The effect of bovine colostrum supplementation on levels of secretory immunoglobulin-A (S-IgA) in saliva of elite athletes, non-exercising controls and non-exercising older adults

A project completed as fulfilment of the
requirements of a doctoral thesis in clinical nutrition

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Abstract

Secretory immunoglobulin-A (S-IgA) in saliva may reflect levels of immune defence at other mucosal sites. Reduced levels of salivary S-IgA have been associated with an increased risk for upper respiratory symptoms (URS) in athletes. Previously, the consumption of a nutrition supplement, bovine colostrum (BC) by distance runners, was shown to significantly increase levels of salivary S-IgA compared to baseline; however the mechanism was not known. The immunomodulatory effect of BC is investigated further in these current studies.

Twenty-five swimmers (12 males [M], 13 females [F], age 14-23 years) training at an elite level, 28 lightly-exercising students (9M, 19F, age 18-27 years), and 45 healthy older adults (20M, 20F, age 65-76 years), consumed a supplement of either BC or placebo for ten weeks. Saliva samples were collected at baseline, weekly for four weeks during supplementation and post-supplementation. Blood samples were collected at baseline, monthly during supplementation and post-supplementation.

No significant changes were seen in levels of S-IgA in either BC or placebo groups within any of the cohorts. There was a trend towards a significant difference in URS reportage between BC and placebo groups in the swimmers cohort, but not in the students or older adults. There was also a trend towards a difference in the number of swimmers reporting URS. Fewer numbers of swimmers consuming BC reported URS compared the placebo (P=0.062) after consuming BC for four weeks compared to those consuming the placebo. Post-exercise plasma cortisol results were significantly reduced in the BC subgroup compared to the placebo (P=0.004).

These results do not support the findings of previous intervention studies investigating the immunomodulatory effect of BC in athletes. However the reduced reportage of URS, among swimmers consuming the BC supplement, suggested there was some benefit to their health. A possible explanation is that BC has impacted on non-infectious causes of URS. Growth factors present in

BC may enhance intestinal repair which could be advantageous to athletes recovering from bouts of prolonged intensive exercise. The effect of gastrointestinal disturbances on local and systemic immunity may be minimised which benefits immune protection. However an inconsistent effect of BC supplementation on immune protection in athletes means further research is still required. In these studies there was no benefit to immune protection in the student or older adult cohort. Further investigation into the safety of BC for all population groups is still required.

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Introduction

The aim of these current studies was to further investigate the role of a bovine colostrum supplement in mucosal immune protection in other potentially immune depressed groups.

Two studies were planned with three different cohorts (two cohorts in Study 1 and one cohort in Study 2), the sample size for each cohort was based on a power calculation carried out with the results from the previous study involving middle-aged recreational runners [1]. The study design involved saliva and blood sampling as well as the self-recording of wellness symptoms and activity or training, and the monitoring of dietary intake. A large number of saliva samples were provided by the participants at specified sampling times at their training locations from three different Auckland swimming pools, University or home. To ensure standardised sampling procedures were followed research assistants (RAs) were hired to assist with the collection of saliva, aliquotting to microtubes and freezing. Assistance was sought to centrifuge blood, aliquot and freeze the plasma. Further assistance was also sought to issue the trial supplement and collect dietary and anthropometric data. This was especially important during the study with older adults where each participant was visited at their home or place of work.

The planning, co-ordinating and project managing of the two studies was my responsibility which included:

- Development of the research proposal in conjunction with my supervisors
- Preparation of two ethics applications for the different studies
- Recruitment and training of the participants
- Employment and training of the research assistants
- Collection and storage of saliva samples and co-ordination of RAs to assist
- Co-ordination of blood collection with participants, coaches, phlebotomists (Diagnostic Medlab, Massey University Health Centre) and timely delivery of blood samples to LabPlus for analysis

- Co-ordination of blood centrifugation, storage and delivery of plasma samples to the appropriate laboratory for analysis
- Performing cytokine analysis: interleukin (IL)-6, IL-1ra and interferon-alpha at Massey University (Albany campus)
- Issuing of supplement, collection of unconsumed supplement and determining compliance
- Liaison with Fonterra for delivery of supplement and the issuing of ID codes in a double blinded manner
- Liaison with Fonterra for test results of supplement
- Performing dietary interviews, co-ordinating data entry of dietary data and performing quality control of all dietary data
- Checking and 'data-cleaning' all immunological and biochemical data
- Performing statistical analysis of data
- Providing written study summary to participants and updates to ethics committee
- Preparing regular reports for sponsor (with Frank's help)
- Presenting results at conferences which included:
    - Self-reported upper respiratory symptoms were reduced among swimmers who consumed a bovine colostrum supplement, oral presentation, 'New Image'-Colostrum seminar, Rotorua, 2008
    - Reduced reporting of upper respiratory symptoms among swimmers following bovine colostrum supplementation. Oral presentation 'Australian Association of Exercise and Sport Science', conference, September 2006.
    - The effects of ageing on the homing ability of lymphocyte subsets in older adults. Poster presentation to 'Development and Function of Secondary Lymphoid Tissues' conference, Pasteur Institute, Paris, 2006.
    - The Effect of Bovine Colostrum Supplementation on Salivary IgA in Recreational Runners. Poster presentation to 'Nutrigenomics' conference, Auckland, 2006.

- The Effect of Bovine Colostrum Supplementation on Salivary IgA in Distance Runners. Oral presentation to the 'National Sport Science' conference, Kuala Lumpur, 2004.

- Writing thesis

My PhD supervisors provided assistance during the project planning stage and gave support and feedback during the trials and the preparation of the thesis:

- Dr Frank Cross (Frank taught me how to do an enzyme-linked immunoassay and assisted with all report writing)
- Dr Clare Wall (Clare helped with saliva and dietary collection during the older adult study)

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- Dr Ajmol Ali (Aj helped with the early morning saliva sampling of the swimmers and volunteered as a participant in the student cohort)
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The research assistants performed the following tasks:

- Janie Proctor (assisted with saliva sampling and blood centrifuging for both Study 1 and 2, performed data entry for most of the wellness and training records for Study 1 and most of the dietary data)
- Christel Dunshea-Mooij (assisted with saliva sampling, anthropometric measurements and diet interviews in Study 2)
- Catherine Murray (assisted with saliva sampling, anthropometric measurements and diet interviews in Study 2)
- Jane Fenton (assisted with saliva sampling, anthropometric measurements and diet interviews in Study 1 and 2, performed most of the data entry of wellness and activity records for Study 2)
- Pam Van Horst (assisted with saliva sampling for Study 1 and 2, anthropometric measurements and diet interviews in Study 2)
- Renee Prideaux (assisted with saliva sampling in Study 1)
- Claire Svendsen (assisted with saliva sampling in Study 1)

- Cindy Claassens (anthropometric measurements and diet interviews in Study 2)

In summary laboratory analysis of samples collected for this study were performed as listed below:

- Blood samples were analysed at LabPlus for full blood count, liver function analysis and cell phenotyping
- Plasma samples were analysed for levels of cytokines by me at the nutrition laboratory, Albany campus, Massey University
- Plasma samples were analysed for immunolgobulins, albumin, cortisol, C-reactive protein and cortisol by Dr Phil Pearce, nutrition laboratory, Palmerston North campus, Massey University
- Saliva samples were analysed for immunolgobulins, albumin and osmolality by Dr Phil Pearce, nutrition laboratory, Palmerston North campus, Massey University

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List of Abbreviations used

Abbreviation	Definition
AF	Activity factor
Alb	Albumin
APC	Antigen presenting cell
Ave	Average
BALT	Bronchial associated lymphoid tissue
Base	Baseline
BC	Bovine colostrum
B-cell	B-lymphocyte
BM	Body mass
BMR	Basal metabolic rate
CD	Cluster of differentiation number
CHO g/kgBM	Grams of carbohydrate per kilogram of bodymass
CHO/kgBM/day	Carbohydrate per kilogram body mass per day
CMIS	Common mucosal immune system
CTL	Cytotoxic T-cell
diff	Difference
Drink g/day	Grams of drink consumed per day
EDEE	Estimated daily energy expenditure
EGF	Epidermal growth factor
F	Female
GALT	Gut associated lymphoid tissue
Gen	Gender
GH	Growth hormone
IFN	Interferon
IFN-α	Interferon-alpha
Ig	Immunoglobulin
IgA	Immunoglobulin A
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IL-1ra	Interleukin-1receptor antagonist
IL-6	Interleukin-6
Intens	Intensity
IQ range	Interquartile range
J-chain	Polypeptide joining chain
LP	Lamina propria
LPS	Lipopolysaccharide
M	Male
MAdCAM-1	Mucosal addressin cell adhesion molecule
MBM	Mature bovine milk
M-cell	Microfold cell
Med	Median
MET	Metabolic equivalent
MHC	Major histocompatibility complex
N/A	Not applicable
ND	Not detected
No Ana.	Analysis not performed
NP	Not possible to calculate
NRV	Nutrient reference value
NSAID	Non-steroid anti-inflammatory drug
OA	Older adults
Osm	Osmolality
PAL	Physical activity level
PIgR	Receptor for polymeric immunoglobulin
Pl	Placebo

List of Abbreviations used (continued)

Abbreviation	Definition
Post	Post-supplementation
PPs	Peyer's patches
Ptn g/kgBM	Grams of protein per kilogram of body mass
RDBPC	Randomised double blind placebo controlled
RDDI	Reported daily dietary intake
RDEI	Reported daily energy intake
RDI	Reported daily intake
SEM	Standard error of the mean
S-IgA	Secretory immunoglobulin A
St	Students
Sw	Swimmers
T-cell	T-lymphocyte
TGF-β	Transforming growth factor beta
UL	Upper level of intake
URS	Upper respiratory symptoms
URT	Upper respiratory tract
URTI	Upper respiratory tract infection
VCAM-1	Vascular adhesion molecule
wks	Weeks

1.0 Literature Review

1.1 Introduction

There is a synergistic relationship between nutrition and immune function; nutrition status affects the risk for infection (as well as severity and duration), while infection affects nutritional status because of the increased nutrient demand of cells involved in the immune response [2]. Immune function is also modulated by both over-nutrition (when sufficient to result in obesity) and under-nutrition. For example protein malnutrition affects the structure and integrity of the gut epithelium; therefore the pattern of antigen presentation to underlying cells could be affected due to increased permeability in the epithelium [3]. Specific micronutrient deficiencies are associated with an impaired immune response increasing risk for infection. Infectious disease increases the risk for under-nutrition due to the high demand for specific nutrients, this is of concern to at-risk population groups in developed countries e.g. older adults [4]. In addition even adequately nourished people may develop certain immunologically based diseases (e.g. Crohn's disease, food allergies etc) the course of which may be altered by dietary manipulations [4]. Some diseases (e.g. rheumatoid arthritis) develop from an overactive immune response resulting in inflammation and tissue damage (processes normally involved in pathogen removal) of a destructive nature which generally do not respond to nutrition interventions [4].

Recently there has been much interest in investigating the effects of food components on improving health in the general population [4]. In the clinical setting human trials have shown that single nutrient supplementation (amino acids, arginine, glutamine, n-3 fatty acids, nucleic acids) can modulate resistance to infection [5]. It is not clear whether these benefits are due to an observed pharmacological effect of the nutrient or as a result of the whole of the clinical nutrition support programme administered to the patient [6]. In the general population it is not known if disease risk such as upper respiratory tract

infection (URTI) can be affected by the whole diet and whether a nutrition intervention can have also have an effect.

URTI are among the most prevalent infections occurring in humans [7], and evidence suggests that they are experienced by adults on average at about two to four infections per person per year [7, 8]. There is a close association between the incidence of URTI and cold weather which places an additional seasonal burden on health services [9]. Lower respiratory tract diseases such as pneumonia also show a similar seasonal pattern [9]. Adults over the age of 65 years experience an increased rate of respiratory tract infection during colder temperatures, are particularly vulnerable to under-nutrition [10], and are thus at increased risk for mortality [9]. Another at-risk population group for URTI appears to be those who undertake prolonged intensive exercise programmes, such as elite and endurance athletes, who, while not considered to be clinically immune-deficient have an increased risk for URTI when there is insufficient recovery from intensive bouts of exercise [11]. This can be further exacerbated by poor nutritional practices [12]. The occurrence of URTI in athletes may be of concern close to competition, when even minor illnesses can affect performance [13].

Biomarkers of immune function

There is much to be learned about the variability of the immune response in apparently healthy people, their susceptibility to infection and the manner in which nutrition may benefit [14]. One of the difficulties in investigating the effect of nutrition on immune function is that not all components of the immune system respond in a dose-dependent manner to a given nutrient [14]. There are many factors that could affect an immune response including nutritional status [15], age [16], gender [17], genotype [14], health status [10] and immunological status (prior exposure to pathogenic organisms) [18]. Certain population groups may have specific/unique health risks and nutritional interventions have received increased research attention for their corrective/alleviative effects. However it is unclear whether individuals who are apparently healthy and have adequate dietary intake of nutrients known to affect immune status, will experience benefit to immune function from further nutrition interventions [14].

Improvement to immune function may only occur if an individual is experiencing physiological stress such as prolonged intensive exercise (e.g. an increased incidence of URTI may occur as a result of changes to mucosal immunity [19]), or they have deficient or excess dietary intake of one or more nutrients. It is also not possible to predict the cumulative effects of several small changes such as improved nutrition, exercise and change in lifestyle.

Measuring a range of immune biomarkers will add validity to any investigation of a nutrition intervention on immune function, especially if there are consistent changes across the range [4]. Individual aspects of innate and adaptive immunity can be measured; *ex vivo* isolated cells studied in culture or, *in vivo* by measuring changes in concentrations of proteins that are relevant to immune function in response to immunological challenges such as vaccines [4]. Changes may be monitored in the peripheral blood in response to an immunological challenge such as severity of infection [14], or physiological stress [20]. However peripheral blood measurements provide limited information as they may be indicative of a response to stimulation of the immune system and may not measure functional changes [4]. Studies with animals can provide more information about the impact on immune function. Cells can be collected from tissues such as lymph nodes and the peritoneal cavity, whereas in humans, blood is usually sampled. Alternatively *in vitro* studies where a specific nutrient is applied to cells under very controlled conditions can also provide valuable information about mechanism [14].

Reviews of markers of immune function that have been used in nutrition intervention studies have been published by Albers *et al.,* 2005 [4] and Calder and Kew, 2002 [14]. The various markers of changes to immune function which have been used to monitor the effect of a nutrition intervention each have their own strength and weakness. In order to assess the usefulness of the various markers available Albers *et al.,* 2005 have applied a rating system using the following criteria:

Criteria for usefulness of markers [4]

Biological relevance	Differentially expressed in normal and high risk diseased individuals Correlates with relevant clinical endpoint Known mechanistic link
Biological sensitivity	Low within-subject variability Low between-subject variability
Feasibility	Validated assay available Robustness of assay Technical feasibility

For the purposes of this review bio-markers used to assess changes in mucosal immunity are of interest and these are listed in Table 1 along with a comment on their usefulness as determined by applying the above criteria. The additional advantage of monitoring changes to mucosal immunity is that non–invasive measures such as urinary and salivary markers can be used in healthy individuals. There can be difficulties in recruiting sufficient participants for a nutrition intervention from the apparently healthy population, particularly if venepuncture is required.

Table 1: Immune parameters that could be monitored to measure changes to mucosal immunity listed in order of usefulness (from Albers *et al*., 2005 [4])

Mucosal immune markers of the gut-associated lymphoid tissue (GALT)		Usefulness as a marker
Salivary and stool Ig	Vaccine specific S-IgA	High: Meets all above criteria except for between-subject variability
	Non specific S-IgA	High: Meets all above criteria except for between-subject variability
Inflammatory markers	Response to attenuated pathogens	Low: Meets all criteria except being differentially expressed in all individuals. Ethical considerations.
	Stool calprotectin	Low: Does not meet biological relevance criteria, meets all others
Integrity of mucosal barrier	Sugar permeability (marker for small molecules)	Low: Does not meet any of the biological relevance criteria. Meets all other criteria
	Bacterial permeability	Low: Only meets feasibility criteria
	Serum endotoxins	Low: Meets some of feasibility criteria

Salivary S-IgA is reported to be a useful indirect marker of gut mucosal immunity as it may reflect levels of IgA secreted at other mucosal surfaces [4]. In addition the amount of S-IgA on the mucosal surface of the respiratory tract can also be indicated by levels in the saliva [21]. A number of methodological issues need to be considered when measuring salivary S-IgA e.g. ensuring sample integrity during collection and storage and ensuring levels of S-IgA are not affected by participant dehydration. These issues were reviewed previously in a study with athletes in their normal training environment [22] (see Appendix 10.1).

Bio-active food components

It is known that under-nutrition can impact on immune function [23] and that certain nutrients are required for the immune system to function e.g. essential amino acids, essential fatty acids (e.g. linoleic acid), folic acid, vitamins A, B6, B12, C,D, E, zinc, copper, iron and selenium [14]. Ideally dietary intake of these nutrients provides an optimal supply. Supplementation can result in excess intake which can have a negative effect on immune function. Excess intake of one nutrient may also affect the intake of another, e.g. zinc and copper [24]. Zinc is not stored in the body; its bioavailability depends on the dietary source. It is poorly absorbed from vegetarian diets and resorption is affected in the presence of other minerals such as copper, calcium and iron [24]. As a result of increased understanding of how certain nutrients can affect health, nutrition guidelines have been established and considerable research has been carried out in the field of nutritional epidemiology in an attempt to understand how food can contribute to disease states [25].

Despite this increasing knowledge, some diseases in the western world are considered to be nearly at epidemic levels, including cancer, obesity, diabetes and cardiovascular diseases all of which involve dysfunction of several biological mechanisms [25]. While dietary intake is likely to be a factor in all of these disease states, it is difficult to determine how a nutrition intervention with a single nutrient would have an effect on improving the health outcome. It is even more difficult to elucidate how a food which contains a mixture of nutrients would have an effect.

Even with these limitations there has recently been much interest in the molecular mechanisms by which food may modulate various physiological processes (including their effect on growth, neurological development and immune function) [25]. As a result the term 'functional food' has emerged which encompasses the health-related benefits of a food or nutrient [26]. An area that has received considerable attention is the health benefit of probiotics, prebiotics (ingredients that have a beneficial effect on the existing gut microflora), synbiotics (both probiotic and prebiotic), and foods supplemented with individual nutrients [26]. Probiotics have been defined as 'living micro-organisms that upon digestion exert health benefits beyond general nutrition'; that is they can positively influence immune function and metabolic processes [27]. Probiotics are generally lactic acid producing bacteria such as lactobacilli and bifidobacteria which are often added to yoghurts and dairy products [27]. The health benefits [28, 29] and risks [30] of probiotics are covered extensively in the literature. Their effect on immune function is included here if it is within the scope of the literature review.

Owing to the complex nature of the mix of nutrients in foods it has been difficult to establish scientific evidence to support efficacy for health claims. As analytical techniques improve, further molecular components are identified that may have health benefits. For example a recent analysis of the bovine milk proteome has identified many new proteins that may be immunologically protective for both the mammary gland and the neonate [31]. In randomised controlled trials investigating the effect of a nutrition intervention it is also necessary to choose a placebo that is sufficiently different in bio-active levels, but is matched in nutrient content, and this can be problematic. In studies investigating the effect of a bovine colostrum (BC) intervention on immune function in athletes, different placebos have been used by different investigators e.g. maltodextrin [32], whey protein [33] and skim milk powder [1]. Whey protein and skim milk powder contain varying levels of bio-actives that may be immunomodulatory and therefore may not be sufficiently different to a BC intervention to identify any effect. Maltodextrin may not provide the same protein content as BC (low levels of protein intake can affect immune function

such as lymphocyte proliferation). Therefore it is necessary: to determine which bio-active component(s) is having an effect on the stimulation of immune function, to establish an effective dose of the food product, to select an appropriate placebo, and select an appropriate outcome variable to predict efficacy as well as determining any effect separate from that of the normal diet [34].

Aim of the Literature Review

The main aim of this literature review is:

- To review the role of nutrition in mucosal immune function in two population groups at risk of depressed mucosal immune function, elite athletes and older adults.

Secondary aims are:

- To provide a broad overview of oropharyngeal (from the mouth to the oesophagus) and gastrointestinal tract mucosal immunology
- To provide an overview of the role of effectors (cells, antibody, cytokines) in immune defence at mucosal surfaces in elite athletes and older adults.
- To elucidate how nutritional supplementation may affect the mucosal immune system, focusing on BC and milk proteins and their immunomodulatory effect.

1.2 The mucosal and systemic immune systems

The immune system has developed during evolution to protect against the large number of infectious micro-organisms to which our bodies are exposed every day. It can be divided into innate and adaptive systems and operates both systemically and locally. Several interdependent defence mechanisms exist that together comprise our immunity against infection. These range from external barriers such as skin and mucous secretions, and innate defence such as phagocytic cells, to specific adaptation to micro-organisms through the action of antibodies and specific antigen-recognising immune cells [35].

1.2.1 Innate immunity

Pathogenic penetration of external barriers results in the activation of the immune system. Invasion is prevented by various factors such as enzymes, lysozymes or through the activity of phagocytic cells. Phagocytic cells have evolved to distinguish micro-organisms from self components through mechanisms such as pattern recognition receptors. These receptors bind to exposed microbial polysaccharide and lipopolysaccharide (LPS) molecules [35]. Phagocytic cells include neutrophils, which make up approximately 60-70% of the leucocytes in the blood-stream, and mononuclear monocytes which settle in tissues as mature macrophages [35].

Neutrophils are produced in large numbers (about 10^{11} cells per day by the bone marrow) to compensate for their short half-life of eight-twelve hours in the peripheral blood [36]. Monocytes modulate the immune/inflammatory response through the action of cytokines. This is in co-operation with the adaptive immune response via major histocompatibility complex (MHC) class-II assisted presentation of antigen to T-lymphocytes (T-cells). Innate immune function is linked to adaptive immunity in an integrated immune response (see Figure 1 and Section 1.2.2).

1.2.2 Adaptive immunity

The main role of lymphocytes is in immune defence where they are predominantly involved in the adaptive immune response. There are two types of lymphocytes (which can have characteristics of specificity and memory), B lymphocytes (B-cells, which can differentiate to immunoglobulin producing plasma cells) and T-cells (T-helper and cytotoxic cells) [37]. Stimulation of T-cells occur through the production of interleukin-1 (IL-1) by macrophages and subsequent defence involves co-ordination of the action of the three main lymphocytes: B-cells, cytotoxic T-cells (CTLs) and T-helper cells [37].

Leucocytes express MHC which assists with the recognition of 'nonself' antigenic fragments as distinct from self proteins. Class I MHC is expressed on virtually all nucleated cells in the body and its main role is to assist in the elimination of cells that have been altered. Antigen presenting cells (APCs) utilise MHC class II molecules to present antigen to the CD4 receptor on T-helper cells, initiating an immune response [38]. Cell mediated immunity includes the activation of macrophages, natural killer cells, antigen specific cytotoxic T-cells and stimulation of cytokine production without the involvement of antibody [38]. The integrated immune response involves activation of B-cells, providing additional controls to the host against harm from an improper immune response [38]. B-cells develop specificity for one antibody which binds to antigen facilitating recognition by phagocytic cells. They generally require activation by T-helper cells to become plasma cells or memory cells (which have a long life and are able to respond quickly to produce immunoglobulin following subsequent exposure to the original antigen). The link between the innate and adaptive immune systems including the role of immunoglobulins (Igs) is shown in Figure 1.

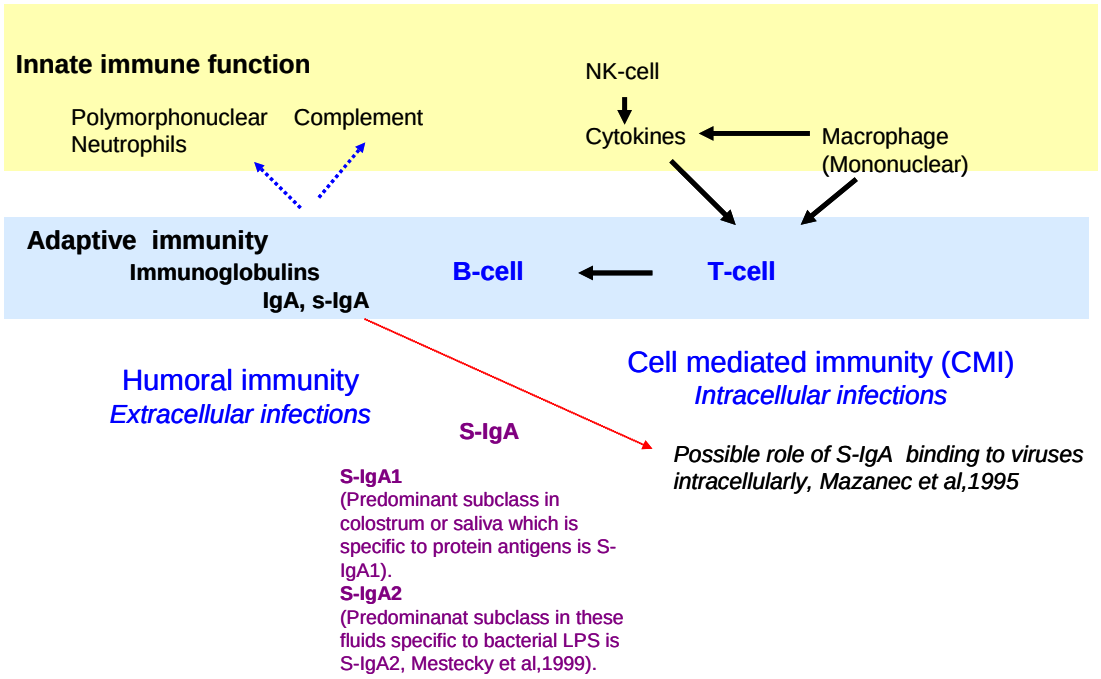


Figure 1. The link between the innate and adaptive immune system (adapted from Roitt and Delves, 2001, [39])

The mucosal immune system

The mucosal immune system forms the largest part of the immune system and may be central to host protection as it defends the intestine, respiratory tract, mouth, eyes and reproductive tract [4]. It consists of organised lymphoid tissue (separate or grouped lymphoid follicles) and various diffuse cellular components (such as antibody-producing cells) dispensed throughout the epithelium and the lamina propria (LP). It is thought these components are all linked in a common mucosal immune system (CMIS) (see Section 1.3.3). The gut-associated lymphoid tissue (GALT) is the largest lymphoid system in the body and may be central to the mucosal-associated lymphoid system because of its exposure to antigens and its influence on the whole of the immune system [40] (see Section 1.3.1).

