered antigens was observed in WPC-fed mice, but with parenterally-administered antigens only secondary responses were elevated by WPC feeding. This latter result further confirms the previous report by Wong & Watson (1995) who demonstrated that WPC-fed BALB/c mice mounted increased secondary, but not primary, serum IgG responses to ovalbumin following intra-peritoneal injection of antigen coupled to dextran sulphate. It remains to be determined whether the increase in secondary serum antibody responses, observed in

WPC-fed mice here, reflects a delayed recirculation of antigen-sensitised lymphocytes from the intestinal mucosa to the peripheral blood pool, or rather represents a different mechanism (such as systemic up-regulation of cellular glutathione levels, which is known to promote increased immune cell function (Bounous *et al.*, 1989a)). However, it is noteworthy that in Chapter 6, WPC was shown to up-regulate intestinal tract antibody responses in mice following oral immunisation (ovalbumin plus cholera toxin) in the absence of concurrent indices of lymphocyte activation from the spleen or peripheral blood.

As previously discussed in Chapter 7, the measurement of whole Ig antibody responses does not differentiate which isotype or isotypes of antibody are predominantly responsible for the observed enhancement of humoral antibody responses. Therefore, in the present chapter specific IgG and IgA antibody levels were measured by ELISA. Specific mucosal IgG and IgA production by mice pre- and non-pre-fed WPC were not significantly different from controls (Figure 8.4). This finding implies that the observed enhancement of humoral responses may have been the result of a collective increase in the levels of all immunoglobulins including not only IgG and IgA but also other immunoglobulins such as IgM. Alternatively, a substantial increase in the level of IgM alone may have occurred. Further investigation into the immunoglobulins produced against various antigens is therefore warranted to fully characterise the specific antibody responses of WPC fed mice.

Recent research has shown some promise for immunomodulatory bovine milk proteins in health applications, including a polymeric casein-based diet (which can reduce inflammatory cytokines in juvenile patients with Crohn's disease (Fell *et al.*, 2000)), and a colostral whey dietary supplement (which has provided some tentative evidence for up-regulation of systemic antibody responses following oral immunisation of adult volunteers with attenuated *S. typhi* (He *et al.*, 2001)). The results of the present study indicate that whey protein concentrate can increase humoral immune responsiveness to T-dependent vaccine antigens, in mice at least, and could thus offer promise as an adjunct dietary treatment to boost post-vaccination responses in humans, particularly among subjects with sub-optimal immunity, for example children and the elderly. Such an action has been demonstrated elsewhere using probiotic lactobacilli as a dietary supplement, with increased immunoresponsiveness to oral rotavirus and *S. typhi*

vaccines (Link-Amster *et al.*, 1994; Isolauri *et al.*, 1995; Fang *et al.*, 2000). Further research will be required to identify the mode of action of immunoenhancement by WPC, and to determine the full impact of dietary WPC on the humoral immune system in humans.

CHAPTER 9

General Discussion

Bovine whey proteins have been studied with increasing intensity since initial demonstrations that they can enhance several parameters of immune function (Bounous & Kongshavn, 1978; Bounous & Kongshavn, 1982; Bounous *et al.*, 1983a). Over the last two decades, several studies have reported the immunomodulatory effects of WPC preparations and individual whey protein isolates, in both *in vivo* dietary studies and *in vitro* cell culture systems (Bounous *et al.*, 1989a; Guimont *et al.*, 1997; Rejman *et al.*, 1992b; Wong & Watson, 1995; Wong *et al.*, 1997c, Zimecki & Kruzel, 2000).

The proteins present in bovine milk are now known to exert immunological effects in non-ruminant mammals. The whey protein fraction, in particular, has been found to contain a variety of immunopotentiating major and minor proteins (reviewed in Section 1.5 and 1.6). Whey proteins have also been demonstrated to impart tumour-retarding properties in both animals and humans (McIntosh *et al.*, 1995; Kennedy *et al.*, 1995; reviewed in Section 1.8) and anti-infection properties in animal model studies (Bounous *et al.*, 1981; Ford *et al.*, 2001; reviewed in Section 1.9). This suggests that this protein source may have a valuable use as a dietary additive to increase immune protection and defence.

Demonstrated benefits of dietary whey protein to the immune system in the murine model may subsequently lead to the development of immune-boosting, nutritionally and physiologically advantageous food supplements suitable for human consumption. Particularly relevant to modern human health is the potential production of whey protein concentrates as dietary adjuvants or immunopotentiators to increase immune responses to currently administered vaccines.

In this study the *in vitro* screening of whey protein components provided a means of rapidly evaluating the immune-enhancing potential of several whey protein components (α-lactalbumin, β-lactoglobulin and lactoferrin). The enhancement of the mitogenic activity of murine splenocytes (Chapter 3) and the phagocytic ability of both murine macrophages and human monocytes (THP-1 cell line) (Chapter 4) by whey protein fractions, highly enriched for β-lactoglobulin or lactoferrin, suggests that these whey proteins possess immune-enhancing potential *in vitro*. The *in vitro* enhancement of phagocytic activity by bovine milk fractions such as casein has been demonstrated in several previous studies (Jolles *et al.*, 1981-1982; Gattegno *et al.*, 1988; Migliore-

Samour *et al.*, 1989), however, this study (Chapter 4) has demonstrated that *in vitro* phagocytic activity can also be enhanced by bovine whey protein fractions.

This study focused on aspects of the immune response that contribute to an acquired protection, especially lymphocyte function and phenotype, antibody production and monocyte uptake of microscopic particles. *In vivo* studies (Chapter 5, 6 and 7) demonstrated that the magnitude of the immunomodulatory effect of whey proteins was greater on antibody responses than on monocyte function and lymphocyte proliferation and phenotypes. The effect of whey proteins on other aspects of immune function (such as polymorphonuclear cells, NK cells and major histocompatibility complex expression/antigen presentation) was not investigated in the present study. However, a limited amount of studies have demonstrated that whey proteins can also modulate parameters of the immune system that were not investigated. For example, whey proteins have been found to modulate natural killer and neutrophil function (Shimizu *et al.*, 1996; Shinoda *et al.*, 1996; Miyauchi *et al.*, 1997; reviewed in Section 1.6.1). Hence, the immunomodulatory effects of whey proteins do not appear to be restricted to lymphocyte and monocyte function.

Whey protein components produced by the NZDB demonstrated greater immune-enhancing potential for both lymphocyte proliferation and phagocytic function *in vitro* when compared to commercially prepared whey protein components (manufactured by ICN) (Chapter 3 and 4). These findings highlight variation in the immunomodulatory properties of whey proteins obtained from different suppliers. The variable immunomodulatory effects of different WPC preparations may be largely due to differences in the original source, composition and processing of the WPC. The breed and/or diet (i.e. pasture or grain) of the cow may affect the levels of certain amino acids present in its milk and alter whey protein compositions (Eigel *et al.*, 1984; Swaisgood, 1996). A prime example of variation in the immunoregulatory properties of milk obtained from different sources is the generation of the bioactive peptide BCM-7 from the gastric digestion of milk produced by *Bos taurus* cattle (A1 β-casein molecule), but not milk produced by *Bos indicus* cattle (A2 β-casein molecule) (reviewed in Section 1.4.3). Detailed comparison of WPC originating from different countries and breeds of cow would be advantageous in assessing the relationship between composition and the immunomodulatory potential of WPC preparations.

Smithers *et al.* (1996) found that a whey product with the lowest biological functionality (measured by the ability of the dietary protein to elicit an immune response to a particular antigen) had been subjected to severe processing, including high heat treatment and mechanical damage. The most efficacious product had received a relatively mild treatment. Bounous & Gold (1991) found the humoral immune response was highest in mice fed a dietary whey protein concentrate exhibiting the highest solubility (undenatured conformation) and the greatest relative concentration of the thermolabile bovine serum albumin and immunoglobulins. Therefore, the biological activity of whey proteins appears to be particularly dependent upon their processing history and ultimately their native conformation (Smithers *et al.*, 1996). The use of large scale, commercial processing techniques can denature any biologically active and possible immunoregulatory proteins present in whey protein preparations (reviewed in Section 1.4.4). Compared to the pasteurisation treatment of the parent milk of the ICN whey protein products, the method used to sterilise the parent milk of the NZDB whey protein products was milder and less likely to induce functional and bioactive changes in the milk protein. The use of non-commercial, small scale processing techniques in the manufacture of the NZDB whey protein preparations may have also retained the immunoregulatory activity of these proteins more than the commercially manufactured ICN whey proteins tested in the same study. Since it is well established that whey protein preparations prepared by different processing techniques and manufactured by different companies do vary in both composition and functionality, continued effort is required to develop and evaluate standard tests to reliably predict the functional, biological and immunoregulatory activity of different whey protein preparations and determine the optimal processing conditions required to produce whey protein preparations with maximal immunoregulatory properties. Seasonal variation and batch to batch variation of whey protein products will also need to be determined.

In addition to processing techniques used to manufacture immunoregulatory whey protein preparations, another important consideration is the combination and ratios of individual whey proteins (e.g. β-lactoglobulin, α-lactalbumin and lactoferrin) present in the final whey protein preparation or isolate. Therefore, differences in the purity of the whey protein isolates tested in Chapters 3 and 4 may have contributed to the observed differences in the immunomodulatory effects of the different whey protein preparations.

For example, β-lactoglobulin (ICN) was 99% pure and β-lactoglobulin (NZDB) was 94% pure. This subtle difference in purity may have resulted in the presence of small proportions of other whey protein fractions or other stimulatory factors in the β-lactoglobulin (NZDB) preparation, that in combination with the variant/s of the β-lactoglobulin protein itself, resulted in a more effective immunostimulation of cell proliferation and phagocytic function of murine cells *in vitro* (Chapter 3 and 4). Previous research by Wong *et al.* (1998) found that of three commercially available β-lactoglobulin preparations (β-lactoglobulin containing variants A and B, purified variant A and purified variant B), the unseparated mixture containing both A and B variants showed the most immunostimulation of murine spleen cells *in vitro*. The importance of the combination of individual whey proteins in whey protein preparations has also been highlighted in several *in vivo* studies (Bounous & Amer, 1988; Bounous *et al.,* 1988a; Bounous *et al.,* 1989a; Wong & Watson, 1995, Chapter 5) and is discussed further below.

In vivo studies presented in this thesis demonstrated for the first time that the short-term feeding of individual whey proteins (α-lactalbumin, β-lactoglobulin and lactoferrin) can significantly enhance humoral immune responses to orally administered antigens (cholera toxin, ovalbumin and polio) in mice (Chapter 5). This discovery supports previous findings that individual dietary whey protein components can enhance humoral immune function (Berthou *et al.*, 1987; Bounous *et al.*, 1988a; Zimecki *et al.*, 1991; Miyauchi *et al.*, 1997). Consistent with Bounous & Kongshavn (1982) and Bounous *et al.*, (1983a), the results of Chapter 5 found dietary α-lactalbumin, β-lactoglobulin and lactoferrin had no significant effect on cell-mediated immune functions such as mitogen-induced cell proliferation and the phagocytic activity of murine peritoneal or peripheral blood cells *ex vivo.* Whey protein components were however, found to exhibit an immune-enhancing effect on lymphocyte proliferation (Chapter 3) and phagocytic activity (Chapter 4) in *in vitro* studies. This finding is similar to previous studies (Berthou *et al.*, 1987; Otani *et al.*, 1995; Monnai *et al.*, 1998) and demonstrates that the immunomodulatory effect of bovine milk protein components on certain parameters of immune function *in vivo* (Chapter 5) can differ from those predicted/identified in *in vitro* models (Chapter 3 and 4). These differences may be due to degradation effects of gut enzymes on the whey proteins when ingested in *in vivo* studies. Indeed, the enzymatic treatment of bovine caseins has been shown to

completely alter their immunomodulatory potential in several *in vitro* studies. For example, the pepsin treatment of casein enhanced the phagocytic function of murine macrophages compared to the intact native protein (Otani & Futakami, 1996) and the trypsin, chymotrypsin and pepsin treatment of caseinoglycopeptide eliminated its suppressive effect on murine lymphocyte proliferation (Otani & Monnai, 1993). Additionally, gut enzymes may release *de novo* immunomodulatory peptides *in vivo* that are not present *in vitro*. Although positive results from an *in vivo* study may offer the strongest evidence that whey proteins will modulate immune function when used as a dietary inclusion, an *in vitro* study may detect subtle differences that an *in vivo* study may miss, and this strengthens the argument for the use of both *in vitro* and *in vivo* research methods when investigating the immunomodulatory properties of milk proteins (Cross & Gill, 2000).