1.2.3 Immunoglobulins (Igs)

Igs (also known as antibodies) are produced by plasma cells in response to an invading micro-organism and have the ability to recognise different antigens. The first encounter usually results in 'memory' for the pathogen so that on subsequent exposure an effective immune response occurs [39]. To ensure

enough antibody can be produced to combat infection, lymphocyte proliferation is triggered by contact with antigen, and that in turn produces a large clone of plasma cells that will make the same antibody for which the parent lymphocyte was programmed [39].

Antibody consists of two identical heavy polypeptide chains and two identical light polypeptide chains which are held together by disulphide bonds. The N-terminal ends of both the heavy and light chains are highly variable whereas the remainder of the chain is constant. The immunoglobulin molecule can be split into fragments; two monovalent antigen binding fragments (Fab) each of which has an antigen combining site and one crystallisable non antigen binding Fc fragment [41]. There are five classes Igs in humans based on their heavy chain constant regions: IgA, IgG, IgM, IgD and IgE.

IgA

IgA is found in human serum as well as in secretions and there are distinct differences between the two. In serum, IgA is found mostly in the monomeric form (80-99%), is a poor activator of complement, and is produced from cells originating in bone marrow [42]. It seems unlikely to be involved in the neutralisation of antigens that breach the epithelial barrier [43]. Cells producing IgA in secretory tissues originate in the mucosal associated lymphoid tissue. The main role of secretory-IgA is to defend the exposed external surfaces against microbial attack e.g. approximately 40mg/kg body mass (BM) of IgA is transported daily through the intestinal crypt epithelium to the mucosal surfaces compared to 30mg/kg BM of IgG [44].

Secretory-IgA

Secretory-IgA (S-IgA) is the main effector in mucosal immunity, with an important role in first line defence. Production of S-IgA by plasma cells occurs locally with only a small amount coming from circulation [42]. It is found in seromucous secretions such as saliva, tears, nasal fluids, sweat, colostrum and secretions of the lung, genitourinary and gastrointestinal tracts. The presence of high levels of S-IgA in secretions relies on a large proportion of antibody-producing cells being committed to the IgA isotype, and the cooperation of the

mucosal B-cells with a transmembrane epithelial glycoprotein known as secretory component (SC) [43, 45] (also known as polymeric immunoglobulin receptor (pIgR) because it mediates the transport of both dimers and polymers of IgA and pentameric IgM) (see Figure 2).

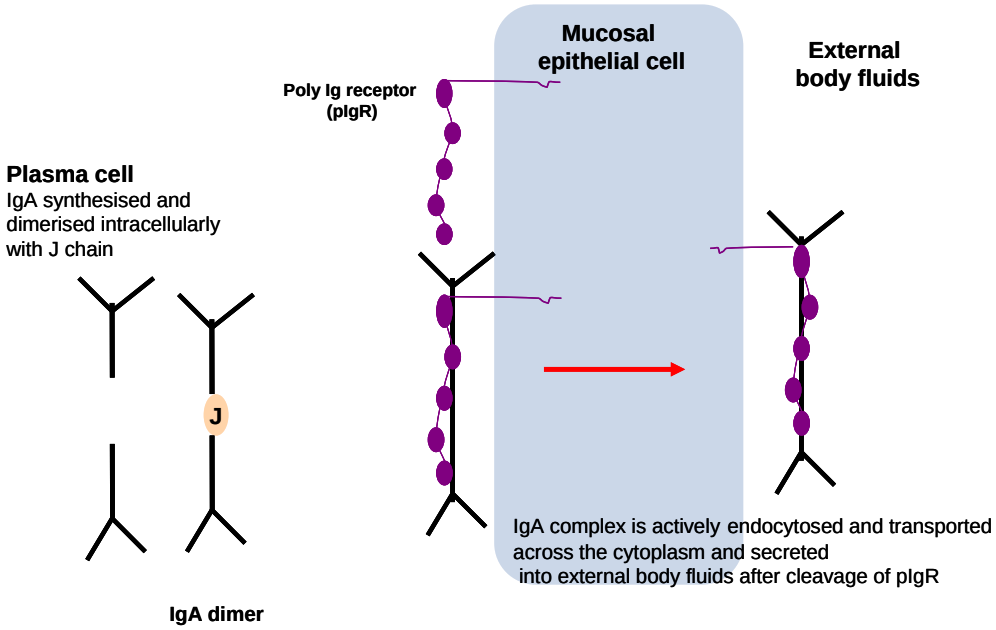


Figure 2: Synthesis and transport of S-IgA across the epithelial membrane (adapted from Roitt and Delves [44])

Successful transport across the epithelium requires intracellular binding of IgA with a cysteine-rich polypeptide known as a polypeptide joining chain (J-chain) [45]. Both the dimeric and polymeric forms of IgA bind strongly through the J-chain to pIgR, then by endocytosis the complex is transported across the cytoplasm and secreted into the external body fluids, after cleavage of the pIgR peptide chain [44] [43]. Immunocytes from all Ig isotypes can produce J-chain but it is only incorporated into dimeric and polymeric IgA (or pentameric IgM); if these are not present then the J-chain is degraded intracellularly [45].

Antimicrobial activity of S-IgA in mucosal secretions

Secretory antibodies (IgA and IgM), have a major protective role in mucosal immune function and continuously efflux into the perimembraneous layer. The main mode of anti-microbial action by S-IgA involves surrounding the microbe in a hydrophilic shell, preventing its adherence to mucosal surfaces [46].

S-IgA has an important function in the clearance of virally infected cells from mucosal surfaces [47]. This is evidenced by increased numbers of S-IgA antibodies appearing in saliva, after virus shedding has ceased, following a viral infection in the respiratory tract [47, 48]. The number of IgA secreting cells correlates with viral resistance [47], enhancing the ability to rapidly produce IgA in response to viral infection. By the removal of viral pathogens S-IgA may prevent reinfection [47]. There are several mechanisms for diminution of viral infectivity by S-IgA [47] including:

- Prevention of attachment of a virus to cellular receptors [46]
- Prevention of penetration of an attached virus into the host cell [46]
- Neutralisation of a virus following penetration of the host cell (recently it has been shown that S-IgA may also neutralise viruses within epithelial cells; transcytosing antiviral S-IgA may inhibit intracellular viral replication [46, 49])

The ability of S-IgA to be selectively transported across mucosal surfaces as well as its inherent antiviral activity [47] could be the main advantages of salivary S-IgA protection in the respiratory tract. Saliva is produced in variable amounts from several glands; parotid gland (40%), submandibular (40%), sublingual (10%) and the minor glands (10%). While the minor salivary glands contribute only 10% of total saliva, levels of S-IgA from this source are higher than in saliva from other glands (accounting for about 25% of the total production of S-IgA) [50].

In the gut, S-IgA has a key role in maintaining homeostasis through its influence on microbial populations and subsequent local and systemic inflammatory responses [51]. S-IgA can act indirectly by promoting uptake of viral antigens via Ig receptors present on microfold cells (M-cells) (see Section 1.3.1) to sub-epithelial macrophages [47]. In IgA-deficient mice that lacked intestinal IgA, expansion of populations of segmented filamentous bacteria and anaerobic micro-organisms was observed, along with induction of germinal centres and activation of local inflammatory and immune responses [51]. From *in vitro* studies it has been shown that dimeric IgA can neutralise lipopolysaccharide (LPS) toxins located in epithelial cells; LPS toxins are produced by gram-

negative bacteria and uptake results in acute local inflammation responses. Therefore through the influence on gut microflora and participation in the neutralisation of permeated LPS, S-IgA could have an important role in minimising local and systemic inflammatory responses.

Recently an immunomodulatory role of S-IgA re-entering the body (through the swallowing of S-IgA complexes) in the induction and the maintenance of further mucosal S-IgA production has been investigated [52]. It was shown that orally administered rS-IgA (comprising a chimera of human secretory component and mouse IgA) induced an immunomodulatory effect in mice [52]. This suggested it may be possible that S-IgA/antigen complexes that are swallowed may be absorbed across M-cells in PPs and trigger mucosal and systemic responses that facilitate the presentation of luminal antigens to dendritic cells. Further research is needed to identify the role of these S-IgA complexes in immunomodulation.

Secretory IgA subclasses

Production of IgA subclasses varies at different sites: IgA1 secreting subclasses are the most frequent in the aerodigestive tract (75-93%), including the bronchial mucosal tissues, whereas the IgA2 subclass predominates in the bowel [43]. The ratio of S-IgA1 to S-IgA2 in the upper respiratory tract is approximately 60:40 possibly as a result of higher exposure to viral antigens [43]. In addition, the dispersal of food antigens may also affect the ratios of subclasses e.g. in the jejeunum the immunocytes are mainly the IgA1 subclass and in the bowel they are mainly IgA2 [45]. Further it is thought that LPS has a role in subclass-switching as bacterial overgrowth of bypassed jejeunal segments results in increased levels of IgA2 [45].

Differentiation of B-cells to IgA-producing plasma cells

Terminal differentiation of B-cells to antibody-producing plasma cells occurs under the influence of T-helper cells. T-helper cells include; T-helper type-2 cells (Th2) which produce IgA-stimulating cytokines interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-13 (IL-13), and T-helper type-1 cells (Th1) which produce IgA-inhibiting cytokines,

interferon-gamma (IFN-γ), tumour necrosis factor-beta (TNF-β) and interleukin-2 (IL-2). The interaction between T-and B-cells and the balance of cytokines controls IgA production [53] [42].

IgG

In humans IgG is the most abundant immunoglobulin in circulation and in nonmucosal tissues (about 75% of Igs). IgG readily diffuses to extra-vascular body spaces and is important in neutralising bacterial toxins and enhancing phagocytosis of microorganisms [44]. IgG has the ability to pass through the placenta unlike other immunoglobulins and has a key role in the development of the infants' immune system. A diverse range of leucocyte receptors are able to bind to the Fc region of IgG which enables it to participate in various functions such as phagocytosis, placental transport, B-cell regulation and antibody dependent cellular cytoxicity [44].

At mucosal sites there are low numbers of IgG plasma cells. In the respiratory tract both locally produced IgG [54] and serum derived IgG have an important protective role [55]. The presence of IgG in saliva is thought to result from passive transudation rather than active transport. Levels of both IgG and albumin in saliva can be considered markers of the degree of transudation particularly in inflammatory states such as stomatitis associated with denture wearing [56] and dehydration when saliva flow rate is altered [55]. Ageing also appears to affect saliva IgG secretion rates to specific pathogens (e.g. *Streptococcus mutans*) which could contribute to impaired mucosal protection of the respiratory tract [57].

IgM

Approximately 5-10% of immunoglobulins are IgM molecules. IgM is made up of polymers of immunoglobulin, has a high molecular weight, does not readily diffuse into tissue and does not pass across the human placenta. Polymers of immunoglobulin are linked by disulphide bonds to pentameric (which may incorporate a single J-chain) and hexameric forms [44]. About 20% of IgM is in the hexameric form, has no J-chain and is more able to activate the complement pathway than the pentameric form. While IgM is found mainly in

serum, some pentameric forms include J-chain so it also has an important role as a secretory Ig. Lower levels of IgM are secreted into mucosal tissues mainly because of the lower proportions of IgM producing cells, however a compensatory increase in IgM secretion occurs in IgA deficient individuals [43]. During infections salivary IgM is expressed early on in the immune response and may appear again after subsequent exposure to the antigen [44, 54]. IgM antibodies have high valency enabling them to bind to antigens that have multiple epitopes and because of their early appearance in circulation during the immune response may have an important role combating bacterial infections [44].

IgD

IgD comprises approximately 2% of Igs and is nearly always co-expressed with IgM. It's main function is to signal that the young spleen-located B-cell is ready for activation in order to participate in the immune response [44]. IgD exists also in the secreted form as a monomer, which is readily degradable and has a short half life in plasma [44]. IgD producing immunocytes are rarely found in the gastrointestinal tract. However 1-2% of immunocytes found in the upper autodigestive (such as salivary and lacrimal glands) are IgD producing cells [58]. Microbial populations in the respiratory tract produce IgD binding factor and may have an influence on the IgD response at this location [58].

IgE

IgE is present in very low concentrations in serum, is synthesised by only a small number of plasma cells. Its main physiological role appears to be in the immune response to pathogens such as parasitic worms and protozoan parasites [44]. It may also facilitate protection from pathogen entry at anatomical sites by triggering an inflammatory response [44]. Although present in small quantities IgE can trigger very powerful immune reactions. After contact with antigen IgE binds with mast cells, this is followed by recruitment of antimicrobial agents and the release of inflammatory mediators. In atopic individuals hypersensitive responses such as asthma, hay fever and food allergies may be mediated by IgE [41].

1.3 Protection at the mucosal surface

About 50-60% of the body's total immune protection underlies the mucosal surfaces of the upper and lower respiratory tract, while the small intestine and colon account for about 70-80% of antibody production (mostly IgA) by the body [53]. The most effective method of inducing IgA production at distal mucosal surfaces (e.g. the oropharyngeal region) may be through the stimulation of induction sites found in mucosal associated lymphoid tissue. It is possible that gastric or oral/nasal immunisation can lead to antibody production by plasma cells in the minor salivary glands [59], which has contributed to the theory that a common mucosal immune system (CMIS) exists (see Section 1.3.3).

The protective barrier also includes extrinsic mechanisms such as the mucous coat, glycocalyx, resident micro-flora, peristalsis, proteolytic gastrointestinal secretions and various other humoral factors [53]. Of equal importance to the host is that immunological unresponsiveness (or tolerance) as well as immune protection of the mucosal immune system occurs through natural exposure to antigens [42]. Cytokines and chemokines produced by $CD4^+$ and $CD8^+$ subsets and APCs (such as dendritic cells, macrophages and B-cells) as well as by non-classical APCs (epithelial cells) participate in the balance between mucosal tolerance and active immunity [42].

1.3.1 Intestinal mucosal immune system

The intestinal mucosal immune system in the human gut provides a large surface area ($300m^2$) for digestion and absorption of nutrients. It is composed of a single layer of epithelial cells and is sealed by tight junctions that are effective at excluding peptides and macromolecules with antigenic properties [60, 61]. Beneath the mucosal epithelium lies the LP; they contain various immunocompetent cells including dendritic cells, macrophages and lymphocytes which form a functional unit with the epithelial cell layer [61] (see Figure 3). Non-immunological factors such as gastric acid, pancreatic juice, bile, motility, mucus, glycocalyx and cell turnover also protect against harmful agents [61].

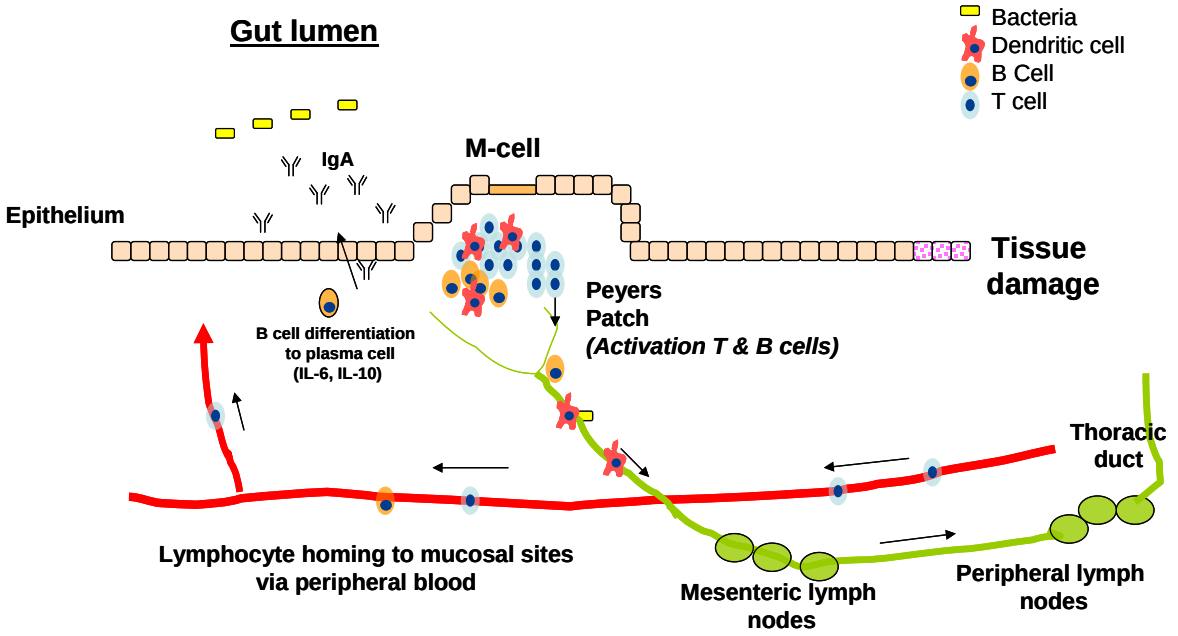


Figure 3: Mucosal immune system in the GALT (adapted from Suzuki et *al*. 2007 [62] and Macpherson and Smith, 2006 [63])

Absorptive epithelium

Absorptive columnar epithelial cells (enterocytes) make up the majority of cells in the epithelial layer. Their surface has numerous microvilli which are covered in glycocalyx and mucus. At the apical end they are connected by junctional complexes (tight junctions) to neighbouring cells important in separating the external and internal environments [60]. The glycocalyx contains various enzymes and nonenzymatic proteins (peptidases, receptors and transport proteins) which are necessary for digestion and absorption [61]. The main functions of the glycocalyx are to prevent the uptake of antigens and pathogens by enterocytes, and also to provide a degradative environment that promotes digestion and absorption of nutrients. The mucus layer is made up of 1% mucin, 1% free protein, and more than 95% water. It contains albumin, immunoglobulin (mostly S-IgA), lysozyme, lactoferrin and epidermal growth factor (EGF) [61]. Enterocytes can also endocytose small amounts of intact proteins and peptides preventing the transepithelial transport of antigens [60].

Should a pathogen invade the epithelium, further protection is provided by the epithelial cells, limiting damage to tiny lesions [61]. The repair process tends to occur in three phases. A rapid initial response called restitution occurs where

cells move rapidly, within the first hour of damage to cover the denuded area [64]. A slower process of cell proliferation and differentiation occurs over the next two days, and finally the mucosal membranes re-establish [64]. This latter stage is important to restoring gut integrity and mucosal defence, failure to do so can result in further inflammation [64].

Several factors stimulate this process, including peptide growth factors, a number of which are present in the gastrointestinal lumen [65]. Salivary glands secrete EGF which are also present in foods such as bovine milk [65]. Receptors for peptide growth factors are on the baso-lateral membrane of the mucosal cells, not the apical membranes, making it difficult for the ligand to reach the receptor [65]. In adults with damage to the gut, permeability is increased; and in inflammatory states it is thought there may be a shift in receptor distribution to the apical membranes [65].

Appropriately activated immune cells may assist in intestinal renewal by producing damage-healing soluble factors, such as transforming growth factor-β (TGF-β). These molecules have multiple roles and are involved in maintaining gut homeostasis and mucosal tolerance (including the down-regulation of the mucosal immune system in response to commensal flora). TGF-β is involved in isotype switching of B-cells to plasma IgA-producing cells. IgA has a crucial role in maintaining gut homeostasis where it influences microbial populations. Disturbance of homeostatic control is likely to result in activation of both local and systemic immunity [51]. The role of TGF-β in tissue repair is to stimulate fibroblast division and to lay down extracellular matrix once inflammation has stopped [66]. TGF-β also suppresses the expression of surface Igs by B-cells, and decreases the secretion of IgG and IgM, but is stimulatory for some immune cells e.g. induction of monocyte secretion of IL-1, IL-6 and TNF [67]. TGF-β1 may have a role in leucocyte migration by upregulating α4 integrin-mediated leucocyte adhesion [68].

Gut-associated lymphoid tissue (GALT)

GALT (made up of germinal centres and the PPs) sits below specialised epithelial cells [4]. PPs are sites of immune induction; they are located on the

anti-mesenteric side of the small intestine and consist of groups of mucosal lymphoid follicles coated with a single cell layer complex, the follicle associated epithelium. Antigen enters PPs across specialised epithelial cells (known as M-cells) to antigen presenting cells which then stimulate antigen-sensitive lymphocytes.

The epithelial layer overlying the follicle associated epithelium is designed to allow access of macromolecules and micro-organisms to the local epithelial surfaces and to promote their uptake by trans-epithelial transport [60]. M-cells, the main feature of follicle associated epithelium, are joined to neighbouring cells by tight junctions and provide openings in the epithelial barrier through vesicular transport [60]. They lack rigid internal cytoskeletons and are easily deformed by lymphoid cells migrating into the epithelium, a pocket (microfold) develops below the M-cell where lymphoid cells enter and leave without disruption to the cell membrane (see Figure 4).

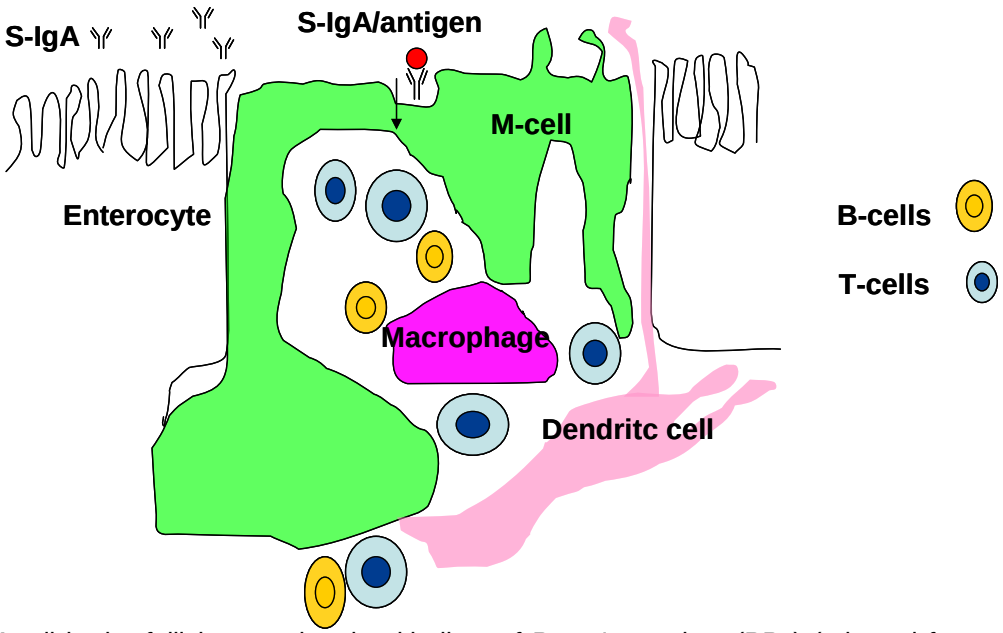


Figure 4: M-cell in the follicle associated epithelium of Peyer's patches (PPs) (adapted from Kato and Owen, 1999 [61])

M-cells have an important role in immunological function. They are involved in sampling foreign material on the epithelial surface and receptor mediated uptake of antigen, but they are not involved in antigen processing [45]. There are no closely packed microvilli on the apical surfaces of M-cells (as seen on other enterocytes). This enables them to respond rapidly to the adherence of

micro-organisms [60]. Uptake of micro-organisms, macromolecules and particles is through endocytosis, these materials are delivered to the intraepithelial pocket by the transepithelial vesicular transport system [60].

Antigen transported through the M-cells to dendritic and macrophage cells is processed and presented to CD4+ T-cells and naïve B-cells, which later become the cytokine-producing cells and plasma cells required for mucosal immune protection [53]. The high-endothelial venule cells in PPs express mucosal addressin cellular adhesion molecule (MAdCAM-1) which directs the migration of naive T- and B-cells through the interaction with L-selectin (a lymph-node homing receptor expressed on lymphocytes) and the integrin α4β7 [53].

1.3.2 Mucosal protection of the upper respiratory tract

Immune protection against antigens begins in the oral mucosa (which form part of the oropharyngeal region of the upper respiratory tract) and is by both non specific (lysozyme, lactoferrin, lactoperoxidase and various anti-microbial peptides) and specific (immune cells, S-IgA, and other immunoglobulin isotypes) defence mechanisms. Saliva contains approximately 10^8 bacteria/mL and in the oral cavity there generally are more than 400 bacterial species [50]. Therefore the oral mucosal immune system must cope with an enormous antigenic challenge without damaging the host [50].

Although the oral cavity displays similarities with other parts of the mucosal immune system, some immune mechanisms differ from those found elsewhere. Local immune responses include those that are part of the secretory immune system from the major and minor salivary glands [50]. There is also a protective role of the systemic immune system, where if the mucosal site is not able to clear the antigen then locally activated T- and B-cells may release cytokines that interact with macrophages and neutrophils resulting in local inflammation [50]. However, the exact relationship between alveolar bronchial lymphocytes and macrophages and circulating lymphocytes is still being investigated [59].

Similar structures to GALT are found in bronchial associated lymphoid tissue (BALT). There is evidence of communication between BALT and distant mucosal sites via the circulatory system involving predominantly a gut to bronchus flow of cells from PPs [53]. Antigen-reactive T-cells and B-cells from the PPs can populate the bronchial mucosal tissues to modulate immunity [59]. The proposed existence of a CMIS has prompted research into development of vaccines that can be administered at distal mucosal sites. In addition there has been recent interest in the role of nutrition on stimulation of GALT as it appears the route (parenteral or enteral) of nutrition can affect the histology, cytokine levels, IgA levels and effectiveness of intestinal respiratory immunity [53].

1.3.3 Common mucosal immune system (CMIS)

Evidence that a CMIS exists can be seen in the similarities of tissues and cells throughout mucosal-associated lymphoid tissue. Additionally immunisation at one mucosal site leads to the appearance of a detectable immune response at a distant site [47, 59]. Animal studies have shown that immunisation in GALT results in specific IgA-producing cells that migrate to the respiratory tract [53]. This suggests that locally-stimulated lymphocytes traffick to distant sites where they reside as IgA-producing plasma cells or as memory B-cells [47]. Nasal and oral immunisation generate IgA responses in diverse sites including gastrointestinal and bronchial tracts, and in secretory glands such as salivary, parotid, lachrymal, cervical and mammary glands [69]. It has also been suggested that enteral stimulation of the production of salivary S-IgA occurs only from the submandibular and sublingual glands, and not from parotid secretions [70]. This indicates that B-cell induction in GALT can result in an IgA response from salivary glands. Cells processed within GALT may home to intestinal and extra-intestinal sites and induce specific immunity, but it is still not clear how B-cell homing is regulated [53].

Nutrition has an important role in the maintenance of host mucosal immune defences. Evidence from parenterally-fed patients indicates an increased risk of infection, particularly pneumonia and intra-abdominal abscesses [71]. Lack of enteral stimulation in parenterally fed patients is associated with an increase

in respiratory infections due to an impaired IgA-mediated mucosal immunity [72]. In mice after four to five days of parenteral feeding intraluminal IgA levels decreased, and GALT atrophy occurred in conjunction with a drop in respiratory tract IgA levels [53]. Levels of all markers were restored when enteral feeding was reintroduced [53]. Without enteral feeding, the co-ordinated system of sensitisation, distribution and interaction of T- and B-cells, important in the production of IgA, the maintenance of normal gut cytokines and the regulation of endothelial markers, can be affected [71, 73]. This indicates that salivary S-IgA levels may reflect changes in intestinal levels of S-IgA following disturbances to immune homeostasis. Changes in levels of salivary S-IgA could therefore be a useful non-invasive marker of the effect of a nutrition intervention.

There may also be other stimulatory effects of nutrition on levels of S-IgA. In parenterally fed mice there was a reduction in GALT T- and B-cell numbers as well as in the expression of MAdCAM-1 [74], which was replicated when a blockade of L-selectin and α4β7 expression occurred. However when the mice were enterally fed (chow-fed), normal levels of intestinal and respiratory S-IgA were maintained despite the blockade [74]. Enteral feeding did not maintain GALT cellular mass in the presence of the blockade, intestinal and respiratory S-IgA levels were maintained [74]. An overview of the interaction of homing receptors on lymphocytes and various cellular adhesion molecules at the mucosal effector site is presented next in Section 1.3.4.

1.3.4 Lymphocyte trafficking

Migration of lymphocytes from the blood-stream into secondary lymphoid tissue is essential for maintenance of both the innate and adaptive immune systems and for protection against pathogens [75]. Lymphocytes mostly migrate through high endothelial venule cells regulated in part by a sequence of lymphocyte/endothelial adhesion interactions (mainly α4 integrins, α4β1 and α4β7) and the appropriate tissue ligand vascular-adhesion molecule-1 (VCAM-1) [75]. Expression of α4 integrins during cell migration can be rapidly affected by chemical stimuli e.g. LPS down-regulates expression on B1 cells [76] and

TGF-β1 can upregulate α4 integrin-dependent adhesion of different leucocytes [68]. Tissue-selective expression of endothelial adhesion molecules that bind to ligands on circulating lymphocytes partly control migration [75].

Naïve T and B-cells that have been sensitised to antigen in PPs become effector and memory cells and migrate to the mesenteric lymph nodes, the thoracic duct and back to intestinal LP through the action of MAdCAM-1 (a ligand for L-selectin and α4β7 found on naïve lymphocytes) located on the high-endothelial venule cells of PPs [53]. Trafficking properties differ between naïve and memory cells in that naïve cells are homogenous in their homing, which facilitates their entry into the lymphoid system. Memory cells are heterogeneous in their trafficking enabling them to home to various mucosal sites [69]. For example, T-cells that have memory for intestinal antigens express α4β7 and traffic to intestinal PPs and LP through their interaction with MAdCAM-1, whereas T-cells that have memory for systemic antigens traffic to non-intestinal inflammatory sites often using α4β1 interactions with VCAM-1, present at sites of inflammation but not intestinal tissues [69].

The expression of homing receptors on B-cells is not as well defined especially on circulating memory B-cell populations. Naïve and memory B-cells expressing α4β7 (such as B-cell blasts and plasma cells producing IgA found in the LP, and B-cell blasts in GALT, PPs and appendix) but not α4β7- memory B-cells, bind MAdCAM-1 [69] Approximately two-thirds of S-IgA+ B-cells express intestinal α4β7 and one-third are α4β7-. This suggests that about a third of the S-IgA+ B-cells lack the ability to enter the intestinal LP. These cells may therefore preferentially home to other mucosal tissues which produce IgA but do not express high levels of MAdCAM-1 (such as the oral mucosa, BALT, tonsils or adenoids and genitourinary tract) [69]. Both α4β+ and α4β7- B-cells bind to VCAM-1 which could also facilitate their recruitment to extra-intestinal sites, particularly to tissues in the CMIS that are expressing VCAM-1 e.g. salivary glands and oral mucosa [69].

1.4 Aetiology of upper respiratory tract infections

URTI are commonly caused by viral infection; the majority of viruses enter the body via the respiratory tract where they infect selected cell types, replicate within those cells and are able to infect other cells [48]. The incidence of the common cold and influenza increases during the winter possibly because of a cooling of the nasal passages and associated weakening of immune defences [77].

Over 200 viruses may cause URTIs including: orthomyxoviruses (influenza), paramyxoviruses (respiratory synctial virus), parainfluenza, coronaviruses, picornaviruses, rhinoviruses, herpes viruses and adenoviruses [77]. Mild upper respiratory illness (the common cold) is mostly caused by rhinoviruses (about 50% of all cases [7]) and coronaviruses [78] [79]. The primary site of replication for rhinoviruses is the nasal mucosa and infection appears to be restricted to the upper respiratory tract. Rhinoviruses bind to intercellular adhesion molecule-1 which is on the apical surface of epithelial cells suggesting trans-epithelial transport is not required for infection. Temperatures in the upper respiratory tract (about 33 °C) are also optimal for virus survival and replication [80]. Viral shedding in nasal secretions can reflect the extent of the infection. If swallowed however, rhinoviruses do not survive passage through the gastrointestinal tract because of their sensitivity to reduced pH.

S-IgA as the main effector of mucosal immune protection has a multifunctional role in preventing URTI. In athletes, reduced levels of salivary S-IgA have been observed immediately after prolonged intensive bouts of exercise [81]. If the post-exercise recovery period is inadequate (i.e. short duration, insufficient nutrient replenishment, inadequate sleep) the cumulative effect over a training season can result in S-IgA levels remaining depressed [82]. Increased risk for URTI was observed in elite swimmers with reduced levels of S-IgA which supports the importance of the role of mucosal protection in prevention of URTI [83]. Older adults also experience an increased incidence of URTI thought to be due to immunosenescence. The increased incidence of infectious diseases

such as URTI in older adults is an important health concern because of the associated increased morbidity and mortality rates [84].

Typically upper respiratory symptoms (URS) begin within one to two days after infection and last one to two weeks. Symptoms are experienced as a result of the disturbance of the normal functioning of cells. As a consequence of local inflammation and systemic aches, fever and fatigue can persist long after the URS disappear; actual clearance of the virus can take longer [48]. Confirmation of URS as URTI is difficult and in clinical trials generally occurs by physician diagnosis or through self-recorded wellness records both of which are problematic. Verification requires serological investigation which is very rarely performed as it is expensive and not practical for large numbers of participants [48]. This is a difficulty in determining the effect of an intervention on a specific immune response and associating any effect with a health outcome such as the incidence of URTI. For these reasons some researchers exclude self-recorded URS days if the duration is less than two consecutive days [85, 86]

Reliability and validation of self reporting of URS

In longitudinal studies, when investigating the effect of a nutrition intervention on changes to an immune marker of mucosal immunity (such as salivary S-IgA), monitoring the incidence of URS is important as viral infection of the upper respiratory tract is accompanied by changes in levels of salivary S-IgA. It has been shown that the immune response to a viral infection results in increased salivary S-IgA levels about six days before the first URS appear, and levels remain elevated before returning to previous levels about 10 days later [48]. Independent subjective evaluation of URS by a physician could be helpful in qualifying symptoms but may not be possible in longitudinal studies, therefore the reliability and accuracy of self-recording of URS is important [8]. There is no ‘gold standard’ questionnaire for assessing symptoms of URTI, as they vary considerably between individuals in intensity and duration [8]. The most commonly used severity rating system was developed in the 1960s and is based on a simple sum of severity points (0=absent, 1=mild, 2=moderate, 3=severe) [8]. It has several limitations including lack of assessment of the

effect on quality of life, and for the athlete the impact of URTI on the ability to continue training.

Questionnaires used to evaluate the effect of a nutrition intervention or physical stress on URS have been criticised for the subjective nature of reportage. This is because of the inability of the individual to distinguish between symptoms due to viral infection or due to other factors such as inhalation of air pollutants [4]. A questionnaire with 44 items (including 32 symptom-based items, and 10 functional quality of life items) has been validated by determining the importance of symptoms to patients [8]. While a questionnaire of this length would not be practical in longitudinal nutrition studies it may be useful for validating a shorter questionnaire. A combination of immune parameter measurements and a questionnaire (or daily wellness records) may provide a better understanding of the changes to immune function and health outcomes that may be a result of a nutrition intervention [4].

As the yearly incidence of URTI is between two to four episodes per person, nutrition intervention trials need to be of sufficient length to detect potential differences (e.g. at least six months) [4]. Interventions of this length may be problematic with respect to compliance of consumption of the intervention. In addition large numbers of subjects studied over a long period of time are required to investigate the effects of a nutrition intervention on infection risk. For example Albers *et al.,* 2005, suggest at least 50 participants for a placebo and 50 in a control group [4], but numbers depend on the desired study end point and need to be statistically determined in order to establish a significant difference in effect. Recruitment for intervention studies to reduce incidence of illness may introduce bias as those participants who experience a higher incidence of URTI could be more likely to volunteer making it difficult to recruit a representative sample [8].

1.5 Effect of exercise on mucosal immunity

It is generally accepted that athletes participating in prolonged bouts of intensive exercise may experience periods of under-performance associated with upper respiratory illness [87-89]. In competition a reduction of hundredths of a second in could be the difference between silver and gold medal standard performance [13]. Moderate levels of exercise, are considered to be of benefit to immune function and athletes who do experience a higher frequency of URTI are not considered to be clinically immune-deficient [88]. Athletes reporting URS do not always present with the classical URTI symptoms, and severity and duration may not be enough to stop training. However symptoms that become persistent and recurring are characteristic in athletes experiencing the over-training syndrome [90]. Insufficient recovery between exercise bouts may result in a chronically depressed immune system [11, 91], putting the athlete at increased risk for URTI [90, 91]. But, even in over-trained athletes exhibiting depressed immune function, an increase in infection rate is not always observed [92].

1.5.1 The effect of exercise-induced stress on immune function

Research suggests that short term (4 weeks) high intensity exercise, and medium term (8 to 15 weeks) moderate intensity training do not significantly alter immune function. It is high intensity training for greater than six months which may result in immune depression as has been indicated by a reduction in natural killer cell numbers [91] and depressed salivary S-IgA levels [82]. Exercise intensity is also an important factor in lymphocyte redox status and apoptotic processes [93]. In normally sedentary males (aged 19 to 23 years) intensive exercise reduced lymphocyte glutathione peroxidase content, raised oxidative stress-induced phosphatidyl serine exposure and DNA fragmentation in lymphocytes [93], while moderate exercise increased glutathione peroxidase content improving resistance to damage from oxidative stress [93]. However, adaptations of the immune system to long-term training by endurance and ultra-endurance athletes also occurs as NK and lymphocyte cell functions are enhanced compared to sedentary individuals [91].

In athletes experiencing recurring URS, it is possible that the viral infection has not cleared, or reactivation is occurring rather than that a new primary infection is taking hold. Reactivation may be caused by disturbance of previously latent Epstein-Barr virus, found within immortalised B-cells. These cells are under the tight control of Epstein-Barr virus-specific CTLs; transient suppression of CTLs cells allows transformation of the B-cells and expression of Epstein-Barr viral-DNA, followed by an increased incidence of URS. In a cohort of elite male swimmers a high proportion experienced latent Epstein-Barr viral shedding which appeared to precede the appearance of URS [48]. In this cohort of swimmers low baseline levels of salivary S-IgA (probably as a result of sub-clinical immune dysregulation) may also have contributed to the incidence of URS [48].

Immune cell concentrations and functions are affected acutely by exercise for up to 72 hours, and during this time a dormant pathogen can take hold [94] or exposure to a new pathogen [95] could result in URTI. Reported changes to immune cell concentrations in response to acute bouts of exercise have been summarised and published by Lim and MacKinnon, 2006 [91]. Immune system changes included leucocytosis (53 to 261%), monocytosis (32 to 396%), granulocytosis (32 to 396%), neutrophilia (78 to 260%) and lymphopaenia (-19 to -60%), all of which lasted for more than an hour after exercise [91]. Lymphocyte concentrations decreased post-exercise (but returned to normal resting levels within 6 to 24 hours); changes were seen in natural killer cells (-60 to -80%), B-cells (-8 to -40%), T-cells (-14 to -50%), T-suppressor cells (-40 to -60%) and T-helper cells (-5 to -52%) [91].

Neutrophil and monocyte numbers are either not affected or are increased after acute bouts of exercise. After a marathon race it was shown that exhaustive exercise in ten male runners (aged 31$\pm$5 years) induced increases in neutrophil and monocyte numbers. This was accompanied by larger increases in anti-inflammatory cytokines (as indicated by increases in plasma IL-6 and IL-10) and antioxidant mechanisms suggestive of a protective mechanism against exercise-induced oxidative stress [96]. Cellular functions may also be altered by prolonged periods of intensive exercise, for example reduced neutrophil

oxidative activity at rest [97], and blunted MHC-II expression and antigen presenting ability in macrophages [98] have been observed.

There is lack of agreement on the source of the temporary increase in numbers of circulating leucocytes in the peripheral blood after exercise. It is possible their origin is from blood vessel walls, lung, gastrointestinal tract or the spleen [92]; because the cells have a short telomere length they are unlikely to have come from the bone marrow or thymus [92]. Conversely Suzuki *et al*., 2003 [96] suggested that increased neutrophil levels seen in marathon runners post-exercise originated from bone marrow due to stimulation by IL-6 and colony stimulating factor. It is also not clear where the leucocytes migrate to when cell numbers decrease; muscle biopsies show that the immune cells do not migrate to muscle tissues to participate in the inflammatory process as was originally thought [92]. It is possible that cells adhere to endothelial vessels, skin, spleen and lungs. While leucocyte numbers may return to baseline levels after a bout of exercise there may be a different response to a second bout especially if insufficient recovery time has been allowed [92].

Additionally, exercise primes circulating leucocytes to respond to physiological stress by increasing the expression of growth factors as well as pro- and anti-inflammatory factors which is is part of the adaptive response to exercise enhancing the athletes' ability to recover from tissue injury [99]. For example following eccentric exercise (where muscles are lengthened to control motion of the body resulting in muscle fibre damage): decreased levels of TNF-α and increased protein synthesis occurred, while in younger adults no change in TNF-α occurred although a number of skeletal muscle immune system interactions were observed [99]. Leucocytes might also have a role as a virtual endocrine organ and supply specific tissues with a variety of mediators that participate not only in the immune response but could participate in other metabolic functions such as bone remodelling [99].

Previously it was proposed immune alterations experienced by athletes were largely a result of muscle trauma stimulating cytokine production that affected immune cell profiles, in particular lymphocyte subsets [90]. Muscle trauma

includes the mechanical disruption of cellular proteins, metabolic stress on sub cellular contents [100] and disruption of biochemical processes involved with cellular remodelling; protein synthesis and degradation [101]. Oxygen consumption increases during exercise and significantly impacts on the production of ROS. However, defence mechanisms such as enzymatic and non-enzymatic (vitamin) activities adapt to the demand of exercise to reduce the effect of reactive oxygen species on muscle cells [100].

Further evidence of an inter-related role of muscle adaptation and immune function is demonstrated by the role muscle has in the production of IL-6. In response to falling glucose levels, IL-6 is produced by the muscle to regulate substrate delivery and maintain metabolic homeostasis [102, 103]. As well as being anti-inflammatory and inducing C-reactive protein release from hepatocytes, IL-6 stimulates an increase in plasma cortisol (a catabolic hormone) production which may also be immunosuppressive. In a group of 14 male cyclists (25$\pm$5 years) cortisol levels were associated with elevated neutrophil levels, indicating cortisol-induced mobilisation of neutrophils from the marginal pool and the bone marrow after a bout of exercise [102]. Elevated cortisol levels affect the redistribution of lymphocyte subsets from the blood to the peripheral tissues, mitogen-induced lymphocyte proliferation and NK killer cell cytotoxic activity [104]. IL-6 also limits the inflammatory response by inducing an increase in levels of IL-10 and IL-1ra and cortisol without any effect on levels of TNF-α, plasma catecholamines, body temperature and heart rate [105].

Recently it was shown that an acute bout of intensified training induced a mild prolonged inflammatory response associated with feelings of fatigue, and sickness-like symptoms in endurance runners [87]. There was a significant elevation in post-exercise levels of IL-6 and TNF-α as well as a decrease in neutrophil function 16 hours after the bout of exercise was performed. The effect of the decrease in neutrophil function and risk for URTI is still unknown [87]. The source of the chronically elevated IL-6 was likely to be from macrophages in response to damaged muscle as well as production by the muscle in response to low muscle glycogen stores [87]. Another study showed

an association of self-rated health and levels of circulating cytokines [106]. Together this highlights the difficulty that exists in identifying which self-reported URS (by athletes) are related to URTI.

The effect of prolonged intensive exercise on levels of salivary S-IgA, IgM and IgG in athletes

Prolonged intensive exercise may reduce resting salivary S-IgA levels; elite swimmers with levels below 40mg/L at the beginning of a training season were more likely to experience URTI during the season [82]. Reduced levels of salivary S-IgA have also been shown to increase the risk for experiencing URS in American footballers [21]. It has been suggested that temporary deficiency of antibodies at the mucosal surfaces could make individuals susceptible to infection from pathogenic viral and bacterial organisms [19, 48, 81, 82].

Levels of S-IgA in the respiratory tract are influenced by several mechanisms including; the regulation of the saliva secretion rate, the rate of S-IgA synthesis (which may take days) and transport across the epithelium (taking minutes) [103]. During exercise the composition and flow rate of saliva are affected by the sympathetic nervous system and hypothalamic-pituitary-adrenocortical (HPA) axis. Salivary glands are innervated by both the parasympathetic and sympathetic nerves. Increased sympathetic nervous system activity in response to exercise stress affects saliva flow rate and increases cortisol production; it has been reported that cortisol plays a role in inhibiting mobilisation of S-IgA across the mucosal epithelium [103]. Alterations to T-cell control in athletes may also affect levels of S-IgA in saliva. It has been proposed that levels of salivary S-IgA may therefore be a surrogate measure of disturbed immunity [82, 94]. An indication of altered T-cell control (reduced IFN-γ production) was seen in athletes experiencing fatigue as a result of prolonged intense periods of exercise [94].

Few studies have investigated the effect of exercise on other salivary immunoglobulins. In a study with 41 elite swimmers and 25 non-exercising controls (coaching and administrative staff) no alterations to S-IgA, salivary IgG and IgM concentrations were found over a training season [54]. Lower levels of

salivary immunoglobulins were identified in the non-exercising controls compared to the swimmers, which was thought to be associated with psychological stress and lifestyle factors [54]. In a 12-week study of elite swimmers small significant increases were identified in levels of post-exercise S-IgA, salivary IgG and IgM which were not associated with the incidence of URTI [107]. In contrast a study of endurance athletes over an eight week period showed a decrease in post-exercise levels of S-IgA, salivary IgG and IgM which was thought to have been associated with a decreased output of saliva [108]. These findings indicate the relationship between salivary immunoglobulins and URTI in athletes is complex and is still poorly understood.

1.5.2 The effect of exercise on the gastrointestinal tract

Gastritis (with a range of upper and lower gastro-intestinal symptoms) occurs at a high rate in endurance athletes, and ischaemic colitis has been observed in female marathon runners as young as 26 years [109]. Distance running is associated with symptoms in the lower gastrointestinal tract whereas cycling appears to be associated with symptoms in both the upper and lower tracts [110]. The gastrointestinal mucosal surface protects the sterile internal environment from contamination by the gut microflora. LPS is a toxin present in the walls of gram-negative bacteria and these bacteria are harmless when they are in the gut. Small amounts of LPS may cross the gut epithelial tissue but are quickly detoxified by the liver, preventing entry into general circulation [91]. During prolonged exercise blood flow to the intestine is reduced by about 80%. This reduction may persist for some time after exercise, possibly resulting in gut ischaemia [109] and hypoxia, which affect the paracellular tight junctions [91] allowing macromolecules to leak through. Increased gut permeability has been observed in athletes in several studies (determined by increased levels of LPS); temporary, transient endotoxaemia has been documented in ultra-marathoners [91], triathletes [111] and marathoners [112].

Higher levels of circulating anti-LPS antibodies occur in trained athletes due to constant exposure to sub-lethal LPS permeation [91]. However, concentrations of anti-LPS antibodies may be reduced for up to 24 hours post-exercise and

insufficient rest between bouts of exercise would affect the time for levels of anti-LPS antibodies to return to normal [91]. As LPS can down-regulate the expression of α4 integrins on leucocytes it may also be possible exercise-induced disturbances of cell-to-cell communication could alter migratory patterns of IgA-producing cells which may impact on production of salivary S-IgA [76]. Increased neutrophil activation has been associated with endotoxaemia experienced in triathletes [111]. Neutrophils may be recruited to the site of gastric mucosal injury, where there is a release of oxidative products to kill bacteria. This can result in further inflammation if it occurs in an uncontrolled manner [113, 114]. The expansion in numbers of circulating neutrophils observed post-exercise could be partly due to LPS permeation in those athletes that experience disruption to intestinal membranes.

The gut mucosal barrier is capable of remaining intact, despite coming in contact with acid and proteolytic enzymes that degrade food, and despite superficial injuries from physical trauma and consumption of substances such as aspirin and alcohol [64]. However, when the mucosal barrier is disturbed, homeostasis is disrupted and many different peptides and growth factors are required to participate in gut repair to restore mucosal integrity [64]. It is not known if supplements, containing various growth factors could also have an effect on physiological processes such as enhancing the repair of exercise-induced intestinal damage. Results from clinical trials indicate BC (which contains many different peptides, nucleotides and growth factors) stimulates bowel growth in patients consuming non-steroidal anti-inflammatory drugs [64, 115] (see Section 1.7.6). In addition supplementation with BC reduces gut permeability in heat-stressed rats probably by preventing leakage through the tight junctions [116].

1.5.3 The effect of exercise on the induction of stress hormones and their effect on immune function

Exercise-induced stress also affects the endocrine system, which is activated to maintain internal homeostasis [117]. During exercise, communication between the brain and the immune system is important and involves activation of the

HPA-axis and the sympathetic branch of the autonomic nervous system [90]. The HPA-axis is activated by release of corticotrophin-releasing hormones during a stress response [90]. The central stress response is activated by a number of neural and blood-borne factors including cytokines, TNF-α, IL-1, IL-6, IL-2 and IFN-γ with IL-1 being the most potent [90]. Activation of either the HPA-axis or the sympathetic nervous system results in the activation of the other system in a feedback loop releasing cortisol into circulation (see Figure 5).

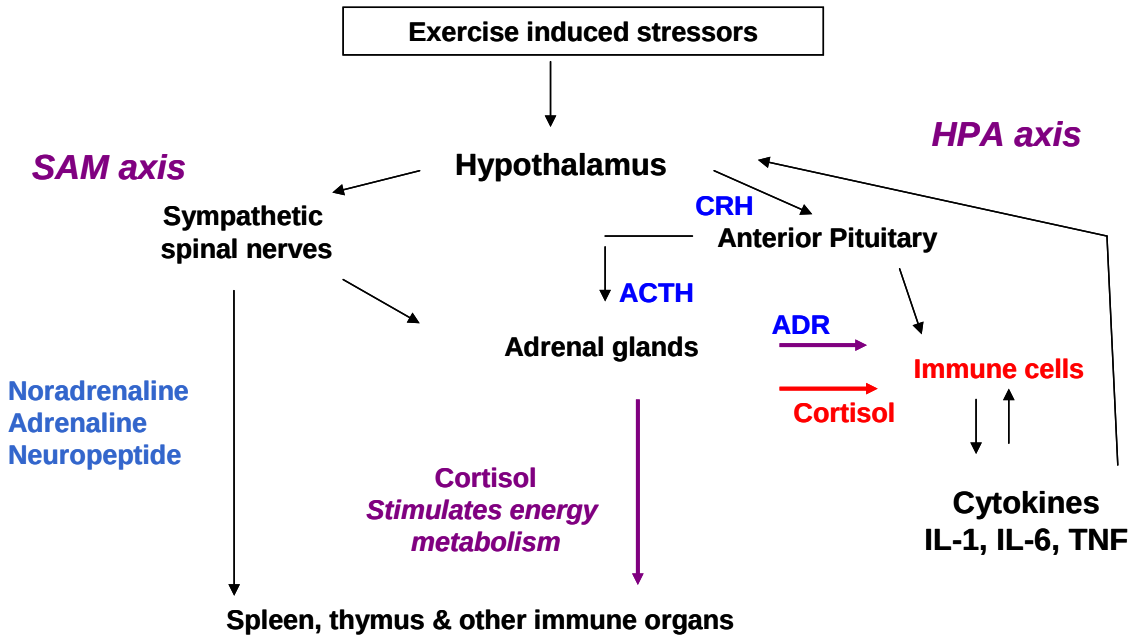


Figure 5: Communication between the brain and the immune system in response to exercise-induced stress (adapted from Armstrong, 2002 [117]). SAM axis=sympathetic-adrenal medullary axis, HPA axis=hypothalamic-pituitary-adrenocortical axis, CRH=corticotrophin-releasing hormone, ACTH=adrenocorticotrophic hormone, ADR=adrenaline

The stress hormones can suppress an immune response and also exaggerate immune inflammatory reactions. Cortisol is anti-inflammatory and restrains the initial inflammatory response in order to prevent further damage. It is also immunosuppressive as it prevents migration of leucocytes to extra-vascular tissues [90]. This reduces the accumulation of monocytes and granulocytes at the site of inflammation, controls cytokine production, and inhibits lymphocyte proliferation, migration and cytotoxicity [90].