The principles governing immunoenhancement by dietary whey protein components and WPC are yet to be completely defined. In mice, dietary protein type had no substantial effect on cell-mediated immune responses (graft-verses-host reactivity, DTH reaction, spleen cell mitogen responses and *Salmonella* infection) but a significant effect on humoral immune responses (measured by plaque-forming cell response) to T-dependent (SRBC) and T-independent (trinitrophenyl-Ficoll) antigens after short term (2 week) exposure (Bounous & Kongshavn, 1985). No significant effect on the phagocytosis of ^{51}Cr-labelled opsonized SRBC was observed between whey protein-fed and non-purified diet-fed mice (Bounous *et al.*, 1981). These findings suggest that the effects of altered dietary protein type on humoral immune responsiveness may be due to changes either in the functional responsiveness of B lymphocytes themselves or in the processes leading to their activation and differentiation in the peripheral lymphoid tissues. Dietary whey protein may alter the humoral immune response by either a central effect on the bone marrow (changing the supply of newly formed B lymphocytes) or a peripheral effect on lymphoid tissues. However, dietary protein type has been shown to have no effect on bone marrow B-lymphocyte genesis (assayed either by pre-B-cell proliferation or by small lymphocyte production) (Bounous *et al.*, 1985). Mice carrying an accessory cell-B cell interaction defect have also been shown to exhibit an enhanced humoral immune response to sheep blood cells following dietary treatment with whey protein, compared to casein or a control (non-purified) diet. This response was less dramatic compared to normal mice, suggesting that although the characteristic response

of dietary protein type may be an intrinsic property of antigen specific B cells, it is more dramatically expressed when T-helper cell derived factors are operational (Bounous *et al.*, 1985). Collectively these findings indicate that the observed effects of altered dietary protein type on humoral immune responsiveness are not exerted centrally on the rate of primary B-lymphocyte production in the bone marrow, but reflect changes on the functional responsiveness of the B lymphocytes themselves in the processes leading to their activation and differentiation in the peripheral lymphoid tissues. In the present study, dietary WPC consistently enhanced both primary and secondary intestinal tract antibody responses to orally-administered antigen, but only enhanced secondary responses to parentally-administered antigens (Chapter 7 and 8). Increases in secondary serum antibody responses may have been due to the delayed recirculation of antigen-sensitised lymphocytes from the intestinal mucosa to the peripheral blood pool or the increased synthesis of glutathione (necessary for lymphocyte proliferation) by systemic lymphocytes via the provision of dietary cysteine (a rate limiting substrate of glutathione synthesis present, at high levels, in WPC) (Bounous *et al.*, 1989a). Kuhara *et al.* (2001) postulated that the inhibition of experimental metastasis by the oral administration of bovine lactoferrin and pepsin hydrolysate of bovine lactoferrin may be due to enhanced cellular immunity mediated by enhanced interleukin-18 production in the intestinal epithelium. Dietary whey proteins may also increase antigen processing and presentation by antigen presenting cells and/or activate cells of the common mucosal immune system, causing more $\alpha4/\beta7$ and $\alpha4/\beta1$ expressing lymphocytes to home to effector tissues. Clearly, further research is required to fully elucidate the mechanisms by which whey proteins modulate the immune system.

The results of this study demonstrated that dietary WPC can significantly enhance gut mucosal antibody responses to orally-administered antigens (cholera toxin and ovalbumin administered in three doses) (Chapter 6). The enhancement of gut mucosal responses to orally-administered antigen in the apparent absence of concurrent indices of lymphocyte activation from the spleen or peripheral blood (Chapter 6), is supported by previous reports that bovine milk proteins act directly on the gut immune tissue (Debabbi *et al.*, 1998; Monnai *et al.*, 1998; Otani *et al.*, 2000; Kuhara *et al.*, 2001). Although the precise mechanisms by which dietary WPC affects localised GALT immune responsiveness are not fully determined, the local (gut mucosal) enhancement of antibody responses (especially in the case of antigen administered via the oral route)

is an important consideration for the potential development of immunopotentiating functional foods based on WPCs, since several human vaccines are administered orally. The ideal vaccination provides host defence at the point of entry of the infectious agent; stimulation of mucosal immunity is therefore an important goal of vaccination against organisms that enter through mucosal surfaces (Janeway *et al.*, 1999). Effective protective immunity against some organisms requires the presence of pre-existing antibody at the time of exposure to the infection. For example, the clinical manifestations of tetanus and diphtheria are due to the effects of extremely powerful exotoxins. Pre-existing antibody against the bacterial exotoxin is necessary to provide a defence against these diseases. Pre-existing antibodies are also required to protect against some intracellular pathogens such as poliomyelitis virus, which infects critical host cells within a short period of entering the body and is not easily controlled by T lymphocytes, once intracellular infection is established (Janeway *et al.*, 1999). Thus the administration of a dietary adjuvant that elevates the production of antibodies at the time of vaccination against specific antigens would likely aid the body in responding to invasion and enhance protection against disease.

Dietary WPC had no significant effect on systemic lymphocyte parameters relevant to antibody production (i.e. lymphocyte proliferative potential, increased helper T-cell and B-cell proportions, or activation) (Chapter 6), but LF-enriched WPC significantly enhanced T and B cell mitogen-induced lymphocyte proliferative potential and the phagocytic ability of murine peritoneal cells compared to dietary WPC alone or control diets (Chapter 7). This finding suggests that variations in the composition of WPC diets can affect the immune system in different ways and supports the hypothesis that among the numerous minor protein and peptide constituents of whey there may be some (such as lactoferrin) that exert specific immunomodulatory effects on the cells of the immune system. While dietary LF-enriched WPC significantly enhanced the proliferative potential of lymphocytes compared to both WPC-fed and control mice (Chapter 7), dietary lactoferrin alone did not affect lymphocyte proliferative potential *in vivo* (Chapter 5). This finding supports the hypothesis that the immune-enhancing property of whey proteins may be synergistic i.e. a result of the combination and mixture of all its constituent proteins rather than one individual whey protein component. Collectively, these results draw attention to the importance of WPC composition and its biological ramifications (Sadler, 1992) and represent a good example of how differing

compositions of WPC can alter the affect of dietary WPC on certain immune parameters.

This study confirmed that in the murine model WPC is able to significantly enhance humoral immune responses to vaccines currently used to immunise humans (polio, flu-virion, tetanus toxoid and diphtheria toxoid) (Chapter 7) and is the first study to demonstrate that dietary WPC can enhance both primary and secondary responses to vaccines in routine medical use. Dietary LF-enriched WPC enhanced both primary and secondary immune responses to oral and systemic antigens to the same degree as dietary WPC alone. Notably, individual whey protein components (α-lactalbumin, β-lactoglobulin and lactoferrin) were only able to enhance secondary immune responses to orally and systemically administered vaccines (Chapter 5). This observation implies that dietary WPC may be more beneficial as an adjuvant to enhance humoral immune responses compared to individual whey protein components alone. This concept is supported by Bounous & Kongshavn (1989) who found that individual major whey components enhanced humoral immune responses (measured by plaque-forming cell response (IgM) to SRBC) to a lesser degree than a total lactalbumin mixture. Furthermore, free amino acid mixes duplicating specific amino acid profiles of various whey proteins were shown to elicit a lower humoral immune response (measured by plaque-forming cell response) to SRBC than intact proteins (Bounous & Kongshavn, 1978; Bounous *et al.*, 1983a). These findings further support the suggestion that the humoral immune-enhancing effect of dietary WPC is associated with its overall plasma amino acid pattern resulting from the contribution of all whey protein components (Bounous *et al.*, 1989a). Alternatively, protein components combined in un-purified whey proteins mixes may counteract each other (with or without influences of other trace factors), hence as bovine whey fractions become progressively more purified and modified, their interactions and immunomodulatory effect may become more apparent (Wong *et al.*, 1997b). Bioactive peptides generated by the *in vitro* enzymatic proteolysis of bovine whey proteins have been identified to have regulatory effects on several aspects of the immune system (Section 1.4.1) and the fractionation of microfiltered whey protein isolate enzymatic digests has been recently found to produce peptides able to stimulate murine splenocyte proliferation at lower concentrations (0.5 – 500 μg/mL) than the total hydrolysates (2000 μg/mL) *in vitro* (Mercier *et al.*, 2004). These findings

indicate that whey proteins not only contain immunomodulatory peptides that can be released by enzymatic digestion but that further fractionation of the whey protein enzymatic digests themselves may also release immunomodulatory short chain (< 5 kDa) and neutral /basic peptides.

An important consideration for the use of WPC as a dietary adjuvant to boost immune responsiveness is the long-term feeding effects of WPC on humoral immune responses to vaccines administered according to the recommended schedules for human health. Previous studies have demonstrated that the immune-enhancing potential of dietary WPC is sustained over a short-term period (6 weeks) in the murine model (Wong & Watson, 1995). The longer-term feeding studies described here have extended the immune-enhancing potential of dietary WPC to a period of 12 weeks (Chapter 6 and 7). The results of these studies established that the long-term feeding (12 weeks) of WPC elicited sustained enhanced primary and secondary humoral immune responses to both oral and systemic vaccines (Chapter 8). This sustained immune enhancement demonstrates that the immunoenhancing effect of dietary WPC is not short-lived and may be expressed as long as the protein is ingested (Chapter 6 and 8). The presence of enhanced mucosal antibody responses to orally-administered antigen after a long period of feeding (3 months) also demonstrates that the immune system does not become 'unresponsive' to the effects of dietary WPC over the time frame investigated (Chapter 6).

To mimic 'Mother's milk' (human) bovine milk-based infant formulae are often supplemented with more whey protein (particularly α-lactalbumin) and lactoferrin (Heine *et al.*, 1991). Therefore, the immune-enhancing potential of LF-enriched WPC preparations provides an opportunity for dairy industries to utilise whey proteins, beyond their nutritional value, in products such as infant formulae. Furthermore, advances in industrial-scale technologies for the cost-effective isolation of α-lactalbumin and lactoferrin (previously expensive to isolate) have now been developed to enable a reliable supply of commercial quantities of bovine whey protein components (Regester *et al.*, 1996; Tomita *et al.*, 2002). Further studies investigating the immunomodulatory properties of WPC diets supplemented with lactoferrin are therefore warranted.

Feeding pre-term infants a whey-predominant formula provides indices of protein utilisation more similar to those obtained with human milk feeding than with cow's milk formula feeding (Janas *et al.*, 1985). Of interest to the regulation of mucosal immunity, lactoferrin can pass intact through the infant gut as evidenced by its presence in the faeces of breast fed infants and can be transported intact from the apical to the basolateral side of intestinal cells *in vitro* (Sfeir *et al.*, 2004). *In vivo* studies have also demonstrated that after oral administration, fragments of lactoferrin survive proteolytic digestion in the small intestine of rats (Kuwata *et al.*, 2001) and substantial amounts of apolactoferrin (20% iron-saturated) and hololactoferrin (100% iron saturated) survive gastric transit in adult humans (Troost *et al.*, 2001). When administered orally, macromolecules such as β-lactoglobulin have been found to empty from the human stomach as an intact protein and cross the epithelial barrier (Mahe *et al.*, 1996). The absorption of large molecules is believed to play a role in different physiological and immunological responses that contribute to oral tolerance and its regulation (Bahna, 1985). However, the role of the β-lactoglobulin molecule itself in different pathways of putative functions such as immune surveillance, metabolic regulation or gastrointestinal disease remains unknown (Sanderson & Walker, 1993). Other milk proteins such as dietary casein have been found to empty from the human stomach in the form of degraded peptides, among which biologically active peptides could be present (Mahe *et al.*, 1996). Indeed, bovine milk peptides such as β-casomorphins, have been detected in the plasma of new born calves after milk intake and in the small intestine of adult humans after ingestion of bovine milk (Svedberg *et al.*, 1985). Consequently, the enzymatic digestion of bovine whey protein preparations may also generate a supply of peptides that could offer immune-enhancing potential in humans (Gardner, 1988; Fiat *et al.*, 1993). These outcomes highlight the suitability of WPC for human consumption, with a possible view to utilising immunoenhancing activity in a functional food format.

Since most human vaccines are administered during the time of infancy, the development of a whey protein-predominant infant formula may provide not only a nutritionally adequate product but also boost immune humoral responses to commonly administered vaccines and feasibly improve their efficacy. The results of this study demonstrate that the pre-feeding of dietary WPC is not necessary to elicit an enhanced mucosal and systemic antibody response to vaccination. Therefore treatment with dietary WPC may begin at the time vaccines are administered. Booster vaccinations

against antigens such as polio viruses, *Clostridium tetani* (tetanus) and *Corynebacterium diphtheriae* (diphtheria) are commonly required throughout childhood and even into adulthood (Janeway *et al.*, 1999) therefore further study investigating the consumption of WPC over a lifetime may prove dietary WPC is efficacious in maintaining enhanced levels of antibodies against these antigens.

This study introduces the possible use of dietary whey proteins as a dietary adjuvant or immunopotentiator to increase responses to vaccines commonly used to immunise humans. While their ultimate clinical utility is unproven, whey proteins have recently been shown to slightly increase humoral (IgA) responses to attenuated *S. typhi* Ty21a oral vaccine in healthy adults aged 20 - 50 years old (He *et al.*, 2001). The majority of dietary whey protein immunoenhancing studies have been conducted in small animal model studies, however several human clinical studies have demonstrated that dietary whey protein may improve the prognosis of human patients with metastatic carcinoma (Kennedy *et al.*, 1995; Bounous, 2000) and HIV seropositive individuals by elevating the level of glutathione in immune cells (Bounous *et al.*, 1993). Additionally, the topical application of a mitogenic bovine whey extract (containing growth factors (GF) such as platelet-derived GF, insulin-like GF-I and -II and transforming GF-β) has also been found to promote repair processes in organotypic *in vitro* models and incisional wounds *in vivo* in rats (Rayner *et al.*, 2000). Bovine colostral products (containing whey) used as mouth rinses in a short-term human study have also indicated a favourable effect by reducing human dental plaque (Loimaranta *et al.*, 1999). Bovine milk whey not only stimulates the proliferation and differentiation of osteoblastic cells *in vitro* (Takada *et al.*, 1996) but also increases bone mineral destiny in healthy adult women (oral dose 200 mg per day) (Aoe *et al.*, 2001) and promotes bone reformation and suppresses bone resorption (while maintaining the balance of bone remodelling) in healthy adult men (oral dose 300 mg per day) (Toba *et al.*, 2001). Collectively these findings demonstrate the diverse immunoregulatory effects of bovine whey protein and support the use of whey proteins as immunoenhancing food supplements suitable for both animal and human consumption, pharmaceutical compositions, anti-tumour therapy, nutritional and intensive care foods. The proposed use of whey proteins as a dietary adjuvant to enhance immune function and vaccine efficacy in humans would undoubtedly be strengthened by future clinical studies confirming the efficacy of WPC to boost humoral immune responses to commonly-used vaccines in humans.

Gaps in the current knowledge of the immunomodulatory effects of whey proteins include the effect of whey proteins on other immune parameters such as cytokine production, NK cell and antigen presenting (eg. dendritic) cell function both *in vitro* and *in vivo*. Information on the effect of whey proteins on these immune parameters may contribute to further understanding of the mechanisms by which whey proteins act on the immune system. Additionally, the *in vivo* fate of immunomodulatory whey proteins is yet to be fully elucidated. Several whey protein-based dietary products available as nutritional supplements in the fitness industry currently appear safe for human consumption (e.g. 100% Whey Protein, Optimum Nutrition, USA and Whey Protein Concentrate, Myopure, Australia). However, the effect of dose, the duration of feeding and the safety of whey proteins in human subjects with immunodeficiency or autoimmune diseases are important considerations when developing immunomodulatory products for human consumption. Further research aimed at fully characterising the immunomodulatory substances present in whey and the manufacturing technologies required to produce them is required. The technological feasibility of producing immunomodulatory whey protein based ingredients/products on a commercial scale also warrants attention.

As consumer demand for natural foods with nutritional and health promoting properties increases, milk and milk by-products are an obvious choice to fill this niche market (Hilliam, 1998). Undoubtedly, whey protein components and WPCs are promising ingredients for the food industry, owing to both their nutritional and functional properties. A great potential exists for whey proteins to be used as specific functional ingredients in various food formulations as an adjuvant or immunopotentiator to boost immune responses to routinely used vaccines. In this context, future research effort has to be focused on not only the development of effective and economical processes for immune enhancing whey protein production but also on the further investigation of currently unresolved questions to fully elucidate the immunomodulatory capabilities of bovine whey.

REFERENCES

Actor J, Hwang S, Olsen M, Zimecki M, Humter R & Kruzel M (2002) Lactoferrin immunomodulation of DTH response in mice. *International Immunopharmacology* **2**, 475-486.

Adamik B, Zimecki M, Wlasczyk A, Berezowicz P & Kubluer A (1998) Lactoferrin effects on the *in vitro* immune response in critically ill patients. *Archivum Immunologiae et Therapice Experimentalis* **46**, 169-176.

Adjei A, Jones J, Enriquez F & Yamamoto M (1999) Dietary nucleotides reduce *Cryptosporidium parvum* infection in dexamethasone immunosuppressed adult mice. *Experimental Parasitology* **92**, 199-208.

Adolfsson O, Huber B & Meydani S (2001) Vitamin E-enhanced interleukin-2 production in old mice: Naive but not memory T cells show increased cell division cycling and interleukin-2-producing capacity. *Journal of Immunology* **167**, 3809-3817.

Aimutis, W (2004) Bioactive properties of milk proteins with particular focus on anticariogenesis. *The Journal of Nutrition* **134,** 986S-995S.

Alexander W (1998) Immunonutrition: The role of ω-3 fatty acids. *Nutrition* **14**, 627-633.

Ambruso D & Johnston R (1981) Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions and an enzymatic generation system. *Journal of Clinical Investigations* **1981**, 352-360.

Anema S (2000) Effect of milk concentration on the irreversible thermal denaturation and disulfide aggregation of β-lactoglobulin. *Journal of Agricultural and Food Chemistry* **48**, 4168-4175.

Anema S & Li Y (2003) Effect of pH on the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. *Journal of Agricultural and Food Chemistry* **51**, 1640-1646.

Aoe S, Toba Y, Yamamura J, Kawakami H, Yahiro H, Kumegawa M, Itabashi A & Takada Y (2001) Controlled trial of the effects of milk basic protein (MBP) supplementation on bone metabolism in healthy adult women. *Bioscience, Biotechnology & Biochemistry* **65**, 913-918.

Aschkenasy A (1973) Differing effects of dietary protein deprivation on the production of rosette-forming cells in the lymph nodes and the spleen and on the levels of serum hemagglutinins. *Immunology* **24**, 617-633.

Azuma N, Mori H, Kaminogawa S & Yamauchi K (1989) Stimulatory effect of human lactoferrin on DNA synthesis in BALB/c 3T3 cells. *Agricultural Biology and Chemistry* **53**, 31-35.

Bagby G, Rigas V & Bennett R (1981) Interaction of lactoferrin, monocytes and T lymphocyte subsets in the regulation of steady state granulopoiesis *in vitro*. *Journal of Clinical Investigations* **68**, 56-63.

Bahana S (1991) Breast milk and special formulas in the prevention of milk allergy. *Advances in Experimental Medicine and Biology* **310**, 445-451.

Balmer S, Scott P & Wharton B (1989) Diet and faecal flora in the new born: Lactoferrin. *Archives of Disease in Childhood* **64**, 1685-1690.

Balmer S & Wharton B (1989) Diet and faecal flora in the new born: Breast milk and infant formula. *Archives of Disease in Childhood* **64**, 1672-1677.

Barnes M, Pearson R, Lee K & Lukes A (1989) Factors contributing to variation in reported milk component percentage in Holstein and Jersey milk. *Journal of Dairy Science* **72**, 1596–1604.

Barness L (1994) Dietary sources of nucleotides - from breast milk to weaning. *Journal of Nutrition* **124**, 128S-130S.

Barta O, Barta V, Crisman M & Akers R (1991) Inhibition of lymphocyte blastogenesis by whey. *American Journal of Veterinary Research* **52**, 247-253.

Baruchel S & Viau G (1996) *In vitro* selective modulation of cellular glutathione by a humanised native milk protein isolate in normal cells and rat mammary carcinoma model. *Anticancer Research* **16**, 1095-1100.

Bates C (1995) Vitamin A. *Lancet* **345**, 31-35.

Baumy J & Brule G (1988) Binding of bivalent cations to alpha-lactalbumin and beta-lactoglobulin: Effect of pH and ionic strength. *Lait* **68**, 33-48.

Beard J (2001) Iron biology in immune function, muscle metabolism and neuronal functioning. *Journal of Nutrition* **131**, 568S-579S.

Belokrylov G, Popova O, Molchanova I, Sorochinskaya E & Anokhina V (1992) Peptides and their constituent amino acids influence the immune response and phagocytosis in different ways. *International Journal of Immunopharmacology* **14**, 1285-1292.

Bengmark S (1998) Immunonutrition: Role of biosurfactants, fibre and probiotic bacteria. *Nutrition* **14**, 585-594.

Bennett R & Davis J (1981) Lactoferrin binding to human peripheral blood cells: an interaction with B-enriched population of lymphocytes and a subpopulation of adherent mononuclear cells. *Journal of Immunology* **127**, 1211-1216.

Berthou J, Migliore-Samour D, Lifchitz A, Delettre J, Floch F & Jolles P (1987) Immunostimulating properties and three dimensional structure of two tripeptides from human and cow caseins. *Federation of European Biochemical Societies Letters* **218**, 55-58.

Birgens H, Karle H, Hansen N & Kristensen L (1984) Lactoferrin receptors in normal and leukemic human blood cells. *Scandinavian Journal of Haematology* **33**, 275-280.

Bistrian B, Blackburn G, Scrimshaw N & Flatt J (1975) Cellular irnmunity in semi-starved states in hospitalised adults. *American Journal of Clinical Nutrition* **28**, 1148-1155.

Bitar D & Bhattacharjee J (1982) Effects of dietary limitations of histidine, methionine, or threonine on the susceptibility of mice to infection with *Salmonella typhimurium* and *Listeria monocytogenes. Nutritional Research* **2**, 309-317.

Blok W, Katan M & van der Meer J (1996) Modulation of inflammation and cytokine production by dietary (n-3) fatty acids. *Journal of Nutrition* **126**, 1515-1533.

Bogden J, Bendich A, Kemp F, Bruening K, Shurnick J, Denny T, Baker H & Louria D (1994) Daily micronutrient supplements enhance delayed hypersensitivity skin test responses in older people. *American Journal of Clinical Nutrition* **60**, 437-447.

Bouhallab S, Molle D & Leonil J (1992) Tryptic hydrolysis of caseinomacropeptide in membrane reactor: Preparation of bioactive peptides. *Biotechnology Letters* **14**, 805-810.

Bounous G (2000) Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. *Anticancer Research* **20**, 4785-4792.

Bounous G & Amer M (1988) The immunoenhancing effect of dietary whey protein concentrate. *Bulletin of the International Dairy Federation* **253**, 44-54.

Bounous G, Baruchel S, Falutz J & Gold P (1993) Whey proteins as a food supplement in HIV-seropositive individuals. *Clinical and Investigative Medicine* **16**, 204-209.

Bounous G, Batist G & Gold P (1989a) Immunoenhancing properties of dietary whey protein mice: Role of glutathione. *Clinical and Investigative Medicine* **12**, 154-161.

Bounous G, Batist G & Gold P (1991) Whey proteins in cancer prevention. *Cancer Letters* **57**, 97-94.

Bounous G, Gervais F, Amer V, Batist G & Gold P (1989b) The influence of dietary whey protein on tissue glutathione and the diseases of ageing. *Clinical and Investigative Medicine* **12**, 343-349.

Bounous G & Gold P (1991) The biological activity of undenatured dietary whey proteins: Role of glutathione. *Clinical and Investigative Medicine* **14**, 296-309.

Bounous G & Kongshavn P (1978) The effect of dietary amino acids on immune reactivity. *Immunology* **35**, 257.

Bounous G & Kongshavn P (1982) Influence of dietary proteins on the immune system of mice. *Journal of Nutrition* **112**, 1747-1755.

Bounous G & Kongshavn P (1985) Differential effect of dietary protein type on the B cell and T cell immune responses in mice. *Journal of Nutrition* **115**, 1403-1408.

Bounous G & Kongshavn P (1989) Influence of protein type in nutritionally adequate diets on the development of immunity. In *Absorption and Utilisation of Amino Acids*, pp. 219-233 [M Freidman, editor]. Florida: CRC Press.

Bounous G, Kongshavn P & Gold P (1988a) The immunoenhancing property of dietary whey protein concentrate. *Clinical and Investigative Medicine* **11**, 271-278.

Bounous G, Kongshavn P, Taveroff A & Gold P (1988b) Evolutionary traits in human proteins. *Medical Hypotheses* **27**, 133-140.

Bounous G, Letourneau L & Kongshavn P (1983a) Influence of dietary protein type on the immune system of mice. *Journal of Nutrition* **113**, 1415-1421.

Bounous G, Letourneau L & Kongshavn P (1983b) The effect of concentration and type of dietary protein hydrolysates on tumour growth in mice. *Clinical and Investigative Medicine* **6**, 197-201.

Bounous G, Papenburg R, Kongshavn P, Gold P & Fleiszer D (1988c) Dietary whey protein inhibits the development of dimethylhydrazine induced malignancy. *Journal of Investigative Medicine* **11**, 213-217.

Bounous G, Shenouda N, Kongshavn P & Osmond D (1985) Mechanism of altered B cell response induced by changes in dietary protein type in mice. *Journal of Nutrition* **115**, 1409-1417.

Bounous G, Stevenson M & Kongshavn P (1981) Influence of dietary lactalbumin hydrolysate on the immune system of mice and resistance to salmonellosis. *The Journal of Infectious Diseases* **144**, 281.

Bourtourault M, Buleon R, Samperez S & Jouan P (1991) Effects of proteins from bovine milk serum on the multiplication of human cancerous cells. *Compte Rendu Des Seances De La Societe De Biologie Filiales* **183**, 319-323.

Britigan B, Serody J, Hayek M, Charniga L & Cohen M (1991) Uptake of lactoferrin by mononuclear phagocytes inhibits their ability to form hydroxyl radicals and protects them from membrane autoperoxidation. *Journal of Immunology* **147**, 4271-4277.

Brix S, Bovetto L, Fritsce R, Barkholt V & Frokiaer H (2003) Immunostimulatory potential of β-lactoglobulin preparations: Effects caused by endotoxin contamination. *Journal of Allergy and Clinical Immunology* **112**, 1216-1222.

Brown E (1984) Interactions of beta-lactoglobulin and alpha-lactalbumin with lipids: A review. *Journal of Dairy Science* **67**, 713-722.

Broxmeyer H, Smithyman A, Eger R, Meyers P & de Sousa M (1978) Identification of lactoferrin as the granulocyte-derived inhibitor of colony stimulating activity production. *Journal of Experimental Medicine* **148**, 1052-1067.

Byatt J, Schmuke J, Comens P, Jonson D & Collier R (1990) The effect of bovine lactoferrin on muscle growth *in vivo* and *in vitro*. *Biochemical and Biophysical Research Communications* **173**, 548-553.

Calder P (1996a) Immunomodulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. *Proceedings of Nutrition Society* **55**, 737-774.

Calder P (1996b) Effects of fatty acids and dietary lipids on cells of the immune system. *Proceedings of Nutritional Society* **55**, 127-150.

Calder P (1998a) Dietary fatty acids and lymphocyte functions. *Proceedings of the Nutrition Society* **57**, 487-502.

Calder P (1998b) Dietary fatty acids and the immune system. *Nutrition Reviews* **56**, S70-S80.

Calder P (1998c) Immunoregulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. *Brazilian Journal of Medical and Biological Research* **31**, 467-490.

Calder P, Yaqoob P, Thies F, Wallace A & Miles E (2002) Fatty acids and lymphocyte functions. *British Journal of Nutrition* **87**, S31-S48.

Capiaumont J, Legrand C, Dousset B, Parmentelot I, Linden G, Bellveille F & Nabet P (1994) Whey and beta-lactoglobulin: 2 milk by-products which can replace foetal calf serum in mouse hybridoma cell culture. *Lait* **74**, 127-137.