Glucocorticoids and catecholamines may suppress cell mediated immunity but boost humoral immunity suggesting an influence on the Th1/Th2 lymphocyte balance and associated cytokine production [90, 92]. The glucocorticoids act

through their cytoplasmic/nuclear receptors on antigen presenting cells to suppress production of IL-12 (the main inducer of Th1 cells), but stimulate IL-4 production (important in up-regulating the Th2 humoral response) [90]. In 14 moderately trained males (aged 24.3±3.6 years) levels of salivary S-IgA were significantly lower and salivary cortisol levels were significantly higher after exercise compared to pre-exercise [118]. This indicated there may have been a suppressive effect of exercise-induced release of cortisol on salivary S-IgA levels. The mechanism for how glucocorticoids affect mucosal immunity in athletes remains unknown.

1.5.4 The effect of exercise and the role of growth factors on immune function

Exercise has an effect on the relationship between inflammatory cytokines and growth factors [99]. The extent of this effect is what confers a health benefit of exercise preparing the athlete for further phusical stress: for example in older adults higher levels of IL-6 were inversely correlated with levels of physical activity, muscle mass, and insulin-like growth factor-1 (IGF-1) [99]. Stress activation of leucocytes therefore could benefit post-stress challenges such as tissue injury [99].

In younger adults brief bouts of heavy exercise were associated with the mobilisation of populations of cells (lymphocytes, monocytes and neutrophils) from the marginating pools, which all secrete a range of cytokines and growth factors [99]. In 11 young men (aged 24±0.9 years) who performed a bout of heavy exercise, the proportion of B-cells expressing growth hormone (GH) and IGF-1 far exceeded the increase in circulating B-cells [99]. In children (12 year old girls and boys), fewer perturbations to the immune system are observed than in young adolescents (14 year old girls and boys) [16]. It is thought that in younger children anabolic mediators such as IGF-1 are particularly sensitive to acute changes in inflammatory cytokines. Therefore in children, where metabolic processes for growth are important, being relatively resistant to major inflammatory responses during and after exercise may be protective to minimise disruption to anabolic mediators [16].

1.6 The effect of ageing on immune function

1.6.1 Respiratory illness in older adults

There are many immunological changes that occur with ageing including an increase in the incidence of respiratory illness [10, 119]. In adults over the age of 65 years, influenza is the fourth leading cause of death, after heart disease, cancer and stroke [120, 121]. The immune remodelling that occurs with ageing can also result in a reduced immune response in some individuals such as antibody response to vaccination. In a population of 210 older adults hospitalised with influenza, 129 had been vaccinated. This may have been due to a suboptimal response to the vaccine [121], or the strain of influenza may have been different to the vaccination. Changes to immune function may be further exacerbated in the frail elderly due to the effects of malnutrition including protein, calorie, vitamin and mineral deficiencies [120].

There has been increased interest in the use of nutrient formulae to enhance immune function in older adults and some studies have shown that supplementation with various nutrients can enhance antibody responses to influenza vaccination in this age group [121]. Reduced incidence of URTI and a higher antibody titre to vaccination were observed in older adults who took a trace element supplement to correct selenium deficiency [121]. In another group of 16 healthy older adults who consumed a nutrition supplement (which included antioxidants, vitamins, minerals [zinc, selenium], and fermentable oligosaccharides) for 183 days, there was less reportage of URS, a better antibody response to influenza (Beijing strain [H1N1]) and greater lymphocyte proliferative response to influenza vaccine than in the placebo group [121].

1.6.2 Immune remodelling during senescence

The immune system is not fully developed at birth and undergoes significant changes as it becomes exposed to microbes and other inhaled and consumed environmental antigens [122]. The gastrointestinal tract is colonised throughout life, and maintenance of mucosal integrity and a balanced flora are essential to

prevent infection [122]. Differentiation of the immune system continues through adolescence, adult life and during senescence when complex remodelling occurs. The function of some immune parameters does not change, for example populations of B-cells [37] and antibody production in secondary responses are not diminished [123]. It is possible that B-cell function may play a key role in successful ageing, as was shown in a study of 513 Swedish octogenarians where survival was associated with higher percentages of cells expressing CD19 (B-cell activation marker) after controlling for age and gender [124]. While immune remodelling during senescence is characterised by thymic involution, the extent of this is varied. This suggests other factors such as nutritional status impact on immune function, and changes in metabolism may also contribute to remodelling of the immune system [122].

The effect of ageing on the innate immune system

In the healthy older adult the main change in innate immune function appears to be a variance in the antigen presenting cells (APC) ability to communicate with T-cells [119]. With ageing the expression of co-stimulatory molecules changes resulting in altered cytokine production by APCs and therefore affecting downstream T-cell function [119]. In addition the number of immunocompetent T-cells is affected by age-related involution of the thymus [119].

The effect of ageing on T-cell function

Impairment of T-cell function is thought to affect the helper CD4 T-cells more than the cytotoxic CD8 T-cells [125]. Evidence for this is the loss of ability of CD4 T-cells to up-regulate telomerase activity (essential for lymphocyte proliferation) [125]. There is a slow decline in proliferation of naïve CD4 T-cells [119] post-thymic involution, suggesting clonal expansion of CD4 T-cells is still occurring in the peripheral circulation [126]. There will be a smaller pool of naïve T-cells that can respond to novel antigens. Deterioration of CD4 T-cell function will impact on protection at mucosal surfaces because of their involvement in the regulation of antibody production and could contribute to autoimmunity in the aged [126]. T-cell subset distribution also changes with ageing. Increased numbers of CD4 cells have been found in nasal associated lymphoid tissue in aged mice compared to young mice, in response to

vaccination [127]. In humans accumulation of $CD4^+$ T-cells has been observed in the air spaces of the lower respiratory tract in healthy older adults [128] which suggests CD4 lymphocyte activation could be protective for normal lung function [129]. This may be an example of immune system remodelling in older adults.

The effect of ageing on B-cells

With ageing the absolute number of B-cells is reduced, but a larger proportion of these remaining cells are memory cells with a noticeable reduction in the naïve B-cell pool (CD27-) [123]. B-cells from older populations have impaired activation and proliferation that may be related to changes in co-stimulatory molecule expression [119]. Primary and secondary responses to vaccination can be impaired especially when T-cell involvement is required, and the specificity and efficacy of antibody production in older individuals is lower than that produced in younger individuals [119]. Changes to B-cell sub-populations observed in older adults include a decrease in B-cells expressing CD5 (considered to be responsible for T-cell independent antibody production), no changes in CD40/CD19 (T-cell dependent), and increases in CD27 (which is a marker of primed or memory cells) and natural killer cells [123]. It is thought that there is increased co-operation between B-cells and natural killer cells, which might be important in regulation of antibody formation in the absence of T-cell control, and might be advantageous to the host in early protection [123]. This immune remodelling also partly explains why antibody production appears to be largely unaffected with ageing, alternatively the early B-cell response may contribute to increased autoimmunity in older adults [123].

The effect of ageing on changing lymphocyte subsets

Ageing increases the CD4/CD8 T-cell ratios in human peripheral blood (and to a lesser extent in the lower respiratory tract) and it is thought that this is genetically controlled [129]. An increase in numbers of CD4 lymphocytes is thought to be due to an accumulation of memory cells and primed T-cells in response to cumulative antigenic stimulation at mucosal surfaces [129]. This may be a protective effect to an ageing immune system as older populations with low levels of CD4 T-cells have an increased mortality risk [129].

The distribution of lymphocyte subsets also changes with age and may be site-specific [130]. In the ageing mouse an increase in the numbers of lymphocytes in the liver, small intestine, colon and appendix was observed along with thymic involution [130]. These are extrathymic sites and as the lymphocyte subsets carried auto-reactivity it was thought the aged mouse may require auto-reactive cells that can act against altered self-cells developing with ageing [130]. The change in distribution of lymphocyte subsets observed in aged mice could therefore be considered to be beneficial to their immune health and could be another example of how the immune system remodels.

1.6.3 Age-related changes to mucosal immunity

Ageing is associated with more infections at mucosal sites such as the respiratory tract, in part because the development of a mucosal response to new antigens is diminished [127]. However the exact nature of the decline in mucosal immunocompetence is still unknown [131]. Changes in salivary gland function occur with ageing and these differences may be important to salivary S-IgA levels. Saliva secretion rates and levels of salivary S-IgA were investigated in 142 older adults (46 men, 96 women) aged 18–82 years [132]. The results indicated there was no impairment of the secretion rate from the minor salivary glands however flow rate of whole unstimulated saliva was reduced with ageing [132]. Concentrations of S-IgA in saliva were also positively correlated with reduced saliva flow rates [132]. This suggests that older adults with lower saliva secretion rates would also experience reduced levels of salivary S-IgA.

In aged mice reduced size of PPs have been observed which may affect oral tolerance and induction of mucosal immunity such as S-IgA production [133]. This may be an example of immune dysregulation that contributes to the decline of mucosal immunity in older adults. Additionally investigations indicate that the homing ability of IgA immunoblasts to the effector site is also compromised with age [134]. This is evidenced by a decreased expression of α4β7 homing receptor on mononuclear cells in aged adult rats compared to young adult rats [135].

In humans age-related changes appear to affect lymphocytes in the LP, but not the ability of intraepithelial lymphocyte to produce IL-2 [122]. Age-related decline in cellular regeneration of those cells with a high turnover rate such as intestinal and colonic mucosal epithelium [136] occurs which in turn has an effect on the ability to repair lesions to the mucosal layer [61]. The gut microenvironment may be further affected by changes to intestinal pH and enzyme functions which favour bacterial overgrowth in the proximal small intestine [136] and also impact on gut motility. Regulated gastrointestinal motility is important in the maintenance of the mucosal barrier [61].

Maintenance of the gut microenvironment requires energy, especially when the host experiences a new infection [122]. When rapid growth or a disease state exists there is an additional nutrient requirement for nucleotides which are essential for rapidly dividing tissue such as the gastrointestinal tract [122]. The immune response may be impaired if these additional nutrient requirements are not met. It is possible that maintenance of the mucosal immune response during ageing is related to a continual need to maintain a response to microbial flora in the gastrointestinal tract. Therefore managing the changes in the gut microenvironment that occur with ageing could have an important role in immune function [122].

The effect of exercise on mucosal immunity in older adults

Moderate levels of exercise may be of benefit to mucosal immunity in older adults. Salivary S-IgA levels and secretion rate were enhanced in 45 older adults (18 men, 27 women) aged 64.9 years $\pm$8.4 years after a twelve month exercise programme [137]. In another study of 274 older adults (114 men, 170 women), aged 71.3$\pm$3.1 years (mean$\pm$SEM), salivary S-IgA levels were significantly enhanced in those who performed more than 7000 steps per day (measured by a pedometer) for 14 days [138]. There were no changes in saliva secretion rate over this period [138]. The effect of exercise training on a health outcome such as the incidence of URTI was not investigated in either study. However the results from these two studies may indicate there is a beneficial effect of moderate exercise (such as performed by exercising free-living individuals) to mucosal immune health.

1.7 The effect of nutrition on mucosal immune protection

There is a synergistic relationship between nutritional status and immune status, both in athletes and older adults [23, 121]. In developing countries, immune function is affected by under-nutrition, but certain population groups are also at risk in developed countries. The rate of infection is higher in older people [10, 128] and this has often been associated with insufficient protein and micronutrient intake [139]. Other at-risk populations include those with eating disorders, alcoholics, premature babies and those with certain diseases [14]. There are multifactorial causes of an altered immune response in athletes and inadequate nutrition is known to have an effect [12].

Nutritional interventions to aid immune function have mostly occurred in the treatment of critically ill and surgical patients (to assist with post-operative recovery as periods of immunosuppression often follow major surgery) [140]. Early nutritional treatment can help to decrease the inflammatory response in critically ill patients and therefore minimise the compensatory mechanisms leading to immunosuppression [140].

Recently there has been interest in optimising immune function in healthy individuals by increasing intake of certain nutrients. The interaction between nutrient intake and immune function is not straight-forward and it cannot be assumed that all functions of the immune system will respond in the same manner to a given nutrient. The role of supplementation in enhancing immune function, especially in healthy individuals, is unclear, and excessive intake of some micronutrients may even be harmful to immune health. More research is needed to better understand an individual's susceptibility to infection, whether enhanced immune function actually improves resistance to infection [14, 141], and the role of a nutrition supplement in maintaining a healthy immune system.

The effect of nutrition on immune protection in athletes

Exercise increases the body's needs for most nutrients; many hormonal and biochemical changes occur with heavy prolonged exercise which will impact on the immune system, and inadequate dietary intake will further exacerbate this

effect. The cumulative effect of exercise on changes to the immune defence system and risk of infection is unknown [11, 141]. Manipulation of training and nutrition practices may improve immune function in the athlete although the role of dietary supplementation in this is still unclear. It is generally accepted that immune function in athletes will be improved by a well-balanced diet [11]. Supplementation with sport drinks (containing carbohydrate and electrolytes) during and after prolonged bouts of exercise reduces levels of exercise-induced stress hormones and cytokines which may benefit immune defence [103, 142].

Many athlete's training diets are considered to be sub-optimal. Those athletes following high protein and low carbohydrate diets, and vegetarians whose diets may be very high in carbohydrate and low in protein, are at risk of impaired immune function [12, 143]. Additionally some athletes follow diets that are low in energy, which are also usually low in dietary fat, and when combined with a heavy training load may modulate immune function. A low intake of dietary fat may affect immune function in several ways. Lymphocytes use fat as a fuel source, and the type and quantity of fat can have an effect on cellular and immune systems at the biochemical and molecular level e.g. cytokine production [104]. Dietary omega-6 fatty acids are thought to increase the levels of proinflammatory cytokines and prostaglandins whereas omega-3 fatty acids decrease these levels [104] (see Section 1.7.4). A summary of the macro and micronutrient deficiencies or excesses that may affect immunity in athletes is found in Table 2.

Table 2: Summary of nutrient deficiencies or excesses that may affect immunity

Nutrient	Possible Mechanism
Carbohydrate status	Important fuel for lymphocytes, neutrophils and macrophages, which have a high metabolic rate [12, 143]
Low plasma glucose during exercise	Raised levels of plasma cortisol, IL-6 and IL-1ra, reduced plasma glutamine and increased post-exercise neutrophilia [144]
Inadequate protein	Detrimentally affects T-cell system, impairs phagocytic function, plasma glutamine levels, salivary S-IgA concentrations, decreases cytokine production, decreases complement formation, [2, 12, 23]
Glutamine deficiency	Used at high rates by leucocytes to provide energy for nucleotide biosynthesis [12, 143, 145]
Excess protein at the expense of carbohydrate	Lowers muscle and plasma glutamine, which is used at high rates and could be considered essential for leucocytes [12]
n3:n6 essential fatty acids	May modulate inflammatory response [146]
Iron deficiency (lost in sweat, urine and faeces)	Affects lymphocyte proliferation (required for DNA synthesis) results in depressed cell-mediated immunity [2, 12, 147]
Zinc deficiency (lost in sweat and urine)	Impaired mitogen-stimulated lymphocyte proliferative responses [12]
Excessive zinc	Impaired T lymphocyte proliferation impairs copper absorption which affects lymphocyte stimulation and antibody formation [12]
Selenium deficiency	Impaired cell-mediated immunity and B-cell function [26]
Vitamin A deficiency	Impaired salivary S-IgA production, decreased mitogen-induced lymphocyte proliferation [2, 12]
Vitamin B6	Affects lymphocyte proliferation and can lead to lymphoid atrophy [148]
Vitamin B12	Essential for nucleic acid synthesis and cell replication [2, 12]
Vitamin D deficiency	Vitamin D acts as a lymphocyte differentiation hormone [2]
Vitamin C deficiency	Possibly affects proliferative response in T lymphocytes [145]
Vitamin E deficiency	Reduces proliferation of T and B lymphocytes and killing power of lymphocytes and leucocytes [12, 143]

The effect of nutrition on immune function in older adults

Immune function is impaired with inadequate nutrition, and older adults may be at risk due to poor dietary practices associated with age-related depression and social changes [15]. To date there has been little research investigating dietary nutrient intake and the effect on immune parameters and clinical outcomes in older adults [121]. Risk for poor nutritional status is higher in this group compared to younger adults due to a number of ageing processes such as decreased lean body mass, decreases in bone density, decreased gastrointestinal efficiency and an increased incidence of illness [149]. Immunological ageing is closely related to nutritional factors [150] and can be classified into three groups, healthy ageing (very healthy individuals with no nutritional deficit), common ageing (various micronutrient deficiencies are found) and pathological ageing (patients with protein-energy deficiencies). In the healthy aged the main changes are to T-cell subsets and not function. In those older adults with micronutrient deficits supplementation with micronutrients often assists immune function [150]. In pathological ageing the immune response is strongly associated with nutritional status; the disease state places increased demand on nutritional reserves, further increasing frailty [150].

1.7.1 The effect of carbohydrate status (from dietary intake) on immune protection

During periods of heavy training athletes should consume enough dietary carbohydrate to cover about 60% of their energy costs [143, 151]. The purpose of this is to replace glycogen stores in the liver and muscle to ensure there is sufficient glucose available for muscle contraction. Glucose is also a major fuel substrate for cells of the immune system (macrophages and lymphocytes) [12]. An increased glycolytic rate is necessary to maintain concentrations of intermediaries for biosynthesis of purine and pyrimidine nucleotides, essential for DNA and mRNA synthesis and necessary for lymphocyte proliferation. If blood glucose levels drop to hypoglycaemic levels it is likely that lymphocyte and macrophage function is impaired. Endurance athletes may experience levels as low as 2.5 mmol/L in the latter stages of competition so this is a

possible mechanism to explain the effect of prolonged intensive exercise affects immune function [12] (see Section 1.5.1 for exercise-induced alterations to immune function).

Diet composition during training may actually affect immune function in some athletes e.g. natural killer cell activity in endurance athletes was enhanced by a carbohydrate-rich diet (65% of dietary energy) but not a fat-rich diet (62% of dietary energy) [11]. Several other studies have shown that when athletes exercise intensively on low carbohydrate diets (<10% of dietary energy) higher levels of stress hormones (adrenaline and cortisol) and cytokines (IL-6, IL-1ra and IL-10) post-exercise are experienced compared to those on normal or high carbohydrate diets [11]. Cortisol promotes gluconeogenesis [152], which has an anti-inflammatory role but is also catabolic and immunosuppressive. This view is supported by a study of 32 male triathletes consuming a high carbohydrate diet (12 grams of carbohydrate per kilogram of body mass per day [CHO/kgBM/day]) for 6 days, and who performed a 1 hour intensive bout of exercise. Salivary cortisol levels were attenuated and salivary S-IgA levels enhanced in the group consuming the 12 grams CHO/kgBM/day compared to the placebo (self-selected dietary intake) [153].

1.7.2 The effect of carbohydrate supplementation on immune protection before, during and after exercise

The benefits of supplementation with carbohydrate drinks (containing 6-7% mix of glucose/sucrose) during exercise to maintain energy and power are now widely accepted [102]. While there are also positive benefits for the immune system there is still no evidence of an effect on the post-exercise levels of salivary S-IgA. Exercise-induced reduction in levels of blood glucose are accompanied by HPA-axis activation, an increase in the release of adrenocorticotrophic hormone and cortisol, an increase in plasma growth hormone and an increase in epinephrine [154]. Plasma cortisol levels have been correlated negatively with plasma glucose in athletes competing in prolonged intensive exercise [144], and prolonged intermittent exercise carried out at high intensity [142]. Cortisol redistributes metabolic fuels at a rate to

enhance cardiovascular activity [117]; and down-regulates the initial inflammatory and immune responses when the athlete has over-reached in order to prevent permanent tissue damage [117].

Carbohydrate ingestion during exercise enhances the ability of athletes to maintain plasma glucose levels. In a study of thirty runners who completed a 2.5 hour run and supplemented with a 6% carbohydrate or placebo solution before during and after the run, post-exercise plasma cortisol and cytokine levels were reduced in the carbohydrate group compared to the placebo group [144]. Concentrations of blood neutrophils and monocytes were also lower in the carbohydrate group, although monocyte and granulocyte oxidative burst activity fell slightly [155]. In another study of 98 marathon runners, plasma levels of cortisol, IL-10, IL-1ra, IL-6 and IL-8 rose strongly after a marathon race irrespective of age and gender. However smaller post-exercise increases in levels of cortisol, IL-10 and IL-1ra occurred in the 48 marathon runners who consumed a carbohydrate drink before during and after the race compared to those consuming a placebo [156]. In a group of 14 male cyclists and triathletes a reduced exercise-induced immune response was measured in those who consumed 6% carbohydrate drink over a four hour training period compared to the placebo. Lower levels of IL-6, cortisol, neutrophils and monocytes were observed in the supplemented group but no effect was observed on levels of lymphocytes or natural killer cells [102].

Soccer players performing intermittent exercise may also experience benefits to immune function from carbohydrate supplementation. Post-exercise levels of IL-6, TNF-α neutrophil trafficking and LPS-stimulated neutrophil degranulation were lower in participants supplemented with carbohydrate drinks compared to placebo in a study of six trained male soccer players [142]. However, when exercise is carried out at moderate intensity (insufficient for an exercise-induced stress response) carbohydrate supplementation has a minor effect on immune function [157]. In a study of 15 elite female rowers who performed exercise at moderate intensity, carbohydrate supplementation had an insignificant effect on the inflammatory response as changes to cytokine levels were minor [157].

1.7.3 The effect of protein status on immune function

Protein deficiency is known to affect immunity [2]; immune defence relies on the rapid replication of immune cells and production of biologically active proteins such as immunoglobulins, acute phase proteins and cytokines [11]. Protein–energy malnutrition has been associated with depression of the number of fully differentiated T-cells, mitogen-induced T-cell response, decreased CD4/CD8 ratio, and impaired phagocytic cell function, cytokine production and complement formation [11].

Athletes

Most athletes increase their energy intake to meet the physiological demands of their sport and easily meet the recommended protein requirements of 1.2-1.4 g/kgBM/day [158]. As a result there is increased dietary intake of all nutrients. However those athletes following food restriction regimes such as those trying to lose weight, vegetarians, and athletes with unbalanced diets (e.g. excessive carbohydrate/protein intake) may be at risk of inadequate dietary protein intake. Athletes participating in sports where leanness or low body mass is thought to be advantageous are most at risk. Energy restriction may also be associated with micronutrient deficiencies and a susceptibility to infection; even a body mass loss of 2kg over 2 weeks adversely affects macrophage phagocytic function [11]. Recently, energy restriction in athletes and associated health problems have been recognised as a sub-clinical condition and called anorexia athletica [11].

Older Adults

Along with age-related-decrease in lean body mass there may also be a reduction in other cells and tissue such as organs, blood components, immune cells (antibodies) and hormones [159]. These changes manifest in a reduction in ability to fight infections and impaired wound-healing [159]. Accompanying a reduction in lean body mass is a decrease in energy requirements as muscle mass is the most metabolically active tissue. Muscle loss may also be due to more sedentary lifestyles and appears to occur rapidly in older adults [159]. It may be more difficult for older adults to maintain nitrogen balance, and a higher

dietary intake of protein compared to younger adults may be necessary. The New Zealand nutrient reference values have been updated in recognition of increased needs [160]. The recommended daily intake of protein for adults over the age of 70 years is 1.07 g/kgBM/day for males and 0.94 g/kgBM/day for females. This compares with the previous recommendation for adults aged 51 to 70 years, of 0.84g/kgBM/day for males and 0.75 g/kgBM/day for females [160].

In older adults with protein energy malnutrition immunity is affected compared to healthy similarly aged adults. Changes have been observed in cell mediated immunity including decreases to a number of T-cell subsets such as $CD3^+$, $CD4^+$, $CD8^+$ and impaired vaccine antibody responses [15, 121]. In elderly subjects with protein energy malnutrition, as indicated by serum albumin levels <30mg/L, lymphopaenia is apparent, in particular peripheral blood counts of $CD4^+$ T-cells <400/mm^3 [15].

There are a number of reasons for reduced protein intake including the cost of good quality meat, older adults being less inclined to cook a balanced meal when living alone, difficulties eating with dentures, and chronic illness [159]. Therefore older adults are the population group at most risk of inadequate protein intake and this may be of concern as decreased immune protection may be placing them at increased risk for infection.

1.7.4 The effect of dietary fat on immune protection

Dietary fat, an essential substrate in the diet is important for energy production and as a major constituent of cell membranes also contributes to general health [12]. It may also aid in the absorption of fat-soluble vitamins (A, D, E, and K) which are important micronutrients for immune function (see Section 1.7.5). Altered ratios of fatty acids in cell membranes can affect membrane fluidity and plasma membrane phospholipids [12]. Membrane phospholipids generate cell-signalling molecules and regulate the activity of some of the proteins involved in signalling mechanisms within immune cells [161].

There are three types of naturally-occurring fatty acids: saturated, cis-monounsaturated and cis-polyunsaturated. Polyunsaturated fats contain double bonds, and two groups are considered essential, and therefore must be obtained from the diet. The first group are the omega-6 fatty acids (n-6) (the double bond occurs at the 6th carbon from the terminal methyl end), and are derived from linoleic acid (18:2). The other group are the omega-3 fatty acids (n-3), derived from α-linolenic acid. Linoleic acid is a precursor for arachidonic acid which is a substrate for eicosanoid production (involved in inflammatory and immune responses), and is important in gene regulation and as a constituent of cell membranes [161]. Dietary linoleic acid is converted to arachidonic acid (20:4n-6), the n-6 fatty acid (known as omega-6), and α-linolenic acid is converted into eicosapentaenoic acid (20:5n-3), docosapentaenoic (22:5n-3) and docosahexaenoic (22:6n-3) (known as omega-3) [161]. The n-6 and n-3 fatty acids compete for the enzymes that metabolise them (see Figure 6). Increasing the dietary consumption of n-3 fatty acids (e.g. through supplementing with fish oil) results in increased n-3 fatty acid in immune cell phospholipids at the expense of incorporation of arachidonic acid [161].

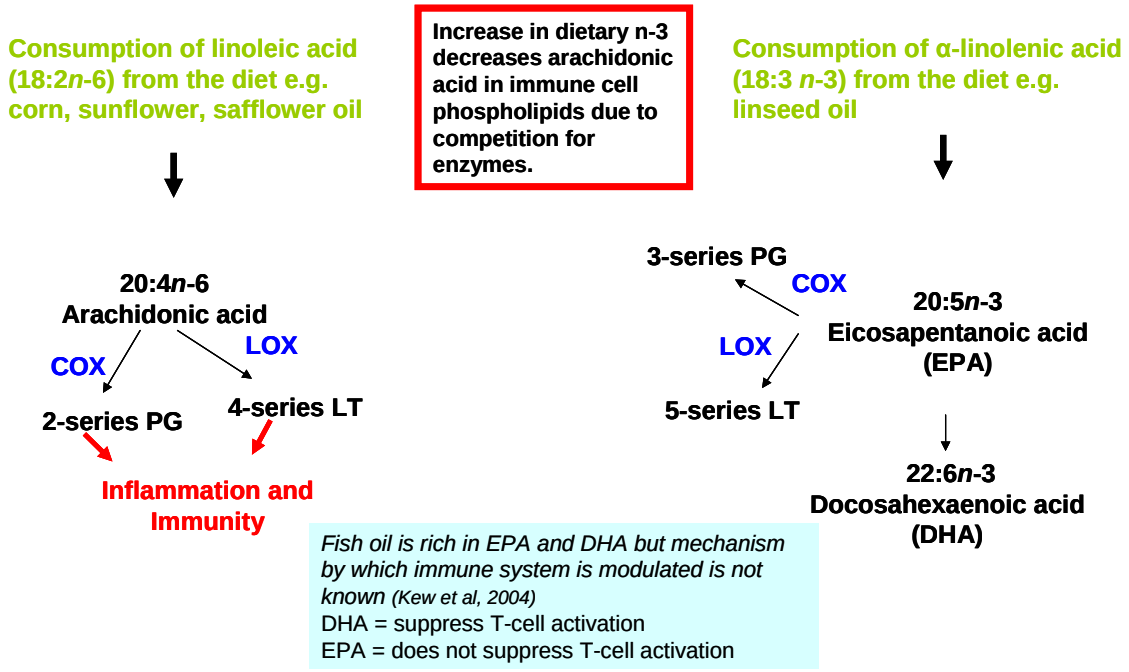


Figure 6: Summary of the biosynthesis and metabolism of long chain polyunsaturated fatty acids that may have an immunomodulatory effect in mammals (adapted from Calder and Grimble, 2002 [161]). PG=prostaglandin, LOX=lipoxygenase, COX=cyclooxygenase, LT=leukotriene

Arachidonic acid is mobilised by a number of enzymes to act as a substrate for cyclooxygenase in order to produce 2-series prostaglandins or for lipoxygenase enzymes to make 4-series leukotrienes [161]. Prostaglandin E_2 is immunosuppressive (suppresses lymphocyte proliferation, natural killer cell activity and TNF-α, IL-1, IL-6, and IFN-γ production) and anti-inflammatory but, does not affect production of IL-4 and IL-10 (Th2 cytokines) and promotes IgE production [161]. Leukotriene B_4 increases vascular permeability and local blood flow, inhibits lymphocyte proliferation and promotes natural killer cell activity; leukotriene may also regulate production of proinflammatory cytokines.

It seems feasible that patients suffering from inflammatory diseases (such as rheumatoid arthritis) as a result of an over-active immune system may benefit from a diet rich in either of these fatty acids [11], for example feeding fish oil (rich in omega–3 fatty acids), can decrease the proportion of arachidonic acid and increase the proportion of omega-3 fatty acids in immune cells [161]. To date results from research indicate that supplementation with eicosapentaenoic or docosahexaenoic fish oil does not affect phagocytosis by monocytes and neutrophils or the expression of adhesion molecules [146]. However a recent study showed a lower level of activation of T-cell function occurred with docosahexaenoic and not eicosapentaenoic acid supplementation [146]. In rats consuming a diet rich in omega-3 fatty acids, a significant reduction in glutamine decarboxylation occurred in the mesenteric lymph node lymphocytes. This may impair the use of glutamine as a fuel source and therefore prevent proliferation of lymphocytes. In humans suppression of T-cell proliferation in response to mitogens has been observed in those who supplemented their diet with large quantities of omega-3 fatty acids [12]. Therefore the benefit of omega-3 supplementation to immune modulation still requires further investigation.

Athletes

Consumption of dietary fat in a balanced diet should meet the needs of athletes. Some surveys indicate that athletes may consume too much fat at the expense of carbohydrate [12]. It is possible that prostaglandin E_2 production by monocytes may be of relevance to athletes as levels can increase at least two-fold following an acute bout of exercise [12], therefore a moderate amount of

omega-3 fatty acids in the diet may be of benefit to counteract the inflammatory response. Lymphocytes and macrophages utilise fatty acids as a fuel source [12]. An athletes' capacity to utilise fat as an energy source increases with endurance training. This may be of benefit to immune cells when blood sugar levels are low. However, it is still recommended that athletes consume a diet where they obtain no more than 30% of their energy from dietary fat. Studies have not consistently reported beneficial results of essential fatty acid supplementation during heavy periods of training [11].

Older adults

There is concern about the effects of high dietary fat intake on hypertension and on cardio-vascular disease in older people. In addition when fatty acids are highly available in the diet they can have a negative effect on immune function, due to their potential to alter cellular membranes and as eicosanoid precursors. Older adults need to reduce their energy intake due to a lower level of activity in order to lower the risk for obesity [162]. Adequate intake levels have been established for polyunsaturated fatty acids as recommended daily intakes can not be determined [160]. Adequate intake level is defined as [160] '*The average daily nutrient intake level based on observed or experimentally-determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate*.' The current adequate intake levels for men and women over 19 years are [160]-

	Men	**Women**
Linoleic	13g	8g
α-Linolenic	1.3g	0.8g
Omega-3	160mg	90mg

More research is needed to determine if there is a benefit of omega fatty acid supplementation to immune function in older adults.

1.7.5 The effect of micronutrient intake on mucosal immune protection

Replenishment of micronutrient levels from daily dietary intake is necessary to meet recommended levels within the body. While only small amounts of

vitamins and minerals are needed they are essential for energy release, metabolic regulation and immune function [12]. Due to the increased dietary intake by athletes in response to energy demands of exercise, any increase in requirement should be met. For older adults there may be an age-related increase in need for some vitamins and minerals particularly given the lower energy needs [162].

Vitamins

Vitamin A and β-carotene

Vitamin A from dietary sources can be ingested as a preformed retinyl ester (which is easily converted to retinol, the main form in circulation) or as a provitamin carotenoid. The preformed vitamin A is found naturally in animal sources whereas the carotenoids are found in fruits and vegetables [163]. A small amount of carotenoids may also be consumed from dairy products and seafood. Vitamin A deficiencies in humans can have a significant effect on immune function e.g. atrophy of the thymus, decreased mitogen-induced lymphocyte proliferation, an increase in adherence of bacteria to respiratory tract epithelial cells and IgA production [143]. In animals, vitamin A deficiencies have also been associated with lower levels of antibodies, interferon-impaired delayed cutaneous hypersensitivity and phagocyte activity [143]. There may be a benefit of supplementation for some individuals e.g. supplementation with β-carotene can increase the number of $CD4^+$ T-helper cells in healthy adults [12]. Conversely excessive intake of vitamin A or β-carotene may be harmful and lead to liver damage [12].

Athletes

The benefit of vitamin A or β-carotene supplementation to immune function in athletes has still not been proven. Many physiological and metabolic processes are up-regulated in response to endurance training including antioxidant systems [12]. An increase in oxygen consumption during exercise leads to an increase in reactive oxygen species production. Exercise-induced up-regulation of antioxidant systems balances the associated oxidative stress, preventing protein oxidation and subsequent muscle damage [164]. It is not known if those

athletes who train extensively such as ultra-marathoners need additional intake from supplements [143]. Most athletes following a balanced diet are likely to meet their vitamin A needs.

Older Adults

Dietary intake of vitamin A appears to be adequate among older adults [165]. It is possible that antioxidant supplementation may help protect against the free radical-generated degenerative effects that occur with ageing. High plasma concentrations of β-carotene (from dietary sources) were associated with fewer acute respiratory infections but not reduced severity in an observational study of non-institutionalised adults over the age of 60 [166]. The mechanism was not known and caution was recommended when using β-carotene supplements because increased risk for mortality has also been reported after supplementation [166]. Further, excess consumption of vitamin A is of concern because of possible hepatotoxic effects [167], and an increased risk of bone loss [168].

Vitamin B6

Vitamin B6 deficiencies may affect lymphocyte numbers and result in lymphoid tissue atrophy and an increase in oral infections [148]. Deficiencies may be common in healthy self-sufficient home-living older adults and may impact on the age-related decline in immune function [15]. Supplementation with vitamin B6 enhanced lymphocyte proliferation in older adults compared to those who were vitamin B6-deficient [15]. A vitamin B6 depletion-repletion study indicated that the daily requirement for vitamin B6 to return plasma levels to normal were 1.90mg for women and 1.96mg for men [162]. The recently issued dietary reference guidelines for New Zealanders have been adjusted to reflect the increased vitamin B6 need for older adults [160]. It is important that older adults are encouraged to include generous amounts of fruits and vegetables in their diet to meet their increased need for vitamin B6.

For adults > 70years	**Men**		**Women**	
	EAR	**RDI**	**EAR**	**RDI**
RDI for vitamin B6	1.4	1.7	1.3	1.5

EAR=Estimated average requirement

RDI=Recommended daily intake=EAR + 2 SD_{EAR}

Vitamin B12

Vitamin B12 can act as a co-enzyme in a number of reactions including the oxidation of fatty acids and the synthesis of RNA and DNA [12]. The natural source of Vitamin B12 (cyanocobalamin) is of animal origin, therefore vegetarians and those avoiding dairy products are at risk of being vitamin B12-deficient [12]. It can only be absorbed from the gut in the presence of intrinsic factor and lack of this factor results in pernicious anaemia, a condition associated with a reduction in phagocytic and bactericidal capacity of neutrophils [12]. Vitamin B12 deficiencies may also impair antibody production [12].

Athletes

Athletes rarely experience vitamin B12 deficiencies as their requirement is small.

Older adults

It is thought that atrophy of gastric mucosa as a result of disease is the most likely cause of vitamin B12 deficiencies in older adults rather than being a result of the ageing process [162].

Vitamin C

Vitamin C is a water-soluble vitamin with antioxidant activities (which could potentially neutralise exercise-induced generation of reactive oxygen species [143]). Vitamin C may also enhance T-cell proliferation [12], antibody production, lymphocyte proliferation and numbers of specific leucocytes [26]. Vitamin C supplementation is widely practised by the general population as it is believed that it reduces the incidence of URTI, however scientific evidence for this is lacking. Routine large daily doses of vitamin C are not recommended due to possible adverse effects such as diarrhoea, joint pain, and exacerbation of gout and kidney stone formation [12, 104].

Athletes

The evidence of benefit of Vitamin C supplementation to immune health in athletes is inconsistent. The incidence of URTI in ultra-endurance athletes may be reduced with vitamin C supplementation [143] but this effect has not been shown in the general population [12]. Daily supplementation with 600mg of vitamin C was associated with fewer athletes reporting URS 14 days after a 90km ultra-marathon [169]. However, another study failed to show an association of vitamin C supplementation with oxidative stress and immune changes in carbohydrate-fed ultramarathoners [170]. The immunomodulatory effect of vitamin C supplementation in athletes requires further research [170].

Older Adults

In older adults vitamin C has an important role as an antioxidant and as catalyst for hydroxylation of proline and lysine needed for collagen production (necessary to make new tissue and heal wounds) [149]. Reduction in vitamin C intake is associated with increased illness, hospitalisation and institutionalisation in older adults [149]. Lowered intake has been associated with a number of chronic illnesses including lung diseases, however deficiencies are easily reversed with adequate intake of fruits and vegetables [149]. Requirements for dietary vitamin C appear to be unchanged with ageing and there is no indication that dietary intake is inadequate in New Zealand in older adults [162].

Vitamin D

Vitamin D includes a group of prohormones and the active form D_3 (calcitrol) has an important role in calcium homeostasis. Receptors for calcitrol have been found on macrophages, monocytes, and dendritic cells indicating the hormone also has a key role in the immune system [171]. Vitamin D appears to stimulate differentiation and maturation of monocytes and inhibit the differentiation and maturation of dendritic cells [171]. It also appears to diminish T-cell proliferation and down-regulate the production of cytokines needed for the Th1 response but upregulate those needed for the Th2 response, as well as TGF-β (a key regulator of the switch of plasma cells to IgA producing cells). When vitamin D has been administered with vaccines an enhanced mucosal

immune response has been induced [171, 172] as indicated by increased serum IgA antibody [171].

Vitamin E

Vitamin E is an antioxidant and includes at least eight structural isomers of tocopherol. In cell membranes vitamin E is the main chain breaking antioxidant, and is able to convert superoxide, hydroxyl and lipid peroxyl radicals to less reactive forms [164]. Vitamin E also appears to be synergistically linked to other antioxidants such as vitamin C [164]. Supplementation has improved some immune functions although the mechanism by which it does this is not clear [165]. It may augment the immune response by altering cytokine generation from T-cells or macrophages [165] or by playing a role in the differentiation of immature T-cells in the thymus [26]. Supplementation with vitamin E has been associated with reduced free radical and reduced prostaglandin production by monocytes [15].

Athletes

Athletes rarely experience vitamin E deficiencies, however it is likely that the athletic population consumes large doses of vitamin E supplements as it has been hypothesised that vitamin E counteracts the effects of oxidative stress [12]. Ironman triathletes who supplemented with vitamin E for two months (800 IU/day) did not experience any change in plasma cytokines, blood leucocyte subsets or salivary S-IgA out-put [173]. Unexpectedly, an increase in lipid peroxidation and inflammation was observed during exercise in these athletes suggesting increased oxidative stress occurred [173]. Therefore further research is required into the long-term effect of vitamin E supplementation on health in athletes and routine large doses are not recommended.

Older Adults

Vitamin E deficiencies were found to occur at a low rate (<1%) in the aged population in the ‘Study of Nutrition in the Elderly in Europe’ survey [15]. In addition intake of vitamin E from dietary sources was found to be adequate amongst older adults in New Zealand in the ‘Mosgiel Community Study of Health and Nutrition in Old Age’ [162].

Vitamin E treated subjects have shown some improvement in self-reported illnesses [174]. In a long term randomised double blind study, subjects aged >65 years received various vitamin E doses (60, 200 or 800 IU) for 235 days. The larger doses of 200 and 800 IU increased the antibody response to hepatitis B vaccine; there was also 30% less self-reporting of illness in the vitamin E supplemented group compared to the placebo control group, however the clinical trial was not powered for statistical significance [174]. It is still unclear whether the requirement for Vitamin E intake changes with ageing.

Minerals

Iron

Abnormal iron status can lead to sub-optimal immune function as iron is involved in a number of cellular processes [147]. Iron deficiency affects immune cell proliferation and lymphocyte signalling pathways. It is also possible that the adaptive immune response depends on the release of iron by macrophages for optimal lymphocyte activation [147].

Athletes

Iron deficiencies are prevalent in the non-exercising population (as high as 25% of the world's population) and the occurrence in athletes is thought to be at about the same rate [12]. However groups within the athletic population (such as male and female endurance athletes, vegetarians and menstruating females) may be at a greater risk for iron deficiency [12], therefore dietary requirements in this group may be higher than in the non-exercising population [11].

The acute phase response to exercise-induced stress results in an increased production of IL-1 which stimulates the release of lactoferrin by granulocytes. Lactoferrin chelates iron from transferrin lowering the overall circulating levels of free iron, and as free iron is necessary to bacterial growth this may be of benefit to the immune system [12]. Conversely, supplementing with iron during periods of infection may actually increase iron availability for bacterial growth [12]. Iron deficiencies in athletes can also decrease natural killer cell activity and

phagocytic activity of neutrophils. The heavy activity performed by endurance athletes makes them more vulnerable to iron deficiencies due to the losses in sweat, impaired iron gastrointestinal absorption and haemolysis from foot strike. The low bio-availability of iron in vegetarian diets increases the risk for athletes who are vegetarian, and daily iron needs may also be higher in menstruating females [12].

Older adults

After menopause the iron requirements for women change to that of men and iron status can be maintained if dietary intake is adequate [149]. However iron deficiency is thought to be quite common in homebound and institutionalised older adults (males and females). In healthy home bound older women when iron deficiency was observed it was associated with a decline in cell-mediated and innate immunity, which could increase risk for infection [175]. These women were also not experiencing disease or inflammatory conditions or had no known deficiencies in nutrients known to affect immunity which was a strength of this study [175]. The study highlighted the importance of identifying iron deficiencies and treating them in older adults.

Zinc

Zinc is essential for immune function and is involved in a number of enzymic processes e.g. it is a co-factor for terminal deoxynucleotidyl transferase which is required by immature T-cells for proliferation and cytokine production [176]. Zinc deficiency results in lymphoid atrophy, decreased delayed hypersensitivity cutaneous responses, decreased IL-2 production, impaired mitogen-stimulated lymphocyte proliferation and decreased natural killer cell activity [12].

Athletes

Zinc is lost from the body in urine and sweat, and these losses may increase with exercise [12]. Vegetarian athletes and athletes following energy-restricted diets are most at risk for zinc deficiencies, however the benefit of zinc supplementation to immune function in athletes still needs to be determined [12].

Older Adults

Zinc deficiencies are common in older adults and may be associated with age-related changes in immune function (especially cell-mediated immunity); it is possible that other age-related factors such as sub-clinical inflammation impact on the biological effects of zinc [177]. In addition T-cell proliferation in response to zinc status may be stronger in older males than females [176]. Further, ageing appeared to be negatively correlated with serum zinc levels observed in healthy Italian, free-living men (n=45) and women (n=48), aged 55-70 years [177]. In New Zealand dietary intake of zinc was found to be below the recommended levels for New Zealand older adults in the 'Mosgiel Community Study of Health and Nutrition in Old Age' study [162] suggesting a risk for zinc deficiencies.

Selenium

Selenium has a critical role as an antioxidant. It is a co-factor for the antioxidant enzyme glutathione peroxidase [164] which is responsible for removing hydrogen peroxide and other organic hydroperoxides from the cell [164]. Selenium has a role in immune function as well as cancer. It is an active component in a number of enzymes protecting membranes from oxidation [178] and acts synergistically with vitamin E to inhibit lipid peroxidation [179]. Even marginal selenium deficiencies contribute to reduced immune function, some cancers and viral diseases [180]. Supplementation in selenium-replete individuals can improve activation of CTLs in response to tumours and NK–cell activity as well as assist proliferation and differentiation to cytotoxic T-cells in older adults [26].

Athletes

In New Zealand the soil is deficient in selenium therefore dietary intake of selenium may also be sub-optimal particularly in those athletes following energy-restricted diets [178].

Older adults

A study of 178 older German women found well nourished, younger, well educated senior females had inadequate selenium intake [181]. In New

Zealand, older females have also been shown to have inadequate intake and sub optimal selenium status due in part to the low selenium content in soil [178].

1.7.6 The effect of bovine colostrum on mucosal immunity

Recently there has been much interest in the non-nutrient components of foods and the way in which they may enhance immune health. BC is marketed as a health food because it is a plentiful source of immune and growth factors. BC is produced in the first few days post-partum; it contains many bio-active factors and immunoglobulins that provide passive immunity to the new-born calf. There is no transfer of antibody across the placenta in calves (unlike humans) so it is important they are fed BC as soon as possible after birth. A number of factors can affect the quality of BC including age of the cow, older cows produce a greater volume of BC that contains higher levels of immunoglobulin compared to a young cow giving birth for the first time [182]. Additionally the type of immunoglobulin varies from herd to herd depending on the pathogens to which the herd has been exposed. Levels of bio-actives in BC that may be of interest to modulation of immune function for humans also decline in BC over the first few days [183]; for example TGF-β is at its highest in the first 24 hours and declines rapidly after this [182].

To be physiologically active, bio-actives need to pass to the intestine intact, and in levels that will have biological significance. Receptors are found in the mucosa of the calf's small intestine for EGF, TGF-β and IGF-I and IGF-II which suggests these BC molecules are likely to be biologically active here [184] e.g. EGF can stimulate migrational processes in the intestine and IGF-1 stimulates intestinal enzymic systems [184]. There are also several anti-microbial factors important for the newborn: the most important are Igs, lactoferrin, lysozyme and lactoperoxidase [185]. Some of these bio-active factors may pass intact to the human intestine, where they could have a biologically significant role (e.g. Igs) [186]. It is possible other bio-active components also retain biological activity (e.g. growth factors and nucleotides). Additionally some growth factors in BC have a high degree of structural similarity to those found in humans such as TGF-β1 [187].

To date though, a mechanism by which BC could affect the immune system in humans has not been elucidated. In animal models, dietary bovine whey proteins have been shown to elevate secretory immunoglobulin levels in the gut mucosal environment [188, 189]. This suggests the establishment of an enhanced mucosal immune state by bovine milk proteins. In addition BC supplementation in mice was thought to polarise intraepithelial T-cells to Th1 as indicated by impaired IL-4 responses [190]. *In vitro* studies have shown that the addition of TGF-β as found in BC can stimulate human lymphocyte cultures [187]. After supplementation with BC, enhancement of the specific IgA response to a bacterial pathogen was seen in 18 healthy humans (9 females and 9 males aged 20-50 years) [191]. These results indicate an immunomodulatory effect of BC. However BC contains many growth factors that can affect gut development, it is of interest to understand their potential effect on the gastrointestinal tract and GALT in humans.

Comparison of the macronutrient content of BC and MBM

The composition of MBM can be affected by many factors including age and breed of the cow, diet (due to seasonal and regional differences) [192], and stage of lactation [183]. Macronutrient content of BC differs to MBM; e.g. the protein content is higher in BC to meet the demands of the new calf's growth, and lactose and fat content levels are less than MBM [183]. A summary of macronutrient differences between BC and MBM is shown in Table 3.

Table 3: Summary of data for macronutrient content of bovine colostrum (BC) and mature bovine milk (MBM)

	Ontsouka *et al*., 2003		Jensen, 1995	Parrish *et al.*, 1950		Prosser, 2007	
	BC [a] (2days)	MBM [a] (week 4)	MBM [b]	BC[c] (2days)	MBM [c]	BC[d]	MBM [d] (30days)
Dairy cow breed	Holstein-Friesian x Simmental		Holstein-Friesian	Not specified		Not specified	
Dry matter g/Kg	159±16	117±12	-	154	129	153	138
Fat g/L	70.5±8.5	57.6±6.7	35	46	40	56.5	50.0
Protein g/L	52.0±3.2	32.8±1.0	36.1	59	31	46.7	32.7
Lactose g/L	43.9±0.9	49.9±0.5	49	43	50	45.6	50.0
IgG g/L	28.3±5.7	1.5 ± 0.1	-	44 – 65	7.4	-	-

a=Ontsouka *et al.*, 2003 [183], *b*=Jensen, 1995 [193], Holstein dairy cows, *c*=Parrish D N, Wise G H, Hughes J S & Atkeson F W (1950) Properties of the colostrum of the dairy cow V Yield, specific gravity and concentrations of total solids and its various components of colostrum and early milk *Journal of Dairy Science* **33,** 457-465, supplied by Fonterra, *d*=Prosser C, 2007 [194]

These data show there are differences in macronutrient content of BC and MBM between breeds of dairy cows and it could also be assumed there would be differences in the bio-active levels.

In New Zealand, the breeds of cows used in dairying are mostly Holstein-Friesian, Jersey and Holstein-Friesian/Jersey crosses. Compared to the North American Holstein–Friesian cows which can be largely fed in confinement systems, the New Zealand bred Holstein-Friesians are pasture-fed. The New Zealand Holsteins produce a lower volume of milk that has a higher milk fat and protein content, attributable to the pasture feeding of New Zealand Holstein-Friesian cows [195]. It is possible that levels of immunologically bio-active substances in BC milk from the same breed of cow vary as a result of the different feeding practices. This is supported by a study of New Zealand Holstein-Friesian herds which found that macronutrient and immunoglobulin levels in MBM milk are affected by both the stage of lactation and seasonal differences [192].

It has been reported that the Holstein-Friesian breed produces lower levels of immunoglobulins compared to other breeds, and transfer of immunity to calves is considered to be less [196]. A study in North American calves from pure bred Holstein-Friesian dams and Holstein-Friesian/Jersey cross-bred sires found there were higher levels of serum protein and IgG compared to those from pure bred Holstein-Friesian sires. This suggests that the introduction of genes from Jersey breeds conferred improvements in calf immunity [197]. Together this highlights a problem for the comparison of results for research using a BC supplement in human intervention trials.

In New Zealand, bovine milk customarily consumed by humans is normally collected four days postpartum (or after 8 milkings) when the immunoglobulin levels are considered acceptable (Part 4: Raw Milk Acceptance [198]). A recent comparison of macronutrient levels between BC and MBM from dairy cows in New Zealand could not be found in the scientific dairy journals. Data for compositional differences between New Zealand produced BC and MBM was supplied by Fonterra Co-operative Ltd [199] and Prosser 2007 [194]. For this

current investigation it is likely that the BC supplement was produced from milk produced by Holstein-Friesians, Jerseys and Holstein-Friesian/Jersey cross breeds.