Capiaumont J, Ostrovidov S, Legrand C, Belleville F & Nabet P (1996) Bovine whey substitute for FCS in CHO-K1 cell cultures. *In Vitro Cell Development Biology* **32**, 8-12.

Carr R, Webster D, Sadi D & Willams H (1990) Immunomodulation by opioids from dietary casein (exorphins). *Annals New York Academy of Sciences* **594**, 374-376.

Carver J (1994) Dietary nucleotides: Cellular immune, intestinal and hepatic system effects. *Journal of Nutrition* **124**, 144S-148S.

Chandra R (1972) Immunocompetence in undernutrition. *Tropical Pediatrics* **81**, 1194-1200.

Chandra R (1992) Effect of vitamin and trace-element supplementation on immune responses and infection in elderly subjects. *Lancet* **340**, 1124-1127.

Chandra R (1993) Nutrition and the immune system. *Proceedings of the Nutrition Society* **52**, 77.

Chandra R (1996) Nutrition, immunity and infection: From basic knowledge of dietary manipulation of the immune responses to practical application of ameliorating suffering and improving survival. *Proceedings of National Academy of Science* **93**, 14304-14307.

Chandra R (1999) Nutrition and immunology: From the clinic to cellular biology and back again. *Proceedings of the Nutrition Society* **58**, 681-683.

Chew B, Wong T, Shultz T & Magnuson N (1997) Effects of conjugated dienoic derivatives of linoleic acid and β-carotene in modulating lymphocyte and macrophage function. *Anticancer Research* **17**, 1099-1106.

Chin S, Storkson J, Ha Y & Pariza M (1994) Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognised class of anticarcinogens. *Journal Food Composition and Analysis* **5**, 185-197.

Clydesdale F (1997) A proposal for the establishment of scientific criteria for health claims for functional foods. *Nutrition Reviews* **55**, 413-422.

Cole B & Ketterer B (1990) The role of glutathione and glutathione transferases in chemical carcinogenesis. *Critical Reviews in Biochemistry and Molecular Biology* **25**, 47-70.

Cook M & Pariza M (1988) The role of conjugated linoleic acid (CLA) in health. *International Dairy Journal* **8**, 459-462.

Cooray R (1996) Casein effects on the myeloperoxide-mediated oxygen dependent bactericidal activity of bovine neutrophils. *Veterinary Immunology and Immunopathology* **51**, 55-65.

Coste M & Tome D (1991) Milk peptides with physiological activities. Opioid and immunostimulating peptides derived from milk proteins. *Lait* **71**, 241-247.

Coste M, Rochet V, Leonil J, Molle D, Bouhallab S & Tome D (1992) Identification of C-terminal peptides of bovine β-casein that enhance proliferation of rat lymphocytes. *Immunological Letters* **33**, 41-46.

Coutsoudis A, Kiepiela P, Coovadia H & Broughton M (1992) Vitamin A supplementation enhances specific IgG antibody levels and total lymphocyte numbers while improving morbidity in measles. *Pediatric Infectious Disease Journal* **11**, 203-9.

Crago S, Kulhavy R, Prince S & Mestecky J (1981) Inhibition of the pokeweed mitogen-induced response of normal peripheral blood lymphocytes by humoral components of colostrum. *Clinical Experimental Immunology* **45**, 386-392.

Cross M & Gill H (1999) Modulation of immune function by a modified bovine whey protein concentrate. *Immunology and Cell Biology* **77**, 345-350.

Cross M & Gill H (2000) Immunomodulatory properties of milk. *British Journal of Nutrition* **84**, S81-S89.

Cross M & Gill H (2001) Can immunoregulatory lactic acid bacteria be used as dietary supplements to limit allergies? *International Archives of Allergy and Immunology* **125**, 112-119.

Cross M, Stevenson L & Gill H (2001) Anti-allergy properties of fermented foods: an important immunoregulatory mechanism of lactic acid bacteria? *International Immunopharmacology* **1**, 891-901.

Crouch S, Slater K & Fletcher J (1992) Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. *Blood* **80**, 235-240.

Cunningham D, Harrison L & Shultz T (1997) Proliferative responses of normal human mammary and MCF-7 breast cancer cells to linoleic acids, conjugated linoleic acid and eicosanoid synthesis inhibitors in culture. *Anticancer Research* **17**, 197-204.

Cunningham-Rundles S & Ho Lin D (1998) Nutrition and the immune system of the gut. *Nutrition* **14**, 573-579.

Damerji O, Derouiche F, Legrand C, Capiaumont J, Bour J, Maugras M, Belleville F, Nabet P, Paquet D & Linden G (1988) Utilisation of whey as a substitute for foetal calf serum in culture media. *Biotechnology Techniques* **2**, 253-258.

Damodaran S (1996) Amino acids, peptides and proteins. In *Food Chemistry, Third Edition*, p. 321 [O Fennema, editor]. New York: Dekker.

Dannenberg F & Kessler H (1988) Reaction kinetics of the denaturation of whey proteins in milk. *Journal of Food Science* **53**, 258-263.

de la Fuente M, Ferrandez M, Burgos M, Soler A, Prieto A & Miquel J (1998) Immune function in aged women is improved by ingestion of vitamins C and E. *Canadian Journal of Physiology and Pharmacology* **76**, 373-380.

De Sousa M, Breedvelt F, Dynesius-Trentham R, Trentham D & Lum J (1988) Iron, iron-binding proteins and immune cells. *Annals of New York Academy of Science* **526**, 310-322.

de Wit J (1981) Structure and functional behaviour of whey proteins. *Netherlands Milk Dairy Journal* **35**, 47-64.

de Wit J (1989) Functional properties of whey proteins. In *Developments in Dairy Chemistry-4* [P Fox, editor]. London: Elsevier Science Publishers.

de Wit J (1990) Thermal stability and functionality of whey proteins. *Journal of Dairy Science* **73**, 3602-3612.

de Wit J (1998) Marshall Rhone-Poulenc award lecture. Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science* **81**, 597-608.

de Wit J, Klarenbeek G & Adamse M (1986) Evaluation of functional properties of WPCs and WPIs; effects of processing and history and composition. *Netherlands Milk and Dairy Journal* **40**, 41-56.

Debabbi H, Dubarry M, Ranteau M & Tome D (1998) Bovine lactoferrin induces both mucosal and systemic immune responses in mice. *Journal of Dairy Research* **65**, 283-293.

Delafuente J (1991) Nutrients and immune responses. *Rheumatic Disease Clinics of North America* **17**, 203-212.

Demetriades K & McClements D (1998) Influence of pH and heating on the physicochemical properties of whey protein stabilized emulsions containing a non-ionic surfactant. *Journal of Agricultural and Food Chemistry* **46**, 3936-3942.

Derouciche A, Legrand C, Bour J, Capiaumont J, Gelot M, Dousset B, Belleville F, Nabet P & Linden G (1990) Biochemical aspects of a whey fraction capable of promoting hybridoma proliferation. Comparison with foetal calf serum. *Lait* **70**, 313-324.

Dial E & Lichtenberger L (2002) Treatment of *Helicobacter felis*-induced gastritis by lactoferrin. *Biochemistry and Cell Biology* **80**, 158.

Dickinson E (1995) Properties of emulsions stabilized with milk proteins: Overview of some recent developments. *Journal of Dairy Science* **80**, 2607-2619.

Djeha A & Brock J (1992) Effect of transferrin, lactoferrin and chelated iron on human T lymphocytes. *British Journal Of Haematology* **80**, 235-241.

Dunbar A, Priebe I, Belford D & Gooddard C (1999) Identification of betacellulin as a major peptide growth factor in milk: purification, characterisation and molecular cloning of bovine betacellulin. *Biochemical Journal* **344**, 713-721.

Duncan R & McArthur W (1981) Lactoferrin-mediated modulation of mononuclear cell activities. *Cellular Immunology* **63**, 308-320.

Eigel W, Butler J, Ernstrom C, Farrell H, Harwalkar V, Jenness R & Whitney R (1984) Nomenclature of proteins of cow's milk: Fifth revision. *Journal of Dairy Science* **67**, 1599-1631.

Elliott A, Dhakal A, Datta N & Deeth H (2003) Heat-induced changes in UHT milks - Part 1. *Australian Journal of Dairy Technology* **58**, 3-10.

Elliott R & Martin J (1984) Dietary protein: a trigger of insulin-dependent diabetes in the BB rat? *Diabetologia* **26**, 297-299.

Elitsur Y & Luk G (1991) Beta-casomorphin (BCM) and human colonic lamina propria lymphocyte proliferation. *Clinical and Experimental Immunology* **85**, 493-497.

Elitsur Y, Neace C, Liu X, Dosescu J & Moshier J (1997) Vitamin A and retinoic acids immunomodulation on human gut lymphocytes. *Immunopharmacology* **35**, 247-253.

Ellison R & Giehl T (1991) Killing of gram-negative bacteria by lactoferrin and lysozyme. *Journal of Clinical Investigations* **88**, 1080-1891.

Elson C & Dertzbaugh M (1994) Mucosal adjuvants. In *Handbook of Mucosal Immunity*, pp. 391-402 [P Ogra, J Mestecky and M Lamm, editors]. USA: Academic Press.

Enomoto A, Konishi M, Hachimura S & Kaminogawa S (1993) Milk whey protein fed as a constituent of the diet induced both oral tolerance and a systemic humoral response, while heat denatured whey protein induced only oral tolerance. *Clinical Immunology and Immunopathology* **66**, 136-142.

Etzel M (2004) Manufacture and use of dairy protein fractions. *The Journal of Nutrition* **134**, 996S-1002S.

Faith R, Liang S, Murgo A & Plotnikoff N (1984) Neuroimmunomodulation with encephalins: enhancement of human natural killer (NK) cell activity *in vitro*. *Clinical Immunology and Immunopathology* **31**, 412-418.

Fang H, Elina T, Heikki A & Seppo S (2000) Modulation of humoral immune response through probiotic intake. *Federation of European Microbiological Societies Immunology and Medical Microbiology* **29**, 47-52.

Fell J, Paintin M, Arnaud-Battandier F, Beattie R, Hollis A, Kitching P, Donnet-Hughes A, MacDonald T & Walker-Smith J (2000) Mucosal healing and a fall in mucosal pro-inflammatory cytokine mRNA induced by a specific oral polymeric diet in paediatric Crohn's disease. *Alimentary Pharmacology and Therapeutics* **14**, 281-289.

Fiat A, Levy-Tolediano S, Caen J & Jolles P (1989) Biologically active peptides of casein and lactoferrin implicated in platelet function. *Journal of Dairy Research* **56**, 351-355.

Fiat A, Migliore-Samour D & Jolles P (1993) Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. *Journal of Dairy Science* **76**, 301-310.

Fidelus R & Tsan M (1986) Enhancement of intracellular glutathione promotes lymphocyte activation by mitogens. *Cellular Immunology* **97**, 155-163.

Fidelus R & Tsan M (1987) Glutathione and lymphocyte activation: A function of ageing and auto-immune disease. *Immunology* **61**, 503-508.

Field C (2000) Use of T-cell function to determine the effect of physiologically active food components. *American Journal of Clinical Nutrition* **71**, 1720S-1725S.

Field C, Johnson I & Schley P (2002) Nutrients and their role in host resistance to infection. *Journal of Leukocyte Biology* **71**, 16-32.

Field C, McBurney M, Massimino S, Haymak M & Sunvold G (1999) The fermentable fibre content of the diet alters the function and composition of canine gut-associated lymphoid tissue. *Veterinary Immunology and Immunopathology* **72**, 325-341.

Fleet J (1995) A new role for lactoferrin: DNA binding and transcription activation. *Nutrition Reviews* **53**, 226-227.

Floyd C, Ota D, Corriere J, Dudriek S & Copeland E (1983) Effect of serum source on protein-free diet lymphocyte blastogenesis. *Journal of Surgical Research* **35**, 243-248.

Ford J, Wong C & Colditz I (2001) Effects of dietary protein types on immune responses and levels of infection with *Eimeria vermiformis* in mice. *Immunology and Cell Biology* **79**, 23-28.

Fraker P, King L, Laakko T & Vollmer T (2000) The dynamic link between the integrity of the immune system and zinc status. *Journal of Nutrition* **130**, 1399S-1406S.

Francis G, Regester G, Webb H & Ballard F (1995) Extraction from cheese whey by cation-exchange chromatography of factors that stimulate the growth of mammalian cells. *Journal of Dairy Science* **78**, 1209-1218.

Friedman A, Halevy O, Schrift M, Arazi Y & Sklan D (1994) Retinoic acid receptor-alpha gene expression is modulated by dietary vitamin A and by retinoic acid in chicken T lymphocytes. *Cellular Immunology* **152**, 240-248.

Fuller R & Gibson G (1997) Modification of the intestinal microflora using probiotics and prebiotics. *Gastroenterology* **32**, 28-31.

Furukawa T, Meydani S & Blumberg J (1987) Reversal of age-associated decline immune responsiveness by dietary glutathione supplementation in mice. *Mechanisms of Ageing and Development* **38**, 107-117.

Gardner M (1988) Gastrointestinal absorption of intact proteins. *Annual Review of Nutrition* **8**, 329.

Gattegno L, Migliore-Samour D, Saffar L & Jolles P (1988) Enhancement of phagocytic activity of human monocytic-macrophagic cells by immunostimulating peptides from human casein. *Immunology Letters* **18**, 27-32.

Gershwin, M (1985) Protein. In *Nutrition and Immunity*, pp. 156-189. Orlando: Academic Press.

Gibson G (1999) Dietary modulation of the human gut microflora using the prebiotics oligofructose and insulin. *Journal of Nutrition* **129**, 1438S-1441S.

Gibson G & Roberfroid M (1995) Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *Journal of Nutrition* **125**, 1401-1412.

Gill H (1998) Stimulation of the immune system by lactic cultures. *International Dairy Journal* **8**, 535-544.

Gill H, Cross M & Rutherfurd K (2001b) Dietary probiotic supplementation enhances natural killer cell activity in the elderly: An investigation of age-related immunological changes. *Journal of Clinical Immunology* **21**, 264-271.

Gill H, Darragh A & Cross M (2001a) Optimising immunity and gut function in the elderly. *Journal of Nutrition Health and Aging* **5**, 80-91.

Gill H, Doull F, Rutherfurd K & Cross M (2000b) Immunoregulatory peptides in milk. *British Journal Of Nutrition* **84**, 111-117.

Gill H, Rutherfurd K, Prasad J & Gopal P (2000a) Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *British Journal of Nutrition* **83**, 167-176.

Gobbetti M, Stepaniak L, De Angelis M, Corsetti A & Di Cagno R (2002) Latent bioactive peptides in milk protein; proteolytic activation and significance in dairy processing. *Critical Reviews in Food Science and Nutrition* **42**, 223-239.

Gogos C, Ginopoulos P, Salsa B, Apostolidou E, Zoumbos N & Kalfarentzos F (1998) Dietary omega-3 polyunsaturated fatty acids plus vitamin E restore immunodeficiency and prolong survival for severely ill patients with generalised malignancy: A randomised control trial. *Cancer letters* **82**, 395-402.

Goldblum R & Goldman A (1994) Immunological components of milk: Formation and function. In *Handbook of Mucosal Immunology*, pp. 643-652 [P Ogra, M Lamm, J McGhee, J Mestecky, W Strober and J Bienenstock, editors]. San Diego: Academic Press.

Goldman A, Chheda S, Keeny S & Schmalstieg F & Schanler R (1994) Immunologic protection of the premature new born by human milk. *Seminars in Perinatology* **18**, 495-501.

Good R, Jose D, Cooper W, Fernandes G & Kramer T (1977) Influence of nutrition on antibody production and cellular immune responses in man, rats, mice and guinea pigs. In *Malnutrition and the Immune Response*, pp. 169-183 [R Suskind, editor]. New York: Raven Press.

Gortler I, Sawatzki G, Schulz-Lell G & Schaub J (1993) Investigations on the *in vitro* and *in vivo* digestibility of bovine lactoferrin. In *New Perspectives in Infant Nutrition Symposium, Antwerp 1992*, pp. 95-99 [B Renner and G Sawatzki, editors]. New York: Thieme Medical Publishers Incorporated.

Gould G (2000) Preservation: past, present and future. *British Medical Bulletin* **56**, 84-96.

GraphPad Prizm® (1999) Graph Pad Prizm® under Microsoft Windows, Version 3.00 USA: GraphPad Software Incorporated.

Grimble R & Grimble G (1998) Immunonutrition: Role of sulphur amino acids, related amino acids and polyamines. *Nutrition* **14**, 605-610.

Gross R & Newberne P (1980) Role of nutrition in immunologic function. *Physiology Review* **60**, 188-302.

Grosse R & Boehmer F, Langen P, Kurtz A, Lehmann W, Mieth M & Wallukat G (1991) Purification, biological assay, an immunoassay of mammary derived growth inhibitor. *Methods in Enzymology* **198**, 425-493.

Guimont C, Marchal E, Girardet J & Linden G (1997) Biologically active factors in bovine milk and dairy by products: Influence on cell culture. *Critical Reviews in Food Science and Nutrition* **37**, 393-410.

Ha Y, Grimm N & Pariza M (1987) Anticarcinogens from ground beef: heat altered derivatives of linoleic acid. *Carcinogenesis* **8**, 1881-1887.

Hakansson T, Shinoda I, Fukuwatri Y & Shimamura S (1995) Apoptosis induced by a human milk protein. *Proceedings of National Academy of Science* **92**, 8064-8068.

Hambraeus L & Lonnerdal B (1994) The physiological role of lactoferrin. In *Indigenous Antimicrobial Agents of Milk*, pp. 97-107. Brussels: International Dairy Federation.

Hansen R (1974) Milk in human nutrition. In *Lactation-A Comprehensive Treatise VIII. Nutrition and Biochemistry of Milk/Maintenance.* New York: Academic Press.

Hanson L, Ahlstedt S, Anderson B, Carlsson B, Fallstrom S, Mellander L, Porras O, Soderstrom T & Eden C (1985) Protective factors in milk and the development of the immune system. *Pediatrics* **75**, 172-176.

Hanson L, Brinton C, Carlsson B, Dahlgren U, Mellander l, Sutton A & Soderstrom T (1982) The mucosal immune response in the neonate. *Acta Paediatrica Scandanavia* **296**, 53-55.

Haque Z & Kinsella J (1988). Interaction between heated κ-casein and β-lactoglobulin: predominance of hydrophobic interactions in the initial stages of complex formation. *Journal of Dairy Research* **55**, 67-80.

Hashizume S, Kouroda K & Murakami H (1983) Identification of lactoferrin as an essential growth factor for human monocytic cell lines in serum-free medium. *Biochimica et Biophysica Acta* **763**, 377-382.

Havea P, Singh H & Creamer L (2000) Formation of new protein structures in heated mixtures of bovine serum albumin and α-lactalbumin. *Journal of Agricultural and Food Chemistry* **48**, 1548-1556.

He F, Tuomola E, Arvilommi H & Salmenin S (2001) Modulation of human humoral immune response through orally administered bovine colostrum. *Federation of European Microbiological Societies Immunology and Medical Microbiology* **31**, 93-96.

He J & Furmanski P (1995) Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. *Nature* **373**, 721-724.

Heine W, Klein P & Reeds J (1991) The importance of alpha-lactalbumin in infant nutrition. *Journal of Nutrition* **121**, 277.

Hemly R, Kulhler B & Siviski P (1979) Absorption spectra for the complexes from vitamin A and beta-lactoglobulin. *Biophysics* **28**, 447-451.

Hernandez-Trujillo V, Nguyen W, Belleau J, Jeng M, Conley M & Lew D (2004) Cow's milk allergy in a patient with hyper-IgE syndrome. *Annals of Allergy Asthma and Immunology* **92**, 469-474.

Hilliam M (1998) The market for functional foods. *International Dairy Journal* **8**, 349-353.

Hoffman-Goetz L, Bell R & Keir R (1985) Effect of protein on malnutrition and interleukin-1 on *in vitro* rabbit lymphocyte mitogenesis. *Nutrition Research* **5**, 769-780.

Holt C, McPhail D, Nylander T, Otte J, Ipsen R, Bauer R, Ogendal L, Olieman K, de Kruif K, Leonil J, Molle D, Henry G, Maubois J, Perez M, Puyol P, Calvo M, Bury S, Kontopidis G, McNae I, Sawyer L, Ragona L, Zetta L, Molinari H, Klarenbeek B, Jonkman M, Moulin J & Chatterton D (1999a) Apparent chemical composition of nine commercial or semi-commercial whey protein concentrates, isolates and fractions. *International Journal of Food Science and Technology* **34**, 543-556.

Holt C, McPhail D, Nylander T, Otte J, Ipsen R, Bauer R, Ogendal L, Olieman K, de Kruif K, Leonil J, Molle D, Henry G, Maubois J, Perez M, Puyol P, Calvo M, Bury S, Kontopidis G, McNae I, Sawyer L, Ragona L, Zetta L, Molinari H, Klarenbeek B, Jonkman M, Moulin J & Chatterton D (1999b) Some physico-chemical properties of nine commercial or semi-commercial whey protein concentrates, isolates and fractions. *International Journal of Food Science and Technology* **34**, 587-601.

Huffman L (1996) Processing whey protein for use as a food ingredient. *Food Technology* **50**, 49-52.

Hughes D (2001) Dietary carotenoids and human immune function. *Nutrition* **17**, 823-827.

Hugunin A (1987) Applications of U.F. whey protein: Developing new markets. In *Trends in Whey Utilisation.* Brussels: International Dairy Federation.

Huppertz T, Fox P & Kelly A (2004) High pressure treatment of bovine milk: effects on casein micelles and whey proteins. *Dairy Research* **71**, 97-106.

Hutchens T, Henry J & Yip T (1991) Origin of intact lactoferrin and its DNA-binding fragments found in the urine of human-milk fed preterm infants. *Pediatric Research* **29**, 243-250.

Ip C, Chin S, Scimeca J & Pariza M (1991) Mammary cancer prevention by a conjugated dienoic derivative of linoleic acid. *Cancer Research* **51**, 6118-6124.

Isamida T, Tanaka T, Omata Y, Yamauchi K, Shimazaki K & Saito A (1998) Protective effect of lactoferricin against *Toxoplasma gondii* infection in mice. *Journal of Veterinary Medical Science* **60**, 241-244.

Isolauri E, Joensus J, Suomalainen H, Luomala M & Vesikari T (1995) Improved immunogenicity of oral D XRRV reabsorbent rotavirus vaccine by *Lactobacillus casei* GG. *Vaccine* **13**, 310-312.

Janas L, Pacciano M & Hatch T (1985) Indices of protein metabolism in term infants fed human milk, whey predominant formula, or cow's milk formula. *Pediatrics* **75**, 775-784.

Janeway C, Travers P, Walport M & Capra J (1999) *Immunobiology, Fourth Edition*. London: Elsevier Science.

Jaziri M, Migliore-Samour D, Casabianca-Pignede M, Keddad K, Morgat J & Jolles P (1992) Specific binding sites on human phagocytic blood cells for gly-leu-phe and val-glu-pro-ile-pro-tyr, immunostimulating peptides from human milk proteins. *Biochimica et Biophysica Acta* **1160**, 251-261.

Jenness R (1979) Comparative aspects of milk proteins. *Journal of Dairy Research* **46**, 197-210.

Ji T & Haque Z (2003) Cheddar whey processing and source. I: Effect on composition and functional properties of whey protein concentrates. *International Journal of Food Science and Technology* **38**, 453-461.

John A & Bell J (1976) Amino acid requirements of the growing mouse. *Journal of Nutrition* **106**, 1361-1367.

Jolles P, Fiat A, Migliore-Samour D, Drouet L & Caen J (1993) Peptides from milk proteins implicated in anti-thrombosis and immunomodulation. In *New Perspectives in Infant Nutrition Symposium, Antwerp 1992* [B Renner and G Sawatzki, editors]. New York: Thieme Medical Publishers Incorporated.

Jolles P, Parker F, Floch F, Migliore D, Alliel P, Zerial A & Werner G (1981-1982) Immunostimulating substances from human casein. *Journal of Immunopharmacology* **3**, 363-369.

Juto P (1985) Human milk stimulates B-cell function. *Archives of Disease in Childhood* **60**, 610-613.

Jyonouchi H (1994) Nucleotide actions on humoral immune responses. *Journal of Nutrition* **124**, 138S-142S.

Jyonouchi H, Sun S, Winship T & Kuchan M (2001) Dietary ribonucleotides modulate type 1 and type 2 T-helper cell responses against ovalbumin in young BALB/c mice. *Journal of Nutrition* **131**, 1165-1170.

Kaila M, Isolauri E, Soppi E, Virtanen E, Laine S & Arvilommi H (1992) Enhancement of the circulating antibody secreting cell response in human diarrhoea by a human lactobacillus strain. *Pediatric Research* **32**, 141-144.

Kanyshkova T, Buneva V & Nevinsky G (2001) Lactoferrin and its biological functions. *Biochemistry* **66**, 1-7.

Kayser H & Meisel H (1996) Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins. *Federation of European Biochemical Societies Letters* **383**, 18-20.

Kennedy R, Konok G, Bounous G, Baruchel S & Lee T (1995) The use of a whey protein concentrate in the treatment of patients with metastatic carcinoma: A phase I-II clinical study. *Anticancer Research* **15**, 2643-2650.

Kijlstra A & Jeurissen S (1982) Modulation of classical C3 convertase of complement by tear lactoferrin. *Immunology* **47**, 263-270.

Kilara A (1994) Whey protein functionality. In *Protein Functionality in Food Systems* [N Hettiarachchy & G Ziegler, editors]. New York: Marcel Dekker Incorporated.

Kilara A & Panyam D (2003) Peptides from milk proteins and their properties. *Critical Reviews in Food Science and Nutrition* **43**, 607-633.

Kinsella J & Whitehead D (1989) Protein in whey: chemical, physical and functional properties. *Advances in Food Nutrition Research* **33**, 343-438.

Klaus G & Humphrey J (1986) A re-evaluation of the role of C3 in B-cell activation. *Immunology Today* **167**, 163-165.

Kobayashi T, Ohmori T, Yanani M, Kawanashi G, Yoshikai Y & Nomoto K (1991) Protective effect of orally administering immune milk on endogenous infection in X-irradiated mice. *Agricultural Biology and Chemistry* **55**, 2265-2272.

Kobayashi T, Yamamoto M, Hiroi T, McGhee J, Takeshita Y & Kiyono H (1998) Arginine enhances induction of T-helper 1 and T-helper 2 cytokine synthesis by Peyer's patch T cells and antigen-specific mucosal immune response. *Bioscience, Biotechnology & Biochemistry* **62**, 2334-2340.

Kodama M, Kodama T, Murakami M & Kodama M (1994) Autoimmune disease and allergy are controlled by vitamin C treatment. *In Vitro* **8**, 251-257.

Korhonen H (1995) Whey as a raw material for development of new products for human nutrition and health. In *Proceedings of the Nordisk Jordbrugsforskning/Nordic Council of Ministers-seminar*, pp. 207-219. Finland: Nordisk Jordbrugsforskning.