Bio-active growth factors

There are many bio-active growth factors in BC, a list of some of the growth factors that may be of importance to human health, and their typical levels in BC and MBM is included in Table 4.

Table 4: Typical levels of peptide growth factors in BC and MBM

Growth Factor	BC	MBM
IGF-1 (µg/L) [185]	50-2000	<10
IGF-2 (µg/L) [185]	200-600	<10
Insulin (µg/L) [185]	160-320	46
*TGF-β1 (ng/mL) [182]	53.7	
*TGF-β2 (µg/mL) [182]	40.4	
EGF (µg/L) [65]	?	?
TGF-α [65]	?	?
Platelet-derived growth factor [65]	?	?
Vascular endothelial growth factor [65]	?	?

* I day postpartum

Transforming growth factor β family

Transforming growth factors have an important role in a number of cellular functions, including cell growth, differentiation, apoptosis and migration [200, 201]. They also have an important role in embryogenesis, tissue repair, formation of bone and cartilage, regulation of the immune system (especially tolerance induction and maintenance [202]), wound healing and extracellular matrix formation [182, 185]. Three isoforms are expressed in mammals: TGF-β1, TGF-β2 and TGF-β3 which all function mutually exclusively *in vivo* [182]. For example in TGF-β1 knockout mice death occurs soon after birth due to a wasting syndrome; however TGF-β2 knockout mice die from impaired cardiovascular function and lung development [182]. TGF-βs in bovine and porcine colostrum are mostly in the latent form; compared to about 50% of human TGF-βs being active [182]. The predominant form found in BC is TGF-β2, whereas in human colostrum the predominant form is TGF-β1 [182]. *In vitro*, BC TGF-β2 increases IgA secretion by LPS stimulated mesenteric lymph node cells. It was speculated that induction of IgA isotype expression in the intestine may be a specific role for TGF-βs in the neonate [182]. BC-derived

TGF-βs also show similarity with corresponding human TGF-βs; e.g. the amino acid sequence of TGF-β2 is identical to that in humans [185]. In addition, bovine milk-derived TGF-βs have been shown to cross-react with human lymphocytes *in vitro* [187]. Together this suggests that TGF-β from BC may be physiologically active in the human gut.

TGF-β is also expressed in the gastrointestinal tract but it is mainly in the superficial zones where it inhibits cell proliferation once cells have left the crypt region [185]. BC TGF-β has been shown to enhance expression of secretory component (SC) in rat epithelial cells (SC is responsible for the transport of polymeric IgA across epithelial cells into the intestinal lumen) [185]. Therefore TGF- β may be physiologically active in the human gut.

Insulin-like growth factors (IGFs)

IGF-s are multifunctional polypeptides that interact with specific receptors and insulin-like growth factor binding proteins [203]. They are similar in structure to pro-insulin, are able to exert an insulin-like effect at high doses and promote cell proliferation and differentiation [203]. IGF is a hormone that has been identified in the colostrum and milk of several species (see Table 5); the IGF binding proteins appear to have a high degree of similarity between species [185].

Table 5: Comparison of IGF-1 in colostrum and mature milk from different species (Zumkeller,1992 [203])

	IGF-1 (ng/ml)	
Species	Colostrum	Mature milk
Human	8-28	5-10
Cow	100-2000	22-26
Goat	600-660	10-14
Pig	95-357	4-14
Rat	20-40	5-21

In humans circulating IGF-1 is mostly bound to binding proteins [204] and degradation by proteases is required for its release, making it available for metabolic processes such as glucose metabolism [204]. By comparison, in human colostrum (one to three days postpartum) a significant amount of IGF-1 is free (about 73%) [203]. In the human neonate IGF-1 acts locally in the gut as well as systemically suggesting that it is able to pass through the gastrointestinal wall [203]. In the infant, IGFs bind to proliferative intestinal cells

rather than differentiated enterocytes; they appear to be involved in intestinal permeability and mediation of the intestinal response to inflammatory processes [203]. In addition both EGFs and IGFs are important to the neonate as they aid morphological and functional growth and adaptation to a varied diet [203]. Likely benefits of IGFs in colostrum for neonates are in promoting increased turnover of intestinal cells in order to maintain a healthier gut and increased nutrient intake and or immunological performance [185]. Bovine IGF-1 can survive passage through the gastric environment in adults but it is unknown if it has a similar growth-enhancing effect on the gut wall as is seen in the neonate [203].

Epidermal growth factor (EGF) (receptor ligand family)

The poylpeptides in this family bind to the same EGF receptor, and the most important members are EGF and TGF-α [185]. Epidermal growth factor is produced by salivary glands and the duodenum in adult humans but is not present in large amounts in bovine milk. Milk borne EGF is not deactivated under intestinal proteolytic conditions in pre-term infants and adults especially when preserved in the presence of food proteins [65]. However it is unclear what the role of EGF is in the gastrointestinal lumen under normal conditions. EGF receptors are found on the basolateral membrane of the gut epithelial cells but this may vary between species [65]. A possible explanation is that EGF does not have biological activity in the lumen but may act as a luminal surveillance peptide [65]. In this role it would be readily available to stimulate the repair process at the injury site [65]. The concentrations of TGF-α in BC are even lower than EGF and its major role seems to be as a mucosal integrity peptide maintaining the function of epithelium in non-damaged mucosa [65].

Nucleotides

Nucleotides have a key role in nearly all biochemical processes in cell metabolism [205]. The nucleotide content of milk varies at different stages of lactation, reaching maximum levels one day postpartum and then gradually decreasing to about a third of the content after 8 weeks (see Table 6) [205]. Nucleotides are supplied from either biochemical endogenous processes or dietary intake, and are transported across plasma membranes by specified

facilitated diffusion carriers and sodium-dependent transporters [205]. Intestinal mucosae, in particular in the upper intestine, have limited capacity to synthesise the nucleotides needed for optimal function and will activate transcription enzymes necessary for utilising nucleotides from exogenous sources [205]. As an exogenous supply, both nucleotides and nucleosides appear to be important in the regulation of bodily functions, e.g. up-regulation of antibody responses and contribution to iron absorption in the neonate [205].

Table 6: Ribonucleotides (µmol/L) in bovine milk at different stages postpartum, Schlimme *et al*., 2000 [205]

	Time postpartum				
Ribonucleotide	0-1 day	1-2 days	5-10 days	1 month	2 months
Adenosine 5'-monophosphate(AMP)	39.7	61.8	41.9	27.5	20.3
Cytidine 5'-monophosphate (CMP)	31.9	52.5	47.4	33.2	18.9
Guanosine 5'-monophosphate(GMP)	N.D	N.D	N.D	N.D	N.D
Uridine 5'-monophosphate (UMP)	186.3	390.0	31.2	N.D	N.D

N.D=not detected

There is less evidence of the benefit of nucleotide supplementation in adults. Nucleotide requirements increase during periods of stress caused by disease and rapidly dividing tissue (e.g. repair of the gastrointestinal tract), and additional nutritional support may be necessary [122]. Persistent bowel disruption in trauma patients can result in secondary traumas that initiate endotoxaemia [206]. An important therapeutic strategy to preserve bowel mucosa and regeneration of epithelial cells is the use of immunomodulating agents such as ribonucleotides [206].

Stress induced by exercise may also increase the requirements of the immune system for nucleotides. Endurance cyclists who consumed a nucleotide supplement for 60 days experienced attenuated post-exercise salivary cortisol levels and enhanced salivary S-IgA levels [118]. It was thought the nucleotide supplement off-set the effect of training-induced hormonal changes to immune function in the athletes by enhancing protein metabolism [118].

Immunoglobulins

BC Igs appear to have an anti-microbial role in some infective diseases in humans. Oral administration of BC derived IgG preparations reduced and prevented diarrhoea caused by enteropathogens such as rotaviruses and *Escherichia coli* (*E.coli)* [207]. Igs are found in high concentrations in BC (see Table 7) and show a degree of resistance to digestion by gastric and intestinal enzymes in humans. Immunologically active levels of bovine IgG (19±3%) and IgM (19±3%) have been found in the human ileum after oral administration of BC [186]. Interestingly bovine IgA was not detected in the ileum, and one possible explanation is that it may have coated the enterocytes, which could help to prevent adherence of pathogens [186].

Table 7: Typical concentrations of immunoglobulins (Igs) in BC and MBM, Pakkanen and Alto, 1997 [185]

Ig	BC (g/L)	MBM (g/L)
IgG1	52.0-87.0	0.31-0.40
IgG2	1.6-2.1	0.03-0.08
IgM	3.7-6.1	0.03-0.06
IgA	3.2-6.2	0.04-0.06

Since these findings there has been interest in preparing purified fractions of immunoglobulins from BC for clinical use in patients suffering from infections of the gastrointestinal tract [185]. Immunising cows against specific micro-organisms provides BC with specific antibodies. Removal of fat and concentration of BC provides an Ig-rich preparation [208]. Daily consumption of such immune products is required for immune protection [185].

Recently another hypothesis has been proposed for the mechanism by which Igs may offer protection at intestinal mucosal sites. Receptor-mediated protein delivery occurred across mucosal surfaces when adult pigs were fed bovine IgG. The neonatal Fc (fragment of antibody molecule that has no affinity for antigen) receptor transports maternal immunoglobulin across the gut wall in the neonate. Mucosal sites are a common entry point for many pathogens in the neonate so a strong local mucosal immune response is important in the development of protective immunity [209]. The receptor has been found on the surface of gut epithelial cells in adult pigs which also has broad species

specificity for IgG and bound both human and bovine IgG [209]. Other studies have shown that human and mouse neonatal Fc receptor have cross-species affinity; interestingly human neonatal Fc receptor only bound rabbit and guinea pig IgG and not bovine [209].

Other humoral antimicrobial factors

Lactoferrin

Lactoferrin is an anti-microbial component present in both BC and MBM and, has an amino sequence similar to human lactoferrin and transferrin [185] (see Table 8). It is an 80 kDa iron-binding glycoprotein, is active at neutral pH and is able to survive the conditions of the lumen in the presence of secreted bicarbonates [185]. Lactoferrin inhibits the growth of a number of bacterial species. This may be mediated through iron chelation (by this mechanism it reduces the availability of iron for bacterial growth), and modification of bacterial cell membranes resulting in permeability changes [185]. Lactoferrin may also act indirectly by augmentation of T-cell dependent natural killer cell activity [210].

Table 8: Characteristics of humoral anti-microbial factors in BC and MBM, Pakkanen and Alto, 1997 [185]

	Size kDa	BC (mg/mL)	MBM (mg/L)	Sequence identity with molecules in human milk
Lactoferrin	80	1.5-5	0.1	68% sequence identity
Lysozyme	14	0.14-0.7	0.07-0.6	Different to human milk lysozyme
Lactoperoxidase Kussendrager *et al*., 2000 [211]	78	11-45	13-30	Identical with human myelo-, thyro-and eosinophil peroxidase

Lysozyme

Lysozyme is found in many mammalian body fluids, and is antibacterial particularly in the presence of lactoferrin. Its natural substrate is the peptidoglycan layer of the bacterial cell wall, and through degradation results in lysis of the bacteria [185].

Lactoperoxidase

This is a major antibacterial enzyme in BC. Its primary role is to protect the mammary gland against bacterial infection. The lactoperoxidase system is also able to inactivate polio virus and human immune deficiency virus *in vitro* [185]. The amino acid sequence is homologous to human myelo-, thyro- and eosinophil peroxidases [185].

The effect of processing on bio-active levels

Processing is an additional factor that could affect the consistency of bio-active levels in BC from different manufacturers. Fat, casein, lactose and salts may have a protective effect on Igs and growth factors and it is thought their removal during processing may affect the activity and levels of bio-actives in BC [212]. For example, heat treatment and freeze-drying decrease levels of Igs to 75% of original, but do not affect levels of IGF-1 and TGF- β2. The addition of a filtration step in the process, however, can reduce IGF-1 and TGF- β2 by 25% [212]. Pasteurisation does not alter the concentration of IGFs levels. The additional heat treatment for infant formulas destroys proteins [185], and therefore may have an effect on levels of IGF-1. Technological methods are being developed to separate and fractionate the various bio-active proteins and peptides that may be of relevance to human health [213]. This will be important in order to optimise and standardise activity and levels of bio-actives in BC supplements.

In summary, many variables can affect levels of bio-actives in BC, making it difficult to compare studies investigating the effects of BC on immune function in humans. Currently there is no New Zealand food standard or dairy specification that specifies the level of bio-actives required in BC for a health-modulating effect in humans. Therefore it is likely that levels of bio-actives will vary between different brands of commercially available BC and possibly within the same brand if made at different times of the season.

The effect of bovine colostrum on gastrointestinal damage in humans

BC provided a protective effect against non-steroidal anti-inflammatory drug (NSAID) induced gastrointestinal damage in humans. These drugs cause

damage by several mechanisms including reducing prostaglandin levels, mucosal blood flow, stimulating neutrophil activation and possibly stimulating apoptosis [65, 214]. Colostral peptides therefore have the potential to be used as a treatment for a range of gastrointestinal disorders. For example BC enemas have been used to treat patients with ulcerative colitis, and have shown a beneficial reduction in symptoms [215].

The effect of bovine colostrum on immune function in athletes

There have only been a few studies investigating the effect of BC on immune function in athletes and they are summarised in Table 9. In a crossover study of nine male sprinters and jumpers, there was no significant effect of BC on post-exercise salivary S-IgA levels compared to the placebo [32, 216]. In a follow-up study, supplementation with BC in a group of 30 athletes for two weeks resulted in a significant 33% increase in mean levels of salivary S-IgA [216]. There were a number of limitations to this study: there was no discussion of how the quality of the saliva samples was controlled for (e.g. the effects of dehydration and URS symptoms), the athletes were from a range of sports, and the placebo used was maltodextrin (which would not have had the same macro-nutrient profile as the intervention). Nevertheless, as salivary S-IgA plays an important role in mucosal immunity, this is a significant finding and the effect of BC on salivary S-IgA levels needs further investigating in specific sports, in a normal training environment, in different age groups and with various periods of intervention. Because concurrent URS may artificially elevate salivary S-IgA levels and confound the interpretation of diet-mediated effects, it is also important to establish a methodology to control for this variable.

In a previous investigation median levels of salivary S-IgA increased by 79% in distance runners consuming BC after 12 weeks supplementation [1]. The time-dependent change from baseline was statistically significant, even after adjusting for training volume and URS reportage [1]. While the study demonstrated increased salivary S-IgA levels among a cohort of athletes following BC supplementation, physiological interpretation was limited due to the small numbers in the study and the large variability in salivary S-IgA levels.

BC supplementation has been associated with a reduction in the reportage of URS in two studies. In 29 highly trained male cyclists who consumed 10g of BC per day for eight weeks, there was a trend towards a lower incidence of self-recorded URS in those consuming BC compared to the placebo [217]. In a retrospective review of self-recorded URS by 93 physically inactive adult males who consumed 60g per day of BC, it was found that compared to the placebo group (n=81) the BC group reported significantly fewer incidences of URS [33]. The mechanism by which BC may modulate the immune system was not identified.

In a group of 42 competitive cyclists, a small enhancement of performance occurred in the cyclists consuming either 20g or 60g BC per day for eight weeks. Performance improvements were noticed in a work-based time trial following a two-hour training ride at 65% VO_2 max [218]. As there were no differences in plasma IGF-1 levels (it has been thought that IGF-1 levels in BC could enhance anabolic processes in athletes [216]) compared to the placebo group, the proposed mechanism was an enhancement of small intestinal function and improved uptake of nutrients which assisted the recovery process [218].

Several studies have now shown that supplementation with BC does not increase circulating levels of IGF-1 in athletes [216, 219] as first proposed [32]. Another mechanism could be the stimulation of intestinal growth or repair which could modulate mucosal immunity. Several studies of BC supplementation in the clinical situation support this mechanism. NSAIDs affect gut permeability but when consumed together with BC a reduction in intestinal permeability has been observed in patients [214]. Reduced inflammation (determined by reduced levels of the acute phase protein C-reactive protein) has been seen post-operatively in patients consuming BC [220], and after a BC enema a reduction in distal colitis has been noted [215].

A summary of studies investigating the effect of BC on mucosal function including intervention trials with athletes is included in Table 9.

Table 9: Summary of studies investigating the effect of a BC intervention on mucosal function and immunity

Study	Subjects	Study design	Colostrum dosage/day	Intervention period	Response
Roos *et al.,* 1995 [186]	6F, 1M (age 20 to 42 years)	Collection of ileal effluents at 20 minute intervals for 8 hours	400mL 15N labelled BC immunoglobulin concentrates	Single 400mL dose	Recoveries of IgG, IgM, IgA were 19%, 19%, 0% respectively from the ileal effluent
Mero *et al.*, 1997 [32]	9M sprinters and jumpers (age 25±2.5 years)	Randomised, double blind crossover design. 3 x 8 day training treatments separated by 13 days	BC=125mL Bioenervi BC low strength=25mL Bioenervi/100mL of Pl Pl=125mL normal milk whey IGF-1 content 67.6, 13.5, 0 µg respectively	8 days	Serum IGF-1 increased post-training in both Bioenervi doses but not in Pl. No significant changes in salivary S-IgA
Playford *et al.*, 2001 [214]	Study 1: 7M Study 2: 8F, 7M	Study 1: Crossover RDBPC, BC for 7days, NSAID on last 5 days Study 2: Regular NSAID user. Crossover RDBPC. BC for 2 weeks	BC=125mL Bioenervi Pl=Milk whey solution	Study 1: 7days Study 2: 2 weeks	Study 1: Permeability increased with NSAIDS but not when combined with BC Study 2: No effect on permeability in subjects consuming BC
He *et al.*, 2001 [191]	9F, 9M (age 20-50 years)	RDBPC and attenuated salmonella vaccine	BC=100mL Bioenervi Pl=Water coloured with riboflavin	7 days	Trend towards increase in specific IgA among subjects who consumed BC
Mero *et al.,* 2002 [216]	Study 1: 14F, 16M (mean age 21.5 to 22.9 years) active track and field athletes, cross country skiers, orienteers Study 2: 6F, 6M	Study 1: RDBPC Study 2: Blood sampled 60 minutes after ingestion rhIGF-1	Study 1: BC (19)=20g Dynamic Pl (11)=Maltodextrin Study 2: Labelled IGF1	2 weeks	Study 1: Increases in serum IGF-1, salivary IgA. Study 2: IGF-1 not absorbed from BC.
Bolke *et al.*, 2002 [220].	60 patients, 6F, 54M (median age 61years)	RPC to receive preparation 2 days preoperatively	BC=42g Biotest Pl=matched flavoured drink without BC	2 days	BC administration reduced plasma C-reactive protein but not endotoxaemia after elective surgery

Table 9 continued: Summary of studies investigating the effect of a bovine colostrum dietary intervention on mucosal function and immunity

Study	Subjects	Study design	Colostrum dosage/day	Intervention. period	Response
Khan *et al.,* 2002 [215].	14 patients (8F, 6M) aged 16 to 75 years	RDBPC. Daily administration of enema. Also receiving mesalazine	BC=Bioenervi 100mL of 10% solution Pl=Albumin solution	4 weeks	BC enema improved left sided colitis
Kuipers *et al.,* 2002 [219].	9M competitive athletes	Sampling before and after for long term effect and 2 hours after last dose	BC=60g Intact	4 weeks	No increase in serum IGF-1 or IGFBP-3 after 4 weeks intervention and no acute changes seen 2 hours after last dose
Brinkworth & Buckley 2003 [33].	174M, physically inactive, aged 18-35 years	Retrospective analysis of wellness records	BC 60g Intact, n=93, Pl=WP, n=81	8 weeks	Smaller proportion of subjects reported URS if in BC group
Yoshioka *et al.,* 2005 [190].	Murine model	IFN-γ, IL-4, IL-10, intraepithelial lymphocytes, IgA and faecal flora measured after intervention period	BC=Numico Control=Control milk Control=Saline solution	1, 3 , 6 months	Intraepithelial T-cells polarised to Th1 type possibly by direct stimulation of BC on intraepithelial lymphocytes.
Crooks *et al*., 2006 [1].	35 distance runners, 15F, 20M (age 35 to 58 years)	RDBPC. Salivary S-IgA, URS monitored over 12 weeks	BC=10g Immulac Pl=SMP	12 weeks	79% increase n median salivary S-IgA levels. No effect on URS

M=Male
F=Female
BC=Bovine colostrum
Pl=Placebo
RDBPC=Randomised double blind placebo control
RPC=Randomised placebo control
WP=Whey protein
SMP=Skim milk powder

The effect of bovine colostrum on the immune system in older adults

No research into the effect of BC supplementation on immune function in older adults was found in the literature. There is a lack of information on how ageing affects mucosal immune function. The mucosal immune compartment appears to be unaffected but there is an age-related decline in mucosal immune responses in old animals and humans [84]. It may be possible to enhance mucosal immunity in older adults through the use of oral and nasal vaccines. Adjuvants for these vaccines and mucosal immunostimulatory agents have shown promising results in aged rodents, suggesting it is possible to enhance mucosal immunity in older animals [84]. In order to establish a treatment that can be effective in aiding the mucosal response, the mechanisms in the mucosal immune system most affected by ageing need to be understood [84]. This will be especially important for nutritional supplements such as BC which has numerous bio-actives that may have a direct or indirect effect on mucosal immunity.

Safety of bovine colostrum

BC supplementation could also lead to adverse effects. Growth hormones are present in large quantities in BC which are important to the neonate but may be harmful to the older population, in particular when cancer is present [203]. One area of concern is the effect of IGF-1; while it assists maturation in the neonate's intestine it may not have the same effect on the adult intestine and may even hasten senescence [203]. Further, it is known that systemically administered growth factors may induce proliferation of pre-malignant cells in non-target areas that harbour these cells. When growth hormones were administered to aged rats and monkeys, T-cells were stimulated [221]. Autoimmune diseases also occur at a higher rate in older adults compared to younger adults. This is thought to be due to dysregulation of immune function [123]. It is not known how a nutrition supplement with high levels of growth hormones could impact on autoimmunity in older humans. Safety testing must be completed before this becomes a viable supplement [65].

1.8 Assessment of dietary intake

When assessing the effect of a nutrition intervention (such as BC supplementation) on immune function it is necessary to assess dietary intake in order to place any immunomodulatory effect in the context of the whole diet. There are limitations to using all dietary assessment methods, these include: the number of days data is collected, interviewer bias, estimation of portion sizes and deviation from normal eating patterns [222]. The evaluation of dietary intake involves collecting information from participants, interpreting and analysing it and comparing the results with recommended guidelines. The main approaches to measuring dietary intake include diet records, diet recall, diet history and food frequency [223].

Recently, nutrient reference values for Australians and New Zealanders have been revised and published [160]. The recommended daily dietary intakes for adolescents aged 14-18 years, young adults aged 19-30 years and adults aged 51-70 years and over 70 years are summarised in Appendices 10.39 and 10.40. Separate dietary guidelines (for macronutrients, calcium and iron) have been established for athletes in New Zealand and are included in Appendix 10.38.

1.8.1 Dietary assessment methods

Diet history

Diet history is the gold standard method in clinical practice and could also be a useful tool in nutrition intervention trials [222]. The diet history method involves a face to face interview with the researcher, and comparisons of the information collected in a twenty-four hour diet recall with dietary intake over preceding periods [223]. When participants have limited time to participate in a dietary interview, other methods need to be used, such as dietary recording.

Dietary Recording

Weighed records

Each item of food and drink is weighed prior to consumption and any food served and not eaten is also weighed [223]. This method places considerable burden on the participant and may result in poor compliance. The participant may also alter eating patterns to make it easier for recording, inadvertently introducing bias [223].

Estimated records

Food and drink consumed is estimated using household measures e.g. standard measuring spoons and cups, by using dimensions and number of items of pre-determined size or using the purchase unit [222]. Additionally diagrams and photos may be used as aids to quantify portions [223]. While the errors will be greater with this method than using weighed records the burden for the participant is less [223], and is often used when assessing dietary intake in athletes [224]. Generally recording includes weekdays as well as one weekend day to account for different dietary intake patterns at the weekend [222].

Menu Record

This method is qualitative and analysis is by determining the frequency of food eaten or by assigning average weights to portions. The advantage of using this method is that information can be collected on foods eaten and meal patterns over a prolonged period of time [223].

Number of days of dietary recording depends on the individual nutrients

To determine habitual dietary intake for individuals, information is collected over a number of days depending on the degree of precision required. A nomogram to account for intra-individual variation when choosing the number of days for dietary recording was developed by Black, 2001 [223] (see Table 10). In practice three to four days of dietary record-keeping have often been used in sports people to ensure better compliance [224], and this method is considered the most accurate and feasible method for research in athletes [225].

Table 10: Predicted number of days of dietary recording required taking into account intra-individual variation (Black, 2001 [223]) compared to numbers of days required for groups of athletes (Burke, 2001 [224])

	%coefficient of variation ($CV_{w)}$	Days of dietary recording required for 15 to 20% precision for an individual	Days of dietary recording required for 15 to 20% precision for a group of 10-20 athletes (Burke,2001 [224])
Energy	23	7 days	3 days
Macronutrients	20 to 26	At least 7 days	Carbohydrate 4 to 5 days
Micronutrients	30 to 40	Much more than seven days	?

Diet recall

The participant is asked to recall actual food and drink previously consumed on specified days, often the last 24 hours is used, and is known as the 24-hour recall [223]. Portion size is quantified by the researcher using tools for estimating diet records. The advantage of this method is that 24-hour recalls can be by a short interview and large numbers of participants can be included [226]. By this method it is difficult to determine with precision individuals who have low and high dietary intake, but mean dietary intakes for groups will be reliable [226]. Accuracy of diet recalls at the individual level can be improved with repeat interviews at intervals over time [223].

The use of a 24-hour diet recall may be suitable for older adults who have hearing, sight or attention disabilities, especially when repeated recalls are carried out by a trained interviewer [226]. This method has been used to assess nutrient intakes among homebound healthy older adults [227].

A summary of the advantages and disadvantages of the dietary recording and recall methods for intervention trials is included in Table 10.