Korhonen H, Marnila P & Gill H (2000) Milk immunoglobulins and complement factors. *British Journal of Nutrition* **84**, S75-S80.

Korhonen H, Pihlanto-Leppala A, Rantamaki P & Tuomo T (1998) The functional and biological properties of whey proteins: Properties for the development of functional foods. *Agricultural and Food Science in Finland* **7**, 283-296.

Kudoh K, Shimizu J, Wada M, Takita T, Kanke Y & Innami S (1998) Effect of indigestible saccharides on B-lymphocyte response of intestinal mucosa and caecal fermentation in rats. *Journal of Nutritional Science and Vitaminology* **44**, 103-112.

Kuhara T, Ligo M, Ito T, Ushida Y, Sekine K, Terada M, Okamura H & Tsuda H (2001) Orally administered lactoferrin exerts an anti-metastatic effect and enhances production of interleukin-18 in the intestinal epithelium. *Nutrition and Cancer* **38**, 192-199.

Kulczycki A Jr & MacDermott R (1985) Bovine IgG and human immune responses: Con A-induced mitogenesis of human mononuclear cells is suppressed by bovine IgG. *International Archives of Allergy and Applied Immunology* **77**, 255-8.

Kulczycki A Jr, Nash G, Bertovich M, Burack H & MacDermott R (1987) Bovine milk IgG, but not serum IgG, inhibits pokeweed mitogen-induced antibody secretion by human peripheral blood mononuclear cells. *Journal of Clinical Immunology* **1**, 37-45.

Kulkarni A, Rudolph F & Van Buren C (1994) The role of dietary sources of nucleotides in immune function: A review. *Journal of Nutrition* **124**, 1442S-1446S.

Kulmyrzaev A, Bryant C & McClements D (2000) Influence of sucrose on the thermal denaturation, gelation, and emulsion stabilization of whey proteins. *Journal of Agricultural and Food Chemistry* **48,** 1593-1597.

Kuwata H, Yamauchi K, Teraguchi S, Ushida Y, Shimokawa Y, Toida T & Hayasawa H (2001) Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the gastrointestinal tract of adult rats. *The Journal of Nutrition* **131**, 2121-2127.

Lacey J & Wilmore D (1990) Is glutamine a conditionally essential amino acid? *Nutrition Reviews* **48**, 297-309.

Laugesen M & Elliott R (2003) Ischaemic heart disease, Type 1 diabetes, and cow's milk A1 β-casein. *The New Zealand Medical Journal* **116,** 1168.

Law A & Leaver J (2000) Effect of pH on the thermal denaturation of whey proteins in milk. *Journal of Agricultural and Food Chemistry* **48**, 6722-679.

Lesourd B (1997) Nutrition and immunity in the elderly: modification of immune responses with nutritional treatments. *American Journal of Clinical Nutrition* **66**, 478S-484S.

Legrand C, Capiaumont J, Belleville F & Nabet P (1993) Comparison of metabolism of hybridoma cells cultured in media supplemented with whey or foetal calf serum. *Biotechnology Progress* **9**, 573-579.

Lehmann W, Widmaier R & Lange P (1989) Response of different mammary epithelial cell lines to a mammary derived growth inhibitor. *Biochimica et Biophysica Acta* **48**, 143-151.

Leveugle B, Mazurier J, Legrand C, Mazurier C, Montreuil J & Spik G (1993) Lactoferrin binding to its platelet receptor inhibits platelet aggregation. *European Journal of Biochemistry* **213**, 1205-1211.

Levy J (1998) Immunonutrition: The paediatric experience. *Nutrition* **14**, 641-647.

Licastro F, Chiricolo M, Mocchegiani E, Fabris N, Zannoti M, Beltrandi E, Mancini R, Parente R, Arena G & Masi M (1994) Oral zinc supplementation in Down's syndrome subjects decreased infections and normalised some humoral and cellular immune parameters. *Journal of Intellectual Disability Research* **38**, 149-62.

Lim B, Yamada K, Nonaka M, Kuramoto Y, Hung P & Sugano M (1997) Dietary fibres modulate indices of intestinal immune functions in rats. *Journal of Nutrition* **127**, 663-667.

Lima M & Kierszenbaum F (1985) Lactoferrin effects on phagocytic function. I: Increased uptake and killing of extracellular parasite by murine macrophages and human monocytes. *Journal of Immunology* **134**, 4176-4183.

Lima M & Kierszenbaum F (1987) Lactoferrin effects on phagocytic cell function. II: The presence of iron is required for lactoferrin molecule to stimulate intracellular killing by macrophages but not enhance uptake of particles and micro-organisms. *Journal of Immunology* **139**, 1647-1651.

Link-Amster H, Rochat F, Saudan K, Migno O & Aeschlimann J (1994) Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *Federation of European Microbiological Societies Immunology and Medical Microbiology* **10**, 55-64.

Lloyd J, Gill H & Husband A (1995) The effect of storage on immunophenotyping of sheep lymphocytes by flow cytometry. *Veterinary Immunology and Immunopathology* **47**, 131-142.

Loimaranta V, Laine M, Soderling E, Vasara E, Rokka S, Marnila P, Korhonen H, Tossavainen O & Tenovuo J (1999) Effects of bovine immune and non-immune whey preparations on the composition and pH responses of human dental plaque. *European Journal of Oral Science* **107**, 224-250.

Lonnerdal B & Iyer S (1995) Lactoferrin: molecular structure and biological function. *Annual Review of Nutrition* **15**, 93-110.

Lonnerdal B (1996) Lactoferrin in milk. *Annales Nestle* **54**, 79-87.

Lorenz E & Good R (1993) Nutritional indicators for cancer prevention-calorie restriction. In *Nutrient Modulation of the Immune Response* [S Cunningham-Rundles, editor]. New York: Marcel Dekker Incorporated.

Low P, Rutherfurd K, Cross M & Gill H (2001) Enhancement of mucosal antibody responses by dietary whey protein concentrate. *Food and Agricultural Immunology* **13**, 255-264.

Magnuson J, Henry J, Yip T & Hutchens T (1990) Structural homology of human, bovine and porcine milk lactoferrins: Evidence for shared antigen determinants. *Pediatric Research* **28**, 176-181.

Mahe S, Roos N, Benamouzig R, Davin L, Luengo C, Gagnon L, Gausserges N, Rautureau J & Tome D (1996) Gastrojejunal kinetics and the digestion of [15N]beta-lactoglobulin and casein in humans: the influence of the nature and quantity of the protein. *The American Journal of Clinical Nutrition* **63**, 546-552.

Majamma H & Isolauri E (1997) Probiotics: a novel approach in the management of food allergy. *Journal of Allergy and Clinical Immunology* **99**, 179-185.

Malave I & Layrisse M (1976) Immune response in malnutrition. Differential effect of dietary protein restriction on the IgM and IgG response to alloantigens. *Cellular Immunology* **21**, 337-343.

Mann P (1978) The effect of various dietary restricted regimes on some immunological parameters of mice. *Growth* **42**, 87-103.

Maruyama S, Mitacki H, Awaya J, Kurono M, Tomizuka N & Suzuki H (1987) Angiotensin I-converting enzyme inhibitory activity of the C-terminal hexapeptide of alpha S1 casein. *Agricultural and Biological Chemistry* **51**, 2557-2561.

Masson P & Heremans J (1971) Lactoferrin in milk from different species. *Comparative Biochemistry and Physiology* **39**, 119-129.

Mati A, Moulti-Mati F, Girardet J, Fokou E, Belleville-Nabet F, Nabet P & Linden G (1993) Mitogenic activity of hydrophobic fractions of proteose-peptone from cow's, ewe's and goat's milk measured with Mark-3 hybridoma culture. *Journal of Dairy Science* **60**, 443-448.

Matsuzaki T, Yamazaki R, Hashimoto S & Yokokura T (1998) The effect of oral feeding of *Lactobacillus casei* strain Shirota on immunoglobulin E production in mice. *Journal of Dairy Science* **81**, 48-53.

Matsuzaki T & Chin J (2000) Modulating immune responses with probiotic bacteria. *Immunology and Cell biology* **78**, 67-73.

Mattsby-Baltzer I, Roseanu A, Motaas C, Elverfors J, Engberg I & Hanson L (1996) Lactoferrin or a fragment thereof inhibits the endotoxin-induced interleukin-6 response in human monocytic cells. *Pediatric Research* **40**, 257-262.

Maubois J & Ollivier G (1997) Extraction of milk proteins. In *Food Proteins and Their Applications*, pp. 579-595 [S Damodaran and A Paraf, editors]. New York: Marcel Dekker Incorporated.

Mazurier J, Legrand C, Hu W, Montreuil J & Spick G (1989) Expression of human lactoferrin receptors in phytohaemagglutinin-stimulated human peripheral blood lymphocytes. *European Journal of Biochemistry* **179**, 481-497.

McDowell E, Keenan K & Huang M (1984) Effect of vitamin A deprivation on hamster tracheal epithelium. A quantitative morphologic study. *Virchows Archives of B-cell Pathology Including Molecular Pathology* **45**, 197-219.

McIntosh G, Regester G, Le Leu R, Royle P & Smithers G (1995) Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. *Journal of Nutrition* **125**, 809-816.

McIntosh G, Royle P, Leu R, Regester G, Johnson M, Grinsted R, Kenward R & Smithers G (1998) Whey proteins as functional food ingredients? *International Dairy Journal* **8**, 425-434.

Meisel H (1998) Overview on milk protein-derived peptides. *International Dairy Journal* **8**, 363-373.

Meisel H & Schlimme E (1990) Milk proteins: Precursors of bioactive peptides. *Trends in Food Science and Technology* **August**, 41-43.

Meister A & Anderson M (1983) Glutathione. *Annual Review of Biochemistry* **52**, 711-760.

Menne E, Guggenbuhl N & Roberfroid M (2000) Fn-type chicory insulin hydrolysate has a prebiotic effect in humans. *Journal of Nutrition* **130**, 1197-1199.

Mercier A, Gauthier S & Fliss I (2004) Immunomodulatory effects of whey proteins and their enzymatic digests. *International Dairy Journal* **14**, 175-183 .

Mestecky J, Abraham R & Ogra P (1994) Common mucosal immune system and strategies for the development of vaccines effective at the mucosal surfaces. In *Handbook of Mucosal Immunity*, pp. 391-402 [P Ogra, J Mestecky and M Lamm, editors]. USA: Academic Press.

Meydani S, Maydani M, Verdon C, Shapiro A, Blumberg J & Hayes K (1986) Vitamin E supplementation suppresses prostaglandin $E1_2$ synthesis and enhances the immune response of aged mice. *Mechanisms of Ageing and Development* **34**, 191-201.

Meydani S (1996) Effect of (n-3) polyunsaturated fatty acids on cytokine production and their biological function. *Nutrition* **12**, S8-S14.

Micke P, Beeh K, Schlaak J & Buhl R (2001) Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. *European Journal of Clinical Investigation* **31**, 171-178.

Migliore-Samour D, Floch F & Jolles P (1989) Biologically active casein peptides implicated in immunomodulation. *Journal of Dairy Research* **56**, 357-362.

Migliore-Samour D & Jolles P (1988) Casein, a prohormone with an immunomodulating role for the newborn? *Experientia* **44**, 188-193.

Migliore-Samour D, Roch-Arveiller M, Tissot M, Jaziri M, Keddad K, Giroud J & Jolles P (1992) Effects of tripeptides derived from milk proteins on polymorphonuclear oxidative and phosphoinositide metabolisms. *Biochemical Pharmacology* **44**, 673-680.

Miles E & Calder P (1988) Modulation of immune function by dietary fatty acids. *Proceedings of the Nutrition Society* **57**, 277-292.

Miller C, Park Y, Pariza M & Cook M (1994) Feeding conjugated linoleic acid to animals partially overcomes catabolic responses to endotoxin injection. *Biochemical and Biophysical Research Communications* **198**, 1107-1112.

Mincheva-Nilsson L, Hammarstrom M, Juto P & Hammarstrom S (1990) Human milk contains proteins that stimulate and suppress T-lymphocyte proliferation. *Clinical Experimental Immunology* **79**, 463-469.

Minehira K, Inoue S, Nonaka M, Osada K, Yamada K & Sugano M (2000) Effects of dietary protein type on oxidised cholesterol-induced alteration in age-related modulation of lipid metabolism and indices of immune function in rats. *Biochimica et Biophysica Acta* **1483**, 141-153.

Miyauchi H, Kaino A, Shinoda I, Fukuwatri Y & Hayasawa H (1997) Immunomodulatory effect of bovine lactoferrin pepsin hydrolysate on murine splenocytes and Peyer's patch cells. *Journal of Dairy Science* **80**, 2330-2339.

Monnai M, Horimoto Y & Otani H (1998) Immunomodificatory effect of dietary bovine kappa-caseinoglycopeptide on serum antibody levels and proliferative responses of lymphocytes in mice. *Milchwissenschaft* **53**, 129-131.

Monnai M & Otani H (1997) Effect of bovine kappa-caseino-glycopepetide on secretion of interleukin-1 family cytokines by P388D1 cells, a line derived from mouse monocyte/macrophage. *Milchwissenschaft* **52**, 192-196.

Morr C, Swenson P & Richter R (1973) Functional characteristics of whey protein concentrates. *Journal of Food Science* **38**, 324-330.

Morr C (1984) Production and use of milk proteins in food. *Food Technology* **38**, 39-48.

Morr C (1985) Manufacture, functionality and utilisation of milk protein products. In *Milk Proteins '84,* p.171. The Netherlands: Pudoc, Wageningen.

Morr C (1992) Improving the texture and functionality of whey protein concentrate. *Food Technology* **January**, 110-113.