Table 11: Summary of dietary analysis methodologies appropriate for use in nutrition intervention trials (adapted from Black, 2001 [223] and Dwyer, 1994 [226])

Record	Use	Advantages	Disadvantages
<u>Diet Record</u>			
Weighed	Favoured where kitchen scales are commonly used Participant records what is eaten at the time of eating Suitable for use under controlled conditions	Good information on individuals Provides feedback Raises awareness Recording error minimised if participants given good instructions Does not rely on memory	High burden for the participant Lower compliance compared to other methods Participant may change eating patterns Covers a limited time Records must be checked and coded in standard way, which is time-consuming and expensive Gender differences, females may be better than males at recording
Estimated	Portions are described in terms of the household measure This method often used in the dietary assessment of athletes in the field	*Same advantages as for weighed records, additionally:* Less burden for the participant and improved compliance compared to weighed records	Moderate burden for participants Less accurate than weighed records Participant may change eating patterns Covers a limited time Difficult to estimate portion sizes Records must be checked and coded in standard way which is time-consuming and expensive Gender differences, females may be better than males at recording
Menu	Qualitative method	Collection of information over long periods of time	No quantitative information collected
<u>Diet Recall</u>			
24 hour recall	Useful method for healthy older adults	Minimal burden for participant More objective than diet history Good reliability between interviewers	Poor information on individual Does not reflect differences between weekend and week days Selective forgetting of foods may occur Interviewer needs to be trained/experienced Relies on memory Portions are estimated Interviews can be time-consuming

1.8.2 Precision and controlling for errors

Errors in collecting dietary data

Errors often arise during record-keeping as many people are only vaguely aware of what they eat and include:

Response biases

The participant may eat differently either consciously or unconsciously to make recording easier, to conform to a better diet or they may eat according to study requirements on recording days [226].

Random errors

The participant may not remember the omission or addition of foods and supplements, may make errors in estimating portion size when foods are not weighed, may make measuring or weighing errors (e.g. heaped vs level spoons, and use non-standard measuring or weighing equipment), may have difficulty estimating quantities and identifying all ingredients when dining out, and the seasonal availability of foods.

Systematic errors

The researcher may introduce systematic errors during the interpretation of estimated diet records. Errors occur when converting household measures into weights and selecting an appropriate substitute. Additionally there are errors associated with the food composition databases: they may not be up to date [228], imported foods may have a different chemical content to New Zealand foods e.g. selenium [163], and the accuracy of chemical analysis e.g. variable vitamin C content in tomatoes [229].

Validation of dietary assessment

To validate the accuracy of a dietary assessment method, reported daily energy intake (RDEI) is determined and compared to estimated daily energy expenditure (EDEE). Under-reporting and over-reporting are common problems during dietary intake assessment and lead to inaccurate assessment of nutrient intake [230]. Further errors can occur when interpreting and converting

anthropometric and activity information to EDEE [223]. A correction factor is established from the difference between RDEI and EDEE and used to account for over- or under-reporting (see Section 1.8.3).

1.8.3 Estimating daily energy expenditure (EDEE)

Direct calorimetry is the most accurate method for determining energy expenditure but is expensive and time consuming to use in the field [231]. Indirect non-calorimetric methods are simpler to perform e.g. the use of activity diaries, measurement of heart rate and dietary intake.

EDEE can be determined by multiplying the estimated basal metabolic rate (BMR) with the appropriate physical activity level (PAL) [231]. BMR is the minimum energy required to maintain the body's vital functions in the waking state [232]. Factors that can affect BMR include age, gender and body size (especially the amount of lean body mass). Different types and amounts of training will also have an effect, as do genetic differences, and with ageing lean body mass declines [233]. One method to estimate BMR is to use prediction equations which take into account variables such as gender [234], height and weight [231].

The suitability of several equations for active individuals has been reviewed; the Cunningham equation best predicted BMR in both active males and females, and the Harris-Benedict (1919) equation was the next best predictor [233]. The Harris-Benedict equation has been used in clinical trials to estimate the energy requirements, e.g. for 459 adults participating in the 'Dietary Approaches to Stop Hypertension' study [235]. Equations published by the World Health Organisation/Food and Agricultural Organisation/United Nations University (WHO/FAO/UNU) are derived from the Schofield equations and are considered to be the best estimate for healthy people [231]. Nutrient reference values that have been established recently for Australia and New Zealand are based on energy expenditure determined using the Schofield equations [160].

Guidelines for PAL values have been established for a range of activities [223], the applicability of these PAL values to activity recorded by athletes has been less well studied. Overestimation of energy expenditure is thought to occur for PAL values over 1.7 [235]. There is some evidence that athletes in normal training will have values around 1.8-2.2 and for extreme activity 2.5-4.0 [223]. Female endurance athletes have been reported to have values between 2.0 [231] and 2.8 [236]. In addition the interpretation of the recorded activity and its coding can introduce further errors [237]. To help overcome these problems a compendium of physical activities has been established to calculate the energy expended in various activities that participants may carry out [237].

1.9 Summary

This review shows that several nutrients and dietary supplements have an immunomodulatory effect, and may impact on mucosal immunity and levels of salivary S-IgA. Functional foods are a complex mix of nutrients and bio-active factors. Isolating the effect of a single nutrient or bio-active is difficult particularly as it may act synergistically with other nutrients or bio-actives. As functional foods are generally natural products, levels of nutrients or bio-actives may also vary between batches. Without standardisation of the specific nutrient(s) or bio-active(s) under investigation comparison of results between studies will be difficult.

In order to elucidate an effect of a functional food in humans randomised clinical trials are necessary. Immune status will be affected by age, gender and prior exposure to pathogenic organisms as well as various physiological stressors e.g. dietary intake, psychological stress and the effect of prolonged intensive exercise [4]. An appropriate immune parameter to monitor in order to prove efficacy is also required. It is not known which parameter will respond in a dose-dependent manner and whether the change will be related to a health improvement [4]. In a previous study, supplementation with BC may have had an immune modulating effect on levels of salivary S-IgA in middle aged distance runners [1]. A review of biomarkers of immune function that may be modulated by a nutrition intervention indicates that salivary S-IgA is a relevant and feasible marker to use [4]. The mechanism by which levels of salivary S-IgA in the distance runners were affected was not known.

Expression of homing integrins on peripheral lymphocytes is reduced in aged rats which could impact on mucosal immunity and S-IgA. It is not known if integrin expression is affected in aged humans, and athletes following intensive exercise regimes. It is also not known whether a nutritional supplement may have an effect on integrin expression. More research is needed to identify appropriate markers of immune function that may change as a result of a nutrition intervention which can be associated with an improvement in health.

2.0 Study aims

Research question

Does supplementation with bovine colostrum enhance levels of salivary S-IgA in all groups of athletes, aged matched controls and older adults? What is the mechanism for the immunomodulatory response?

The primary aim of this study was to further investigate the effects of a BC supplement on levels of salivary S-IgA in elite athletes, their age matched controls and healthy older adults with potentially sub-optimal immunity. The secondary aim of this study was to investigate the mechanism by which a BC supplement could modulate mucosal immune function. The sampling methodology established previously [1] was used to collect saliva samples from participants in their normal training, study or home environment.

To achieve the aims, the following objectives were established:

- Identify the effect of 12 weeks of BC supplement, in the context of the whole of dietary intake, on levels of salivary S-IgA in a group of 50 high performance athletes and 50 non-exercising controls. Within each cohort of 50 there would be 25 taking the BC supplement and 25 taking the placebo
- Identify the effect of 12 weeks of consuming a BC supplement, in the context of the whole of dietary intake, on levels of salivary S-IgA in 50 healthy older adults. Within the cohort there would be 25 taking the BC supplement and 25 taking the 25 placebo
- Monitor daily levels of activity to ensure there were no differences between the BC and placebo groups
- Monitor daily reportage of URS in order to identify any effect on changes in levels of salivary S-IgA

- Perform additional tests on blood samples supplied from a subgroup of 10 from each cohort (a total of 30 participants) to investigate the underlying effect of a BC supplement on levels of salivary S-IgA. The following parameters were measured:
    - Lymphocyte subsets
    - Expression of homing integrin on lymphocyte phenotypes
    - Plasma cortisol levels
    - Cytokines that may have an effect on levels of secretory IgA in saliva

3.0 Methods

The main aim of this study was to determine whether an effect of BC supplementation on levels of salivary S-IgA in marathon runners could be identified in groups with potentially depressed immune systems, such as elite athletes and older adults.

The study was divided into two parts:
Study 1 investigated the effects in a group of young swimmers training at an elite level together with a group of age-matched non-exercising or lightly-exercising controls (exercising for less than one and a half hours per week).
Study 2 investigated the effects in a group of healthy older active adults (able to walk and do light activities such as gardening, housework, shopping) who acted as their own controls.

Saliva and blood samples were collected to establish immunological, biochemical and hormonal status at baseline (Base), during the supplementation period and post-supplementation (Post).

Research assistance

Due to the large number of participants providing saliva and blood samples simultaneously, four research assistants (RAs) were employed. The RAs were nutrition students at Massey University. For study 1, the RAs were trained to assist with aliquotting saliva samples into labelled microtubes and in standard sample storage practices. One research assistant was further trained to help with centrifuging, aliquotting whole blood samples into microtubes and their storage. This research assistant was also trained to interpret and code the food records and enter data into the dietary analysis software. For study 2, the RAs were given additional training to carry out diet recall interviews, collect anthropometric information, as well as collect and aliquot saliva samples and collect and test urine samples with an indicator test strip.

Randomised double-blind placebo controlled design

The study design was a randomised double-blind placebo controlled (RDBPC) trial. Participants were randomly assigned to either BC or placebo in a double-blinded manner. The intervention drink produced by Fonterra (see Section 3.2) was identified with a unique three or four digit randomly generated number by Fonterra's project manager. This number became the identity (ID) code for the participant and enabled confidentiality for all analytical results. The researcher and RAs were blinded to the classification of the ID codes as either BC or placebo. While the assignment of coded product was co-ordinated by Fonterra's project manager (to ensure equal numbers of males and females were included in the BC or placebo group) it was the researcher or RAs who issued the drink and had contact with the participants. Decoding of the intervention occurred after all analytical testing was completed to ensure bias was not introduced by the researcher, RAs or laboratory analysts.

Sample size determination

Sample size for each cohort was estimated by power calculation using the statistical results from the previous study investigating the effect of BC on salivary S-IgA in marathon runners (see Appendix 10.1). To identify a significant difference in levels of salivary S-IgA; 90% power and 5% significance was chosen and this required twenty-five people in each group making a total of fifty participants (BC=25, placebo=25). However it was not known if the effect of BC noted in marathon runners would be observed in all groups of athletes or other groups undergoing changes to immune function such as older adults.

Subgroup sample size determination

A number of immune parameters known to affect immune cell signalling and trafficking were identified and used to assess possible mechanisms of action. Due to cost limitations a few were selected for monitoring in a subgroup of 10 participants from each cohort.

3.1 Study participants

3.1.1 Study 1

Ethical approval for this study was sought and given by the Auckland Ethics Committee (AKX/03/07/182), (see Appendix 10.4).

Swimmers

Twenty-five swimmers (13F, 12M) aged 14-23 years volunteered to take part in the study. These swimmers were undertaking a training programme to compete in the Auckland Swimming Championships, December, 2003 (swimmers from this event were selected to become members of the New Zealand Olympic team competing at Athens, 2004).

Exclusion criteria for this study included:

- Being lactose intolerant or having any other known allergy to cow's milk
- Taking whey protein supplements, or any other immunological modulating supplements such as probiotics
- Receiving treatment for any medical condition diagnosed by a general practitioner (including asthma)

Swimmers were invited to participate in the study after discussing the study with their coach, by an information pamphlet (see Appendix 10.2), through presentations prior to a swim training session, and by sending a letter to their parents if they were under the age of 18 years. All participants were fully informed orally and in writing about the nature of the study, any known risks, and their right to terminate participation at will before signing a formal consent form (see Appendix 10.3).

Initially the aim was to recruit volunteers from one swimming club in order to ensure standardisation of training programmes and training environment. When insufficient numbers volunteered, additional participants were sought from two further clubs, making three swimming clubs in total.

Saliva samples were collected prior to training sessions to avoid the effects of exercise-induced dehydration. Twenty three of the twenty-five participants came from two clubs and saliva samples were collected prior to the morning training session between 5.15am and 5.30am (training started at 5.30am). The swimmers were asked to eat and drink their normal meals prior to training but not to drink within the 30 minutes before they provided a saliva sample. This was requested in order to standardise the time post-prandially prior to providing a saliva sample. It was important that the participants followed their normal routine in order to minimise any disruption of the study protocol. For a few swimmers it was difficult for them to eat before the morning training session as they normally trained without prior drinking or eating. To accommodate two swimmers who could not change their routine, consumption of a sport drink 30 minutes prior to sampling was allowed.

Blood samples were collected after the morning training session between 7.30 and 8.00am. This time was chosen in order to fit in with the swimmers and the phlebotomist on the nominated days. One participant from the placebo group agreed to provide saliva samples and to complete all recording requirements but did not want to provide a blood sample.

For the two participants from the third swimming club it was not possible to collect saliva samples before the morning training session. For these two participants saliva and blood samples were collected between 3.45 and 4.00pm, that is, before their afternoon training session

Training and wellness diaries (see Appendix 10.5 and Section 3.7) were completed daily and a four day diet record completed at week one and a three day diet record completed at week ten of the supplementation period (see Appendix 10.6 and Section 3.9).

Students (age-matched controls)

For comparative purposes volunteers who were non-exercising or lightly exercising university students were also sought. Twenty-eight (19F, 9M) students aged 18-27 years volunteered. Exclusion criteria for this study were the same as listed for the swimmers.

Students were invited to participate in the study through the distribution of an information pamphlet (see Appendix 10.2), posters, through presentations prior to lectures and laboratory sessions and through general e-mail at the Massey University, Albany campus (MUAC). All participants were fully informed orally and in writing about the nature of the study, any known risks, and their right to terminate participation at will before signing a formal consent form (see Appendix 10.3). One student from the placebo group did not want to supply a blood sample but agreed to supply saliva samples and complete all study recording requirements.

Activity and wellness diaries (see Appendix 10.7 and Section 3.7) were completed daily and a four day diet record completed at week one and week ten of the supplementation period (see Appendix 10.6 and Section 3.9).

To fit in with lecture and laboratory timetables saliva sampling was scheduled around lunch time, 11.00am to 1.00pm. The students were also asked to not drink in the 30 minutes prior to providing a saliva sample and to have eaten within the previous two hours.

Blood samples were collected at the Massey University Health Centre between 9.00 am and 12.00pm on the nominated day. Where possible the participants were asked to provide a sample at the same time of day for each of their 5 samples.

3.1.2 Study 2

Ethical approval for this study was sought and given by the Auckland Ethics Committee (AKY/04/10/268) (see Appendix 10.10).

Older Adults

In study 2 forty-five older adults (25F, 20M), aged 65-76 years, who lived on the North Shore and in the north west of Auckland volunteered. This was within the vicinity of the MUAC which was important, as unlike the previous studies the participants did not meet at a central place to provide saliva samples.

The RAs were trained to collect urine samples, test specific gravity using Roche *Combur*^{10}*Tes*t strips, and collect saliva samples from the participants at their homes. The RAs were also trained to show the participants how to complete training and wellness records and how to carry out a twenty-four hour diet recall.

The older adults were invited to participate in the study after presentations and distributing an information pamphlet to ProBuS (acronym of professional and business) clubs, University of the Third Age (U3A) on the North Shore of Auckland, the Point Chevalier Returned Service Association (RSA), by advertising in the North Shore Times, and through contact with a walking group in a suburb of Auckland (Kohimarama) (see Appendix 10.8).

Exclusion criteria for this study included:

After completion of a screening questionnaire (see Appendix 10.11), participants were excluded if they were taking medication for respiratory conditions or digestive problems.

In addition exclusion criteria included:

- Being lactose intolerant or having any other known allergy to cow's milk
- Taking whey protein supplements, or any other immunological modulating supplements such as probiotics
- Not living independently
- Not physically active

All participants were fully informed orally and in writing about the nature of the study any known risks, and their right to terminate participation at will before signing a formal consent form (see Appendix 10.9).

Participants were visited between the hours of 9.00am and 12.00pm on nine occasions over the course of the study and at the same time of day on each visit by the same RA. Saliva samples were collected on nine occasions after the specific gravity of a urine sample was checked. If a specific gravity reading was >1.030 (indicating a dehydrated state) the participant was requested to have a glass of water and wait 30 minutes before providing a saliva sample; this was required with four participants once each as they had forgotten to drink. On subsequent visits they had remembered. Record-keeping in the activity and wellness diaries was also checked at each visit. At week one and week ten of the supplementation period a twenty four hour diet recall interview (see Appendix 10.13) was carried out.

The participants provided blood samples on the nominated day between 8.30am and 1pm at their local diagnostic medical laboratory (DML) or they travelled to the health centre at the MUAC. Where possible the participants provided a blood sample at the same time for each of their five samples. All forty–five participants agreed to provide saliva samples, blood samples and complete all recording requirements.

3.2 Supplement used in both intervention studies

The study participants were given five boxes of supplement, each box containing 28 x 25g sachets. Each participant was asked to take two sachets per day by mixing one sachet in 125mL of cold water in the 250mL drink shaker provided, and drink this blend; they were to do this once in the morning and once in the evening. Five boxes of sachets provided enough supplement for twice-daily consumption for ten weeks.

A drink powder containing BC was the intervention (NZMP Low Protein Colostrum Powder [AN15]) and was formulated in three flavours to provide a choice for the participants (see Appendix 10.14). In the previous study with marathon runners there was no flavour choice, the participants being issued a chocolate flavoured supplement (see Appendix 10.1). The flavour blends were formulated to achieve similar levels for protein, carbohydrate and fat (see Appendix 10.14). The placebo blend contained skim milk powder, matched to the BC blend for equivalent digestible protein content, and fat and carbohydrate levels. The placebo was also formulated into three flavours which were flavour matched to the BC blends. Participants were offered one of the following choices:

All chocolate

An equal mixture of sachets containing chocolate or berry

An equal mixture of sachets containing chocolate, berry or malt.

Microbiological testing of the products was completed by Fonterra, and test results complied with Fonterra's microbiological specification for milk powders prior to the release and distribution of the supplement to Massey University, Albany.

Endogenous bio-active levels were supplied for the following bovine molecules: IgG, IgA, IgM, lactoferrin (Lf), IGF-1, IGF-2, TGF-β1, TGF-β2, and oligosaccharides for each of the flavour blends (see Appendix 10.15). The results indicated there were differences in the bio-active levels between the BC flavour blends (see Table 12, Appendix 10.15). Levels of bio-actives were at

low levels or not detectable for IgA, IgM, Lf, IGF-1 and IGF-2 for all three placebo flavour variants. Levels of TGFβ-1 in the placebo flavour blends were similar to levels in the respective BC flavour blends; but levels of TGFβ-2 were about 45%, 31% and 37% less for chocolate, berry and malt respectively.

Table 12: Bio-active levels for the BC flavour variants

	Malt (BC)	Chocolate (BC)	Berry (BC)	Malt (Pl)	Chocolate (Pl)	Berry (Pl)
IgA mg/g	3.0	3.3	1.7	0.07	<0.01	0.04
IgM mg/g	0.22	0.23	0.09	ND	ND	ND
Lf mg/g	0.2	0.2	0.1	<0.01	<0.01	<0.01
IGF-1 ng/g	53.1	53.7	64.0	2.8	ND	4.5
IGF-2 ng/g	35.3	29.3	31.6	ND	ND	ND
TGFβ-1 ng/g	45.3	19.7	27.7	44.1	21.7	31.0
TGFβ-2 ng/g	162.0	102.7	135.5	101.7	56.8	93.6
Oligosaccharides g/100g	5.92	2.64	3.89	1.52	1.52	3.38

ND=not detected

Flavours and colours were added to achieve blends that were similar in taste and colour as BC powder has a strong distinctive flavour; these blends provided the best combination to minimise the difference between the BC and placebo blends. To a certain extent the participants could choose their flavour combination. Allowing the participants to do this became difficult to manage and not all preferences could be accommodated. Participant preferences had to be anticipated prior to recruiting the participants so that the supplement could be manufactured and packaged in time for the trial, as the supplement blend was specially prepared and was not commercially available.

Assessment of compliance of supplement intake

The daily consumption of the trial drink (either placebo or BC powder) was recorded on the food records, 24 hour diet recalls and included in the dietary analysis for each participant.

At the end of ten weeks supplementation the participants were asked to return all remaining sachets which were counted and the total number of returned sachets subtracted from the total number issued (140 sachets). The difference was used to determine the compliance of supplement intake.

3.3 Study design

3.3.1 Study 1

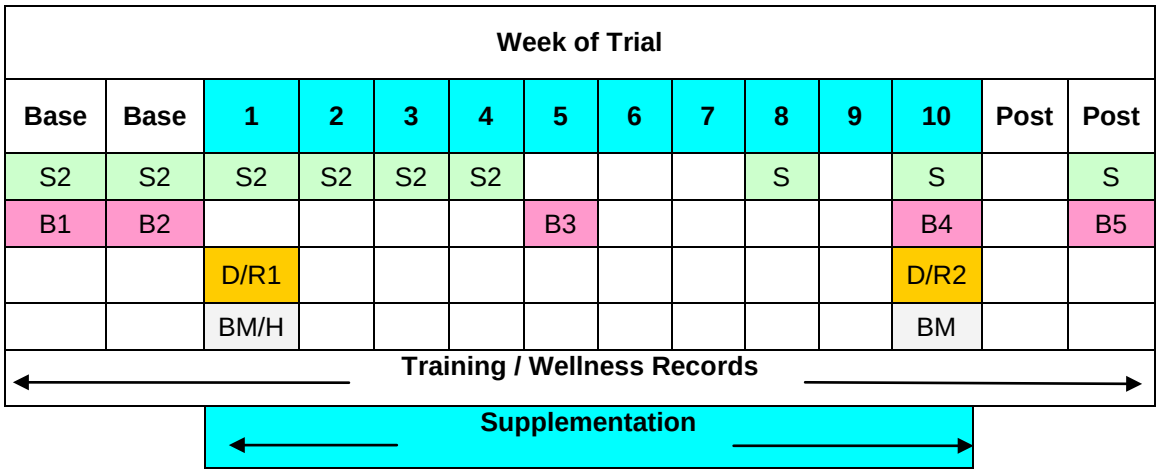
The RDBPC study was initially planned to be 12 weeks (the same length of the trial performed with the marathon runners [see Appendix 10.1]) but was shortened to 10 weeks (see Figure 7), to accommodate the swimmers training cycle leading up to the Auckland swimming championships in the week prior to Christmas, 2003. Due to the time taken to recruit participants there was insufficient time to fit in a 12-week supplementation trial. The students in the control group were no longer on campus after the first week of December as this was their holiday period, and were not available to participate in a supplementation trial. In addition, because the swimming clubs were preparing for the Athens Olympics in early 2004 and did not want to participate in an intervention trial at that time, it was decided that it would be best to shorten the duration of the supplementation period to accommodate as many participants as possible. A previous study investigating the effects of BC supplementation on levels of salivary S-IgA levels in athletes used a dose of 20g/day and found an increase in salivary S-IgA levels occurred after 2 weeks of supplementation [216]. Therefore it was felt that shortening the supplementation period from 12 weeks to 10 weeks should still leave sufficient time to determine whether an immunostimulatory effect of BC does occur. A supplementation period of 10 weeks was used for each of the cohorts, swimmers, students and older adults.

The minimum dose of BC sufficient for eliciting an immunostimulatory effect is unknown as is the length of consumption. The dose of 25g (of BC powder) was chosen for the current studies as it was twice the level consumed in the previous study (see Appendix 10.1). As the supplementation period was to be shortened from the twelve week trial to a ten week trial increasing the dose was considered necessary. For the current studies it was anticipated any change to salivary S-IgA levels as a result of an immunostimulatory effect of BC would occur within the first four weeks of supplementation. Saliva was sampled twice per week at baseline and for the first four weeks of supplementation to monitor changes to salivary S-IgA (see Figure 7). This sampling regime was considered

an acceptable frequency by the swimmers, daily saliva sampling would be ideal but was not possible due to the availability of the participants and the cost of collecting samples. It was hoped that sampling twice per week would be sufficient to identify increases in salivary S-IgA levels in response to URTI.

A further saliva sample was collected one month later (after eight weeks of supplementation), one sample was collected at the end of the ten week supplementation period and one sample two weeks post-intervention. This made a total of 15 saliva samples, and for many of the participants in study 1 this sampling regime was burdensome. Attendance compliance to all sampling sessions was poor. Only one swimmer and ten students attended every sampling session. Because the sampling regime was found to be burdensome and because no significant changes in levels of salivary S-IgA occurred after consuming the BC supplement in either the swimmer or student cohort, the number of saliva samples collected was changed for study 2 (see Figure 8).

Training and wellness diaries were completed each day (see Appendices 10.5 and 10.7) and a four-day diet record completed at weeks one and ten of the intervention for the student cohort (see Appendix 10.6). The swimmers cohort completed a four-day diet record at week one and due to poor record keeping this was reduced to a three-day diet record at week ten. It was felt this would be less burdensome for the swimmers and may encourage better compliance.



S2=Saliva sampled twice per week (IgA, IgM, IgG, albumin, osmolality analysis)
S=Saliva sampled on a single occasion (IgA, IgM, IgG, albumin, osmolality analysis)
B1,2,3,4,5=Blood sampled on a single occasion (IgA, IgM, IgG, albumin, c-reactive protein analysis)
B1,2,5=Blood sampled at base and post-supplementation (Full Blood Count [FBC], Iron studies)
D/R=Diet record (swimmers DR1=4 days, D/R2=3 days, students both D/Rs= 4 days)
BM=Body mass (kg)
H=Height (cm)
B1,2,3,4,5 (swimmers only)=Blood sampled for IGF-1 analysis

Figure 7: Study 1 design: Swimmers and students

3.3.2 Study 2

As for study 1, study 2 was also a RDBPC trial with a ten-week intervention period (see Figure 8). Saliva samples were provided weekly during the baseline period and for the first four weeks of the supplementation period. A single saliva sample was collected in the eighth and tenth weeks of supplementation, and the final sample was collected two-weeks post-supplementation. This made a total of nine saliva samples which most participants supplied.