Morr C & Foegeding E (1990) Composition and functionality of commercial whey and milk protein concentrates and isolates: A status report. *Food Technology* **44**, 100-112.

Morr C & Ha E (1993) Whey protein concentrates and isolates: Processing and functional properties. *Critical Reviews in Food Science and Nutrition* **36**, 431-476.

Morrissey P & Brien N (1998) Dietary antioxidants in health and disease. *International Dairy Journal* **8**, 463-472.

Mottar J, Bassier A, Joniau M & Baert J (1989) Effect of heat induced association of whey proteins and casein micelles on yogurt texture. *Journal of Dairy Science* **72**, 2247-2256.

Mullally M, Meisel H & FitzGerald R (1996) Synthetic peptides corresponding to α-lactalbumin and β-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity. *Biological Chemistry Hoppe-Seyler* **377**, 259-260.

Murakami H (1989) Serum-free media for cultivation of hybridomas. In *Monoclonal Antibodies: Production and Application*, pp. 107-141 [A Mizrahi, editor]. New York: Alan R. Liss.

Nagafuchi S, Katayanagi T, Nakagawa E, Takahashi T, Yajima T, Yonekubo A & Kuwata T (1997) Effects of dietary nucleotides on serum antibody and splenic cytokine production in mice. *Nutrition Research* **17**, 1163-1174.

Newby T, Stokes C & Bourne F (1982) Immunological activities of milk. *Veterinary Immunology and Immunopathology* **3**, 67-94.

Nishiya K & Horwitz D (1982) Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody-dependent cell mediated cytotoxicity. *Journal of Immunology* **129**, 2519-2523.

Noelle R & Lawrence D (1981) Determination of glutathione in lymphocytes and possible association of redox state and proliferative capacity of lymphocytes. *Biochemistry Journal* **198**, 571-579.

O'Brien J & Morrissey P (1989) The Maillard reaction in milk products. *Bulletin of the International Dairy Federation* **238**, 53-61.

Ogra S & Ogra P (1978) Immunological aspects of human colostrum and milk. *Journal of Pediatrics* **92**, 550-555.

Oppenheimer S (2001) Iron and its relation to immunity and infectious disease. *Journal of Nutrition* **131**, 616S-633S.

Oria R, Alvarez-Hemandez X, Liceaga J & Brock J (1988) Uptake and handling of iron from transferrin, lactoferrin and immune complexes by a macrophage cell line. *Biochemical Journal* **252**, 221-225.

Oria R, Ismail M, Sanchez L, Calvo M & Brock J (1993) Effect of heat treatment and other milk proteins on the interaction of lactoferrin with monocytes. *Journal of Dairy Research* **60**, 363-369.

Oster K (1971) Plasmalogen diseases. A new concept of the aetiology of the atherosclerotic process. *American Journal of Clinical Research* **2,** 30-35.

Otani H & Futakami M (1994) Effects of bovine milk proteins on the phagocytic property and formation of nitrite by mouse peritoneal macrophages. *Animal Science and Technology* **65**, 423-431.

Otani H & Futakami M (1996) Modification of nitrite production and phagocytosis of thioglycollate-elicited mouse peritoneal macrophages by bovine casein digests. *Food and Agricultural Immunology* **8**, 59-69.

Otani H, Horimoto Y & Monnai M (1996) Suppression of interleukin-2 receptor expression on mouse $CD4^+$ T cells by bovine kappa-caseinoglycopeptide. *Bioscience, Biotechnology & Biochemistry* **60**, 1017-1019.

Otani H, Kihara Y & Park M (2000) The immunoenhancing property of a dietary casein phosphopeptide preparation in mice. *Food and Agricultural Immunology* **12**, 165-173.

Otani H & Monnai M (1993) Inhibition of proliferative responses of mouse spleen lymphocytes by bovine milk kappa-casein digests. *Food and Agricultural Immunology* **5**, 219-229.

Otani H & Monnai M (1995) Induction of an interleukin-1 receptor antagonist-like component produced from mouse spleen cells by bovine kappa-caseinoglycopeptide. *Bioscience, Biotechnology & Biochemistry* **59**, 1166-1168.

Otani H, Monnai M & Hosono A (1992) Bovine kappa-casein as an inhibitor of the proliferation of mouse splenocytes induced by lipopolysaccharide stimulation. *Milchwissenschaft* **47**, 512-515.

Otani H, Monnai M, Kawasaki Y, Kawakami H & Tanimoto M (1995) Inhibition of mitogen induced proliferative responses of lymphocytes by bovine kappa-caseinoglycopeptides having different carbohydrate chains. *Journal of Dairy Research* **62**, 349-357.

Otani H & Odashima M (1997) Inhibition of proliferative responses of mouse spleen lymphocytes by lacto- and ovotransferrins. *Food and Agricultural Immunology* **9**, 193-201.

Otani H & Yamada Y (1994) Characterization of bovine colostral proteins with inhibitory activity in passive anaphylaxis. *Milchwissenschaft* **49**, 20-24.

Otani H & Yamada Y (1995) Effects of bovine kappa-casein and lactoferrins on several experimental models of allergic disease. *Milchwissenschaft* **50**, 549-553.

Paakkari I, Jarvinen A, Antila P, Mattila M & Pihlanto-Leppala A (1994) Opioid effects of the milk-whey protein derived peptides α- and β-lactorphin. In *β-Casomorphins and Related Peptides. Recent Developments* [V Brantl and H Teschemacher, editors]. New York: Wiley.

Pablo M & Cienfuegos A (2000) Modulatory effects of dietary lipids on immune system functions. *Immunology and Cell Biology* **78**, 31-39.

Palazolo G, Rodriguez F, Farruggia B, Pico G & Delorenzi N (2000) Heat treatment of β-lactoglobulin: structural changes studied by partitioning and fluorescence. *Journal of Agricultural and Food Chemistry* **48**, 3817-3822.

Papenburg R, Bounous G, Fleiszer D & Gold P (1990) Dietary milk proteins inhibit the development of dimethylhydrazine-induced malignancy. *Tumour Biology* **11**, 129-136.

Parker F, Migliore-Samour D, Floch F, Zerial A, Werner G, Jolles J, Casaretto M, Zahn H & Jolles P (1984) Immunostimulating hexapeptide from human casein: Amino acid sequence, synthesis and biological properties. *European Journal of Biochemistry* **145**, 677-682.

Parker N & Goodrum K (1990) A comparison of casein, lactalbumin and soy protein effect on the immune response to a T-dependent antigen. *Nutrition Research* **10**, 781-792.

Parodi P (1996) Milk fat components: possible chemopreventive agents for cancer and other disease. *The Australian Journal of Dairy Technology* **51,** 24-32.

Parodi P (1997) Cow's milk fat components as potential anticarcinogenic agents. *Journal of Nutrition* **127**, 1055-1060.

Parodi P (1998) A role for milk proteins in cancer prevention. *Australian Journal of Dairy Technology* **53**, 37-47.

Parry-Billings M, Evans J, Calder P & Newsholme E (1990) Does glutamine contribute to immunosuppression after major burns? *Lancet* **336**, 523-525.

Peng H, Su S, Tsai J, Tsai L, Kuo H & Kuo S (2004) Effect of ingestion of cow's milk hydrolysed formulas on whey protein-specific Th2 immune responses in naive and sensitised mice. *Clinical and Experimental Allergy* **34**, 663-670.

Penttila I, Zhang M, Bates E, Regester G, Read L & Zola H (2001) Immune modulation in suckling rat pups by a growth factor extract derived from milk whey. *Journal of Dairy Research* **68**, 587-599.

Penn N, Purkins L, Kelleher J, Heatley R, Mascie-Taylor B & Belfield P (1991) The effect of dietary supplementation with vitamins A, C and E on cell-mediated immune function in elderly long-stay patients: a randomized controlled trial. *Age and Ageing* **20**, 169-174.

Perdigon G, Vintini E, Alvarez S, Medina M & Medici M (1999) Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. *Journal of Dairy Science* **82**, 1108-14.

Perez M, Sanchez L, Aranda O, Ena J & Oria R (1992) Effect of beta-lactoglobulin on the activity of pregastric lipase. A possible role for this protein in ruminant milk. *Biochimica et Biophysica Acta* **1123**, 151-155.

Peterson M (2003) *Campylobacter jejuni* enteritis associated with consumption of raw milk. *Environmental Health* **65**, 20-21.

Petro T & Watson R (1982) Dietary stress and development of resistance to *Listeria monocytogenes* in mice. *Journal of Nutrition* **112**, 1498-1505.

Polet H & Spieker-Polet H (1976) Mechanism of the growth-promoting effect of serum albumin on concanavalin A-activated lymphocytes: protective effects of plasma proteins. *Journal of Immunology* **117**, 1275-1281.

Politis I, Zhao X, McBride B & Burton J (1991) Effect of bovine skim milk and whey on monocyte function. *Journal of Dairy Science* **74**, 2467-2471.

Potter J (1996) Nutrition and colorectal cancer. *Cancer Causes and Control* **7**, 127-146.

Prasad A (1988) Zinc and immunity. *Molecular and Cellular Biochemistry* **188**, 63-69.

Rank P (1986) Milk and arteriosclerosis. *Medical Hypotheses* **20,** 317-38.

Rassin D, Gaull G, Raiha N & Heinonen K (1977) Milk protein quantity and quality in low-birth-weight infants. *The Journal of Pediatrics* **90**, 356-360.

Rayner T, Cowin A, Robertson J, Cooter R, Harries R, Regester G, Smithers G, Goddard C & Belford D (2000) Mitogenic whey extract stimulates wound repair activity *in vitro* and promotes healing of rat incisional wounds. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **278**, R1651-R1660.

Regester G, Pearce R, Lee V & Mangino M (1992) Heat related changes to the hydrophobicity of cheese whey correlate with levels of native β-lactoglobulin and α-lactalbumin. *Journal of Dairy Science* **59**, 527-532.

Regester G, McIntosh G, Lee V & Smithers G (1996) Whey proteins as nutritional and functional food ingredients. *Food Australia* **48**, 123-127.

Reiter B (1985) The biological significance of the non-immunoglobulin protective proteins in milk. *Developments in Dairy Chemistry* **3**, 281-336.

Reiter B, Brock J & Steel E (1975) Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. The bacteriostatic effect of lactoferrin on a serum susceptible and serum resistant strain of *E. coli*. *Immunology* **28**, 83-95.

Rejman J & Oliver S (1993) Bimodal effects of lactoferrin on the proliferation of an interleukin-2-dependent cytotoxic T-lymphocyte cell line. *Food and Agricultural Immunology* **5**, 123-128.

Rejman J, Oliver S, Muenchen R & Turner J (1992a) Proliferation of the MAC-T bovine mammary epithelial cell line in the presence of mammary secretion whey proteins. *Cell Biology International Reports* **16**, 993-1001.

Rejman J, Payne K, Torre P, Muenchen R & Oliver S (1992b) Identification of whey proteins that influence bovine blood mononuclear cell proliferation. *Journal of Dairy Science* **75**, 308.

Renard D, Lefevre J, Griffin M & Griffin W (1998) Effects of pH and salt environment on the association of β-lactoglobulin revealed by intrinsic fluorescence studies. *International Journal of Biological Macromolecules.* **22**, 41-49.

Richie E, Hilliard J, Gilmeor R & Gillespie D (1987) Human milk derived lactoferrin inhibits mitogen and alloantigen induced human lymphocyte proliferation. *Journal of Reproductive Immunology* **12**, 137-148.

Roberfroid M (2000) Prebiotics and probiotics: Are they functional foods? *American Journal of Clinical Nutrition* **71**, 1682S-1687S.

Roitt I (1996) *Essential Immunology, Eighth edition.* Oxford: Blackwell Science Ltd.

Rose A, Holt P & Turner K (1982) The effect of low protein diet on the immunogenic activity of murine peritoneal macrophages. *International Archives of Allergy Applied Immunology* **67**, 356-361.

Rose D & Connolly J (1999) Omega-3 fatty acids as cancer chemopreventative agents. *Pharmacology and Therapeutics* **83**, 217-244.

Rosenthal A (1999) Interleukin-2, its receptor and nutrition in older adults; a review. *Journal of Nutrition Health and Aging* **3**, 182-185.

Ross A (1992) Vitamin A status: Relationship to immunity and the antibody response. *Proceedings of the Society of Experimental Biology and Medicine* **200**, 303-320.

Rumore M (1993) Vitamin A as an immunomodulating agent. *Clinical Pharmacology* **12**, 506-514.

Rutherfurd K & Gill H (2000) Peptides affecting coagulation. *British Journal of Nutrition* **84**, S99-S101.

Sadler R (1992) The benefits of dietary whey protein concentrate on the immune response and health. *South African Journal of Dairy Science* **24**, 53-59.

Samelson S, Nelson R & Nyhus L (1985) Protective role of faecal pH in experimental colon carcinogenesis. *Journal of the Royal Society of Medicine* **78**, 230-233.

Sanders M (1998) Overview of functional foods: Emphasis on probiotic bacteria. *International Dairy Journal* **8**, 341-347.

Sanderson I & Walker A (1993) Uptake and transport of macromolecules by the intestine: possible role in clinical disorders (an update). *Gastroenterology* **104**, 622-639.

Sarwar G, L'Abbe M Trick K, Blotting H & Ma C (1999) Influence of feeding alkaline/heat processed proteins on growth and mineral status of rats. *Advances in Experimental Medicine and Biology* **459**, 161-177.

SAS Version 6.12 (1997) SAS System under Microsoft Windows, Cary, NC: SAS Institute Incorporated.

Schanbacher F, Talhouk R, Murray F, Gherman L & Willett L (1998) Milk-borne bioactive peptides. *International Dairy Journal* **8**, 393-403.

Schilling J, Vranjes N, Fierz W, Joller H, Gyurech D, Ludwig E, Marathias K & Geroulanous S (1996) Clinical outcome and immunology of postoperative arginine, omega-3 fatty acids, and nucleotide-enriched enteral feeding: A randomised prospective comparison with standard enteral and low calorie/low fat *i.v.* solutions. *Nutrition* **12**, 432-429.

Schley P & Field C (2002) Immune-enhancing effects of dietary fibres and prebiotics. *British Journal of Nutrition* **87**, S221-S230.

Schmidt R, Packard V & Morris H (1984) Effect of processing on whey protein functionality. *Journal of Dairy Science* **67**, 2723–2733.

Schwager J & Schulze J (1998) Modulation of interleukin production by ascorbic acid. *Veterinary Immunology and Immunopathology* **64**, 45-57.

Sfeir R, Dubarry M, Boyaka P, Rautureau M & Tome D (2004) The mode of oral bovine lactoferrin administration influences mucosal and systemic immune responses in mice. *Journal of Nutrition* **134**, 403-409.

Shah N (2000) Effects of milk-derived bioactives: An overview. *British Journal of Nutrition* **84**, S3-S10.

Shantha N, Ram L, O'Leary J, Hicks C & Decker E (1995) Conjugated linoleic acid concentrations in diary products as affected by processing and storage. *Journal of Food Science* **60**, 696-697.

Shinoda I, Takase M, Fukuwatri Y, Shimamura S, Koller M & Konig W (1996) Effects of lactoferrin and lactoferricin on the release of interleukin-8 from human polymorphonuclear leucocytes. *Bioscience, Biotechnology & Biochemistry* **60**, 521-523.

Shimizu K, Matsuzawa H, Okada K, Tazume S, Dosako S, Kawasaki Y, Hashimoto K & Koga Y (1996) Lactoferrin mediated protection of the host from murine cytomegalovirus infection by a T cell-dependent augmentation of natural killer activity. *Archives of Virology* **141**, 1875-1889.

Smithers G, Ballard F, Copeland A, De Silva K, Dionysius D, Francis G, Goddard C, Grieve P, McIntosh G, Mitchell I, Pearce R & Regester G (1996) New opportunities from the isolation and utilisation of whey proteins. *Journal of Dairy Science* **79**, 1454-1459.

Spreer E & Mixa A (1998) *Milk and Dairy Product Technology.* New York: Marcel Dekker, Incorporated.

Sudharmarajan S, Bennett L, Wheatcroft R & Smithers G (2001) Effects of thermal and chemical modification followed by enzymatic digestion of dairy proteins on recognition by whey protein-specific antibodies. *Australian Journal of Dairy Technology* **56**, 133.

Stark A & Madar Z (2002) Olive oil as a functional food: Epidemiology and nutritional approaches. *Nutrition Reviews* **60**, 170-176.

Steijns J & Hooijdonk A (2000) Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *British Journal of Nutrition* **84**, S11-S17.

Steinhoff U, Senft B & Seyfert H (1994) Lysozyme-encoding bovine cDNAs from neutrophile granulocytes and mammary glands are derived from a different gene than stomach lysozymes. *Gene* **143**, 271-276.

Stelwagen K, Politis I, Guo M, Kindstendt P, Davis S & Farr V (1994) Mammary derived growth inhibitor in bovine milk: effect of milking frequency and somatotropin administration. *Canadian Journal of Animal Science* **74**, 695-698.

Stoeck M, Ruegg C, Miescher S, Carrel S, Cox D, Fliedner V & Alkan S (1989) Comparison of the immunosuppressive properties of milk growth factor and transforming growth factors B1 and B2. *Journal of Immunology* **143**, 3258-3265.

Stoltzner G & Dorsey B (1980) Life-long dietary protein restriction and immune function: responses to mitogens and sheep erythrocytes in BALB/c mice. *American Journal of Clinical Nutrition* 33, 1264-1271.

Stueden W, Slopek S, Mamczar A & Kaczmarek M (1983) Biological aspects of tuftsin activity. *Archivum Immunologiae et Therapiae Experimentalis* **31**, 575-578.

Svedberg J, de Haas J, Leimenstoll F & Teschemacher H (1985) Demonstration of beta-casomorphin immunoreactive materials in *in vitro* digests of bovine milk and in the small intestine contents after bovine milk ingestion in adult humans. *Peptides* **6**, 825.

Swaisgood H (1996) Characteristics of milk. In *Food Chemistry* [O Fennema, editor]. New York: Marcel Dekker Incorporated.

Takada Y, Aoe S & Kimeqawa M (1996) Whey protein stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. *Biochemical and Biophysical Research Communications* **223**, 445-449.

Takahashi T, Nakagawa E, Nara T, Yajima T & Kuwata T (1998) Effects of orally ingested *Bifidobacterium logum* on the mucosal IgA response of mice to dietary antigens. *Bioscience, Biotechnology & Biochemistry* **62**, 10-15.

Talbott M, Miller L & Kerkvliet N (1987) Pyridoxine supplementation: effect on lymphocyte responses in elderly persons. *American Journal of Clinical Nutrition* **46**, 659-664.

Tengerdy R (1989) Vitamin E, immune response, and disease resistance. *Annals of New York Academy of Science* **570**, 335-344.

Tengerdy R (1990) The role of vitamin E in the immune response and disease resistance. *Annals of New York Academy of Science* **587**, 24-33.

Teschemacher H, Koch G & Brantl V (1997) Milk protein-derived opioid receptor ligands. *Biopolymers* **43**, 99-117.

Thibault H, Galan P, Selz F, Preziosi P, Olivier C, Badoual J & Hercberg S (1993) The immune response in iron-deficient young children: effect of iron supplementation on cell-mediated immunity. *European Journal of Pediatrics* **152**, 120-124.

Toba Y, Takada Y, Matsuoka Y, Morita Y, Motouri M, Hirai T, Suguri T, Aoe S, Kawakami H, Kumegawa M, Takeuchi A & Itabashi A (2001) Milk basic protein promotes bone formation suppresses bone resorption in healthy adult men. *Bioscience, Biotechnology & Biochemistry* **65**, 1353-1357.

Tome D & Debabbi H (1998) Physiological effects of milk protein components. *International Dairy Journal* **8**, 383-392.

Tomita M, Wakabayashi H, Yamauchi K, Teraguchi S & Hayasawa H (2002) Bovine lactoferrin and lactoferricin derived from milk: Production and applications. *Biochemistry and Cell Biology* **80**, 109-112.

Tomita M, Bellamy W, Takase M, Wkaby A & Kawase K (1991) Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *Journal of Dairy Science* **74**, 4137-4142.

Torre P & Oliver S (1989) Suppression of mitogenic response of peripheral blood mononuclear cells by bovine mammary secretions. *Journal of Dairy Science* **72**, 219-227.

Torreilles J & Guerin M (1995) Casein-derived peptides promote peroxidase-dependent oxidation of human blood low-density lipoproteins. *Compte Rendu Des Seances De La Societe De Biologie Filiales* **189**, 933-42.

Troost F, Steijns J, Saris W & Brummer R (2001) Gastric digestion of bovine lactoferrin *in vivo* in adults. *The Journal of Nutrition* **131**, 2101-2104.

Turchany J, Aley S & Gillin F (1995) Giardicidal activity of lactoferrin and N-terminal peptides. *Infection and Immunity* **63**, 4450-4452.

van Doorne C, Eling W & Luyken R (1998) Rodent malaria in rats exacerbated by milk protein, attenuated by low protein vegetable diet. *Tropical Medical International Health* **60**, 596-600.

Vanderhoof J (1998) Immunonutrition: The role of carbohydrates. *Nutrition* **14**, 595-598.

Vorland L (1999) Lactoferrin: A multifunctional glycoprotein. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **107**, 971-981.

Wada T, Aiba Y, Shimizu K, Takagi A, Miwa T & Koga Y (1999) The therapeutic effect of bovine lactoferrin in the host infected with *Helicobacter pylori*. *Scandinavian Journal of Gastroenterology* **34**, 238-243.

Wakabayashi H, Takakura N, Kuwata T, Yamauchi K, Teraguchi S & Hayasawa H (2002) Analysis of leucocyte population changes in lactoferrin mice. *Biochemistry and Cell Biology* **80**, 159.

Walstra P & Jenness R (1984) Heating. In *Dairy Chemistry and Physics*. New York: Wiley Interscience.

Wan C, Park C & Lau B (1993) A rapid and simple microfluorometric phagocytosis assay. *Journal of Immunological Methods* **162**, 1-7.

Ward B (1996) Retinol (vitamin A) supplements in the elderly. *Drugs and Aging* **9**, 48-59.

Ward P, Uribe-Luna S & Conneely O (2002) Lactoferrin and host defence. *Biochemistry and Cell Biology* **80**, 95-102.

Waissbluth M & Grieger R (1974) Alkaline denaturation of β-lactoglobulins. Activation parameters and effect on dye binding site. *Biochemistry* **13**, 1285-1288.

Watson D, Francis G & Ballard F (1992) Factors in ruminant colostrum that influence cell growth and murine IgE antibody responses. *Journal of Dairy Research* **59**, 369-380.

Werner G, Floch F, Migliore-Samour D & Jolles P (1986) Immunomodulating peptides. *Experientia* **42**, 521-531.

Wills-Karp M (1999) Immunologic basis of antigen-induced airway hyperresponsiveness. *Annual Review of Immunology* **17**, 255-281.

Wilmore D & Shabert J (1998) Role of glutamine in immunologic responses. *Nutrition* **14**, 618-626.

Wong C, Liu A, Regester G, Francis G & Watson D (1997c) Influence of whey and purified whey proteins on neutrophil functions in sheep. *Journal of Dairy Research* **64**, 281-288.

Wong C, Regester G, Francis G & Watson D (1996c) Immunomodulatory activities of whey fractions in efferent prefemoral lymph of sheep. *Journal of Dairy Research* **63**, 257-267.

Wong C, Seow H, Husband A, Regester G & Watson D (1997b) Effects of purified bovine whey factors on cellular immune functions in ruminants. *Veterinary Immunology and Immunopathology* **56**, 85-96.

Wong C, Seow H, Liu A, Husband A, Smithers G & Watson D (1996a) Modulation of immune responses by bovine beta-casein. *Immunology and Cell Biology* **74**, 323-329.

Wong C & Watson D (1995) Immunomodulatory effects of dietary whey proteins in mice. *Journal of Dairy Science* **62**, 359-368.

Wong C & Watson D (1997) Effects of purified bovine whey factors on cellular immune functions in ruminants. *Veterinary Immunology and Immunopathology* **56**, 85-96.

Wong D, Camirand W & Pavlath A (1996b) Structures and functionalities of milk proteins. *Critical Reviews in Food Science and Nutrition* **36**, 807-844.

Wong K, Middleton N, Montgomery M, Dey M & Carr R (1998) Immunostimulation of murine spleen cells by materials associated with bovine milk protein fractions. *Journal of Dairy Science* **81**, 1825-1832.

Wong M, Chew B, Wong T, Hosick H, Boylston T & Shultz T (1997a) Effects of dietary conjugated linoleic acid on lymphocyte function and growth of mammary tumours in mice. *Anticancer Research* **17**, 987-994.

Yamada K, Matsumara K, Suzaki M, Shirahata S & Murakami H (1991) Stimulation and inhibition of interferon-β production of human diploid fibroblasts by food stuffs. *Agricultural and Biology Chemistry* **55**, 829-832.

Yamauchi K (1992) Biologically functional proteins of milk and peptides derived from milk proteins. *Bulletin of the International Dairy Federation* **272**, 51-58.

Yaqoob P (1998) Monosaturated fats and immune function. *Brazilian Journal of Medical and Biological Research* **31**, 452-465.

Yoshikawa M, Tani F, Yoshimura T & Chiba H (1986) Opioid peptides from milk proteins. *Agricultural and Biological Chemistry* **50**, 2419-2421.

Yun C, Estrada A, Van Kessel A, Gajadhar A, Redmond M & Laarveld B (1998) Immunomodulatory effects of oat B-glucan administered intragastrically or parenterally on mice infected with *Eimeria vermiformis*. *Microbiology and Immunology* **42**, 457-465.

Yun S, Sugita-Konishi Y, Kumagai S & Yamauchi K (1996) Isolation of mitogenic glycophosphopeptides from cheese whey protein concentrate. *Bioscience, Biotechnology and Biochemistry* **60**, 429-433.

Zavizion B, Politis I, Gorewit R, Turner J, Spitzer E & Grosse R (1993) Effect of mammary-derived growth inhibitor on proliferation of MAC-T bovine mammary epithelial cells. *Journal of Dairy Science* **76**, 3721-3726.

Zhang X, Hiller L & Woodward B (2002) The capacity of non-inflammatory (steady-state) dendritic cells to present antigen in the primary response is preserved in acutely protein- or energy-deficient weanling mice. *Journal of Nutrition* **132,** 2748-2756.

Zhang Y & Kolesnick R (1995) Signalling through the sphingomyelin pathway. *Endocrinology* **136**, 4157-4160.

Ziemer C & Gibson G (1998) An overview of probiotics, prebiotics and symbiotics in the functional food concept: Perspectives and future strategies. *International Dairy Journal* **8**, 473-479.

Zimecki M & Kruzel M (2000) Systemic or local co-administration of lactoferrin with sensitising dose of antigen enhances delayed type hypersensitivity in mice. *Immunology Letters* **74**, 183-8.

Zimecki M, Mazurier J, Machnicki M, Wieczorek Z, Montreuil J & Spik G (1991) Immunostimulatory activity of lactotransferrin and maturation of $CD4^-$ $CD8^-$ murine thymocytes. *Immunology Letters* **30**, 119-124.