Activity and wellness diaries (see Appendix 10.12) were completed each day and a 24-hour dietary recall (see Appendix 10.13) was completed at weeks one and ten.

Week of Trial													
Base	**Base**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Post**	**Post**
S	S	S	S	S	S				S		S		S
B1	B2					B3					B4		B5
		24DR									24DR		
		BM/H/W									BM/W		
Activity / Wellness Records													
		Supplementation											

S=Saliva sampled (IgA, IgM, IgG, albumin, osmolality analysis)
B1,2,3,4,5=Blood sampled (IgA, IgM, IgG, albumin, c-reactive protein analysis)
B1,2,5=Blood sampled at base and post-supplementation (Full Blood Count [FBC], iron studies, liver function)
24DR=24 hour diet recall interview
BM=Body mass (kg)
H=Height (cm)
W=Waist measurement (cm)

Figure 8: Study 2 design: Older adults

3.4 Assessment of the primary outcome variable: secretory IgA (S-IgA) in saliva

3.4.1 Saliva collection

Studies 1 and 2

Whole mixed unstimulated saliva samples were stored and analysed according to procedures adapted from the Hunter Immunology Pathology Service, Newcastle, Australia (now called Hunter New England Pathology Service) [55]. Study participants were asked to eat and drink at least 30 minutes before providing a saliva sample. They were instructed to produce a sample by gently spitting saliva collected underneath the tongue into a collection tube. This should provide whole mixed unstimulated saliva. Approximately 1mL of saliva was collected which took between one minute and ten minutes to produce depending on the individual. Saliva was immediately aliquotted into microtubes and placed straight away onto dry ice. The microtubes were held on dry ice until transported to a -80 °C freezer where they were stored until analysis (see Table 14).

3.4.2 Salivary immunoglobulin analysis

Studies 1 and 2

Saliva samples were centrifuged briefly (20 seconds/8228g, at room temperature) to remove particulate matter in an Eppendorf minispin centrifuge prior to analysis. Immunoglobulin and albumin levels in saliva were measured by immunoturbidimetry on a Vitalab, Flexor analyser (Vital Scientific, The Netherlands) using Tina Quant (TQ) (Roche Diagnostics) (see Table 14).

Dehydration and fasting can elevate levels of salivary S-IgA, and this was monitored by measuring salivary albumin and determining osmolality in both study 1 and study 2. Elevated levels of salivary albumin can indicate dehydration, due to altered saliva flow rates, or an increase in tissue permeability. Osmolality measures the total dissolved particles in a solvent. High results for osmolality can indicate dehydration, as saliva flow rate is

reduced, or that there has been recent consumption of a drink with high levels of electrolytes (e.g. a sports drink). Salivary osmolality was measured using a freezing point depression Osmometer (Advanced Digimatic Osmometer 3D2, Advanced Instruments, Massachusetts, USA) (see Table 14). Repeat-measures analysis of variance was performed on the salivary immunoglobulin, albumin and osmolality results to identify time-related changes and differences between the groups.

The salivary S-IgA/osmolality ratio may also be an indicator of hydration status [238]. Analysis of changes in the ratio between the BC and placebo groups within each cohort was investigated. There were no significant differences between the groups. This provided additional evidence that there was no effect of hydration status on salivary S-IgA levels that may have confounded the immunomodulatory effect of the supplement.

As there were no significant differences between the groups within each cohort for these measures of hydration status (salivary albumin, osmolality and salivary S-IgA/osmolality ratio), results of saliva analysis for all participants who met the remaining study criteria were included for statistical comparison.

Study 2: Additional monitoring of hydration status

To control for variations in saliva flow rate due to hydration status specific gravity of urine was measured in the older adults at the time of saliva sampling. This also prompted the participants to remember to have a drink 30-60 minutes before providing a sample. Urine was collected by the participant in a urine collection cup (USL, Unicup) and specific gravity was measured using a ten–patch test strip (Roche, *ComburTest*).

3.5 Assessment of secondary outcome variables - immunological and biochemical parameters

Blood was sampled on five occasions for each cohort during the study, by venepuncture carried out by a registered phlebotomist, into three different Becton Dickson vacutainers according to the sampling plan in Table 13.

Table 13: Sampling plan for whole blood samples

Whole blood samples	Analysis	Blood sample				
Main Group		B1	B2	B3	B4	B5
Whole blood was collected in a 10mL sodium heparinised vacutainer (BD)	IgA, IgG, IgM albumin, c-reactive protein	10	10	10	10	10
Whole blood collected in a 4mL sodium heparinised vacutainer (BD)	Iron studies	4	4	-	-	4
4mL whole blood collected into EDTA vacutainer (BD)	FBC	4	4	-	-	4
Maximum volume of blood collected from each participant		18	18	10	10	18
Subgroup		B1	B2	B3	B4	B5
The above sampling plan was followed with the following additional vacutainers						
Whole blood collected in a 4mL sodium heparinised vacutainer (BD)	Lymphocyte subset analysis	4	4	4	4	4
Whole blood collected in a 4mL EDTA vacutainer (BD)	FBC	-	-	4	4	-
Maximum volume of blood collected from each sub-group participant		22	22	18	18	22

BD=Becton Dickson
FBC=Full blood count
EDTA=ethylene diaminetetraacetic acid
B1=First blood sample, B2=Second blood sample, B3=Third blood sample, B4=Fourth blood sample, B5=Fifth blood sample

Samples were collected twice at baseline (weekly samples), once during the fifth week of the supplement, once during the tenth week of the supplement, and once two weeks post-intervention. The samples were either held at room temperature until couriered to the diagnostic laboratory for analysis or refrigerated until centrifuging. Due to the large number of blood samples

collected at one time a research assistant was employed to help with centrifuging them. Whole blood was centrifuged for ten minutes at 330g at 5ºC; plasma was aliquotted into microtubes and held in a -80°C freezer until analysis for immunological and/or biochemical or hormonal parameters (see Table 14).

Full blood counts were measured to identify any differences between the BC and placebo groups within each cohort that might affect immune function.

Plasma immunoglobulins were measured to identify any effect of consumption of the supplement on immunoglobulin levels within in each cohort

Levels of plasma C-reactive protein were measured to determine whether there were differences between the BC and placebo groups within each cohort as a result of an inflammatory condition.

Liver function tests were performed in the older adult cohort to identify any age related decline in function that may affect immunity.

Plasma levels of IGF-1 were measured in the swimmers cohort to identify any effect of consuming the supplement.

Table 14: Summary of sample collection, storage and analytical procedures for immunological, biochemical and hormonal parameters

Parameter	Participants	Sample	Method of collection	Analysed by	Method of Analysis
S-IgA, IgM, IgG, Albumin	All participants	Saliva	Unstimulated whole mixed saliva immediately aliquotted into microtubes and frozen on dry ice, held at -80ºC until analysed	Nutrition Lab, (MUPN)	Immunoturbidimetric assay. IgA, IgM, IgG, Tina Quant kits, Roche Diagnostics, Vitalab Flexor analyser
Osmolality	All participants	Saliva	Unstimulated whole mixed saliva collected as above	Nutrition Lab, (MUPN)	Freezing point depression (Advanced Digimatic Osmometer 3D2, Advanced Instruments, Massachusetts, USA).
IgA, IgM, IgG, Albumin, c-reactive protein	All participants	Plasma	Whole blood stored at 4ºC until plasma separated by centrifuging in a Biofuge primoR centrifuge (10minutes / 330g / 5ºC), aliquotted into microtubes, held at -80ºC until analysed	Centrifuged by Self & RA, (MUA). Analysed by Nutrition Lab, (MUPN)	Immunoturbidimetric assay. IgA, IgM, IgG, C-reactive protein Tina Quant, Roche Diagnostics. Vitalab Flexor
Cytokines IL-6, IL-1ra, IFN-α	3 subgroups of 10, 1 from each cohort	Plasma	Plasma collected as above	Self (MUA)	ELISA IL-6, DuoSet DY206, RnD systems, IL-1ra DuoSet DY280 RnD systems, Human IFN-α Quantikine kit 41110-2, RnD systems
Lymphocyte phenotyping.	All 3 Subgroups	Blood	4mL heparin vacutainer held at room temperature and analysed within 6 hours of sampling	LabPlus, (ADHB)	CD3 FITC,BD. Cat. No. 349201; CD19 APC,BD, Cat. No. 340437; CD45 PERCP, BD.Cat. No. 347464; CD3 APC,BD.Cat. No. 340440; CD4 FITC,BD, Cat. No. 340133; CD8 FITC,BD, Cat. No. 347313; CD49d PE,Pharmingen, Cat.No.555503. FACS Calibur Flow Cytometer

MUPN=Massey University, Palmerston North
RA=Research Assistant
MUA=Massey University, Albany
ADHB=Auckland District Health Board

Table 14 continued: Summary of sample collection, storage and analytical procedures for immunological biochemical and hormonal parameters

Parameter	Participant	Sample	Method of collection	Analysed by	Method of Analysis
Full blood count: White blood cell count, Red cell count, Platelets	All participants at base and post	Blood	4mL EDTA vacutainer held at room temperature until analysed same day of sampling	LabPlus (ADHB)	Sysmex Automated Haematology Analyser XE-2100
Iron studies: Serum iron, total iron binding capacity, iron saturation, ferritin	All participants at base and post	Blood	4mL heparin vacutainer held at room temperature until analysed same day of sampling	LabPlus (ADHB)	Roche ferritin reagent Kit, Cat No. 166140; Roche Iron reagent kit, Cat No. 1876996; Roche UIBC reagent kit, Cat No. 815156; Hitachi Modular Processor
Liver Function: total protein, globulin, albumin, total bilirubin, Gamma glutamyl transferase (GGT), Alkaline phosphatase (ALP), Aspartate transaminase (AST), Alanine transaminase (ALT)	Older Adults only	Blood	4mL heparin vacutainer held at room temperature until analysed same day of sampling	LabPlus (ADHB)	Analysed on Hitachi Modular Processor; Roche ALP AMP reagent, Cat. No. 2173107; Roche ALT kit, Cat. No.1876805; Roche AST kit, Cat. No.1876848; Roche GGT reagent kit, Cat. No. 2016958; Roche Bil-T reagent kit Cat.No. 2144344/352; Roche Total Protein kit Cat.No. 1970909. Roche Alb Plus Reagent Cat.No.2000903/920
IGF-1	All Swimmers	Plasma	Plasma collected as above	Nutrition Lab (MUPN)	Coated Tube Radioimmunoassay, IGF-1-D-RIA-CT kit, Biosource, Europe, Nivelles, Belgium
Cortisol	All 3 subgroups	Plasma	Plasma collected as above	Nutrition Lab (MUPN)	Coated Tube Radio Immunoassay, Gamma Coat Cortisol I RIA Kit CA-1549, Dia Sorin, Minnesota

MUPN=Massey University, Palmerston North.
MUA=Massey University, Albany
ADHB=Auckland District Health Board

3.6 Investigation into the mechanism by which BC may modulate levels of salivary S-IgA

Additional tests were carried out on blood sampled from the subgroups in order to determine how BC may modulate levels of salivary S-IgA. This included plasma cortisol and cytokine analysis and measurement of various lymphocyte subsets.

Plasma cortisol

Plasma cortisol is affected by diurnal fluctuations [239]. To control for this, sampling time was standardised. To accommodate the swimmers training programmes, sampling occurred between 7.30am to 8.30 am after the morning swim training session. For the students sampling was between 9am to 12 pm to fit in with lecture schedules.

In the swimmers cohort the end of supplementation blood sample was taken pre training and not post-training (the two baseline samples and the sample collected after five weeks of the supplement were collected post-training). It was not possible to collect blood samples pre-training due the limited time before their morning training session. For the blood samples collected at the end of supplementation and post-supplementation, some of the swimmers were sampled later in the day (11am-3pm) as they could not attend the morning training session. As plasma cortisol levels are affected by exercise and are also subject to diurnal fluctuations, the plasma cortisol results from the end of supplementation and final blood samples were excluded from statistical analysis.

Statistical analysis of the plasma cortisol results was therefore undertaken on the baseline results compared to those samples collected after four weeks consumption of the supplement for all cohorts.

Plasma cytokine analysis

Plasma cytokine concentrations were determined using a two step ‘sandwich’ enzyme immunoassay. Capture antibody was coated onto a 96-well

immunoplate (Microlon 600, high affinity flat bottomed plates [Greiner 655061, Germany]) and incubated overnight. Plates were washed with phosphate buffered solution (PBS) (pH 7.2, 0.05% Tween20) and unbound sites were blocked with bovine serum albumin Gibco, 300063-572 (1% in PBS). Pre diluted standards and samples (all samples from the same participant were analysed on the same plate) were added to each well and incubated for two hours, followed by extensive washing and the addition of biotinylated detection anti-body for a further one hour. Streptavidin-conjugated horseradish-peroxidise enzyme (1/2000) was then added for a further thirty minutes before final washing and the addition of the chromogenic substrate 3,3',5,5'-tetramethylybenzidine (TMB). After acidification to stop the reaction the plates were read on a plate reader at 450nm (Fluostar Optima Multifunction Microplate reader-BMG Labtech).

Plasma levels of IL-6, IL-1ra and interferon-alpha (IFN-α) were expressed as pg cytokine/mL, by extrapolating ELISA optical density (OD) values from a standard curve generated using known quantities of serially diluted recombinant human IL-6, IL-1ra and IFN-α (see Table 15). ELISA ODs were obtained as the average of three replicate wells per sample for IL-6 and IL-1ra analysis. Due to the cost of the IFN-α kit two replicate wells were prepared for each sample. The intra-assay coefficient of variation was <10% for each cytokine.

Table 15: Recombinant standards used for the ELISA analysis of the plasma cytokines IL-6, IL-1ra and IFN-α (assay kit details, manufacturer, range of standards used and lower limit of assay)

Cytokine	Assay Kit	Manufacturer	Range of standards used (pg/mL)	Lower limit of assay
IL-6	DuoSet Dy206	RnD systems, Minneapolis	2, 4, 8, 32, 64, 128, 256	2
IL-1ra	DuoSet DY280	RnD systems, Minneapolis	7.5, 15, 30, 60, 120, 480, 960	15
IFN-α	41110-2 Five pre-coated microplates	RnD systems, Minneapolis	12.5, 25, 50, 100, 200, 500	25

Measurement of lymphocyte subsets

The mechanism by which a nutrition supplement such as BC may modulate levels of salivary S-IgA is not known. One possible way is that the homing ability of lymphocytes involved in antibody production at mucosal surfaces is affected. In rodent models it is known there is a reduced percentage of circulating lymphocytes expressing alpha integrins in aged animals [135]. It is not known if expression of alpha integrins on human lymphocytes cells is also affected by ageing or by other physiological stressors such as prolonged intensive exercise. The aim of measuring the expression of alpha integrins on lymphocytes in this study was to investigate whether ageing and the physiological stress of prolonged intensive exercise would have an effect, and whether there were differences in the BC group compared to the placebo group.

The following list of lymphocyte subsets were measured by LabPlus, Auckland hospital (LPHAEIDFPR). Levels were determined in swimmers, students and older adults at baseline and after supplementation with BC and compared to those consuming a placebo:

CD3 (T-cells)
CD3/CD49d (T-cells co-expressing the α4 integrin)
CD3/CD4 (T-helper cells)
CD3/CD4/CD49d (T-helper cells co-expressing the α4 integrin)
CD3/CD8 (T-cytotoxic cells)
CD3/CD8/CD49d (T-cytotoxic cells co-expressing the α4 integrin)
CD19 (B-cells)
CD19/49d (B-cells co-expressing the α4 integrin)

Leucocyte populations in peripheral blood were stained with various combinations of a monoclonal antibody matrix to allow gating of lymphocytes, T-cells and B-cells. The surface markers were interchanged to allow further phenotypic analysis of T-helper and T-cytotoxic cells.

Four fluorochromes were used, which allowed four different conjugated antibodies to be analysed simultaneously on all cell types. This enabled the

identification of the percentage of cells also expressing the homing integrin CD49d:

CD3 (fluorescein isothyocyanate [FITC]),
CD19 (allophycocyanin [APC])
CD45 (peridinin chlorophyll protein [PERCP])
CD3 (APC)
CD4 (FITC)
CD8 (FITC)
CD49d (phycoerythrin, PE)

FACS analysis was performed on a FACScalibur flow cytometer (Becton Dickson) and analysed using Delphic software.

Example of calculations applied to flow cytometric data

Lymphocytes were identified on the basis of their physical granularity determined by measuring the side scatter (SSC) and by positive staining for CD45. Among these lymphocyte-like cells, gating for those cells that were CD3-positive and co-expressing CD4 (T-helper cells) occurred and were expressed as a percentage of the total lymphocytes (36.04%). Of these cells, those that were positive for CD3/CD4 were further gated to find those cells that were also expressing CD49d, and these were expressed as a percentage of total lymphocytes (29.7%).

An example for the flow cytometric analysis of CD4/CD49d T-cells for one older adult is shown in Figure 9.

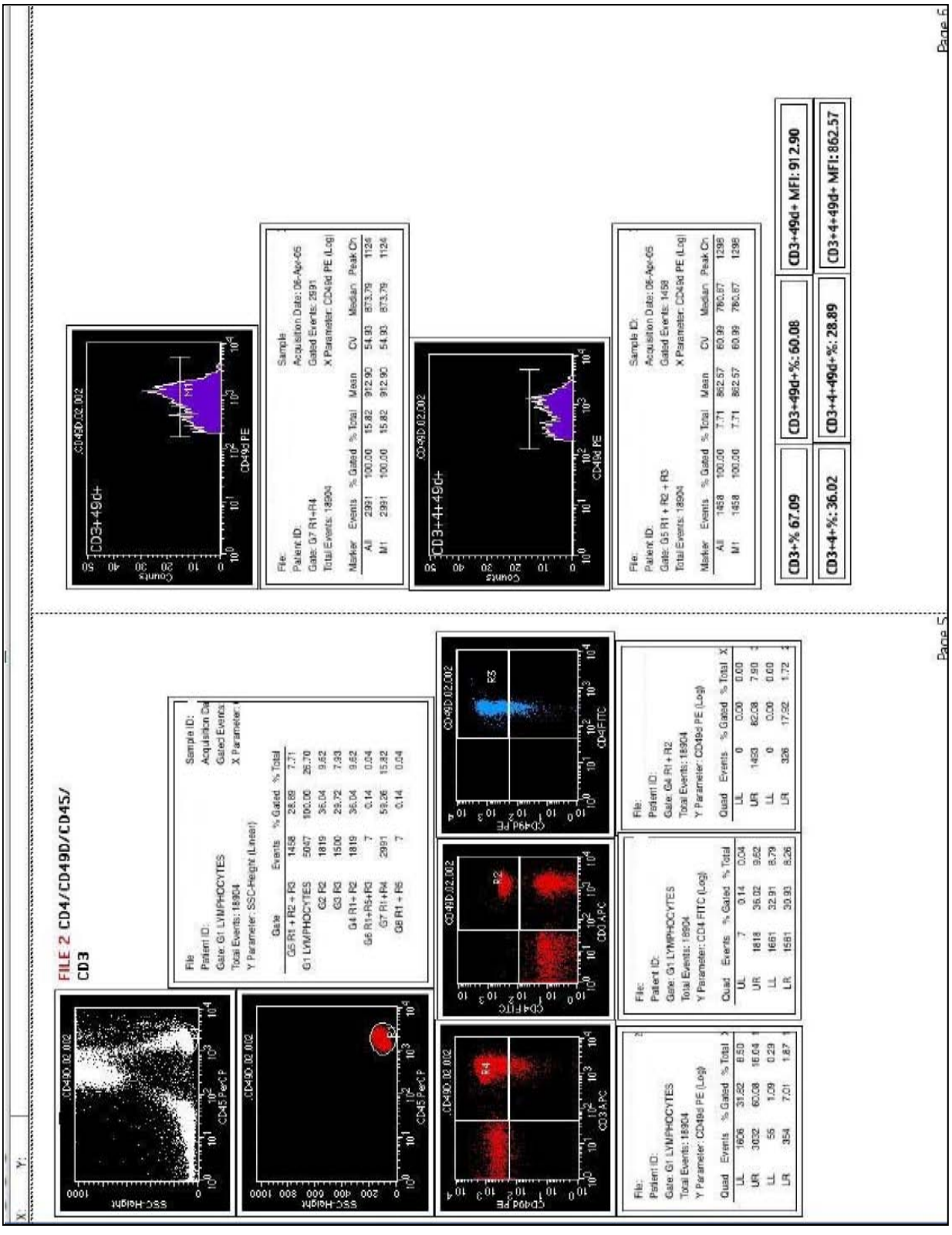


Figure 9: Example of flow cytometry analysis for T-cells expressing CD4 and CD49d in blood sampled from an older adult

MFI analysis

In the above example the mean fluorescent intensity (MFI) of those gated cells that were expressing CD3/CD4/CD49d was determined as 862.57 arbitrary units.

Absolute numbers of cells

Absolute numbers of cells expressing an antibody marker were determined by multiplying the relative percentage of the lymphocyte subset by the absolute number of lymphocytes in the same blood sample. Absolute numbers were supplied by LabPlus as part of the standard full blood count procedure (blood samples were analysed on a Sysmex Automated Haematology Analyser (ES-2100) (Sysmex Corporation, Japan). Values for absolute numbers for each subset were calculated in a spread sheet.

For the blood sample from this older adult, the absolute number of lymphocytes was measured as 1.75×10^9/L, the following calculations were applied:

- The calculated absolute number of CD3/CD4 cells=36.04/100 x (1.75×10^9/L)=6.3×10^8/L
- The calculated absolute number of CD3/CD4/CD49d cells=29.72/100 x (1.75×10^9/L)=5.1×10^8/L.

Comparison of expression of CD49d between cohorts

For comparative purposes between the cohorts, the percentage of CD3/CD4 cells that were also expressing CD49d was determined by dividing the absolute number of cells expressing CD3/CD4/CD49d by the absolute number of cells expressing CD3/CD4.

For this blood sample:

- (5.1×10^8/L) / (6.3×10^8/L)=81.0%

Thus for this older adult, 81.0% of the T-helper cells were also expressing CD49d.

This calculation was applied to each of the lymphocyte phenotypes and for each participant in the subgroups from studies 1 and 2. Comparisons were made between the cohorts for each of the lymphocyte subsets listed above.

3.7 Training/activity and wellness records

Daily wellness and time spent in training or being active were recorded in the appropriate space in the same diary. Participants were requested to fill out the diaries daily for fourteen weeks. This included the two weeks when saliva and blood was sampled at baseline and the two weeks post-supplementation. The diaries were similar in format for each cohort but were changed slightly to accommodate the higher level of training for the athletes and printed in larger font for the older adults.

3.7.1 Training/activity records

Study 1

The swimmers recorded the time spent (in minutes) in each training session as well as each session of additional physical activity (such as gymnasium workouts) and, rated the intensity from 1 to 3 (see sample diary in Appendix 10.5).

1=Mostly endurance
2=A mixture of speed and endurance
3=Mostly speed, high intensity.

The students recorded the time spent (in minutes) in each session of physical activity (such as gymnasium workouts, walking) and rated the intensity from 1 to 3 (see sample diary in Appendix 10.7).

1=Gentle
2=Moderate
3=Very strenuous

Study 2

The older adults recorded the time spent (in minutes) in each activity (such as walking, golf, fishing, gardening or gymnasium workouts) and rated the intensity from 1 to 3 (see sample diary in Appendix 10.12).

Conversion of training/activity to an activity factor (AF)

To control for the chronic and acute effect of repeated bouts of prolonged intensive exercise on several immune parameters including levels of salivary S-IgA [240] [94], each participant's daily activities were converted to a weekly mean activity factor (AF) as outlined below.

The daily activity recorded by each participant was converted to a daily AF by:

- Converting the time spent (in minutes per day) to a proportion (Pr), for example 30 minutes of activity=30minutes/1440minutes (1440minutes in 24hr)=0.02.
- Allocating a metabolic equivalent (one metabolic equivalent, 1MET, is defined for the average adult as the energy expenditure when sitting quietly=3.5mL O_2 kg^{-1} /min-1 or 4.184 kJ / kg^{-1}/ h^{-1}) to each activity for each participant using the multiples in the Compendium of Physical Activities published by Ainsworth *et al.,* 1993 [237].

The different types of activities that were reported by the participants in this study and the intensity ratings are listed in appendix 10.19. The application of the appropriate metabolic equivalent (MET) multiple (M) is listed alongside each activity.

The following steps were followed to calculate an average mean weekly AF for either the BC or placebo group -

- For each activity the proportion of time spent in the activity (Pr) was multiplied by the M to establish an AF i.e. AF=Pr x M
- For each participant a daily AF was determined by summing AF_{ai}…AF_{az} (see example below).
- A weekly AF was calculated by summing the daily AFs.
- A mean weekly AF was determined for each participant. This was used to apply a Physical Activity Level (PAL) for each participant to estimate daily energy expenditure (see Section 3.8). The mean weekly AF was

also used for comparative purposes to determine whether there were differences between the BC and placebo groups within each cohort.

EXAMPLE: To convert reported daily activity into an activity factor (AF):

Participant 1234 (Student)
From the self-recorded activity record:
Monday
Social netball played for 30 minutes/intensity level 2
Walked 60 minutes/intensity level 2

For the first activity recorded (social netball played for 30 minutes/intensity level 2):

Convert the time spent in the activity into a proportion

Pr=30minutes/1440minutes
Pr=0.02

Determine the MET multiple (M)

M for social netball/intensity level 2=7 (see Appendix 10.19)
M=7

Calculate the activity factor (AF_{a1}) for this activity

AF_{a1}=Pr x M
$AF_{a1=}$0.02 x 7
$AF_{a1=}$0.14

For the second activity recorded (walked for 60 minutes, intensity level 2):

Pr=60minutes/1440 minutes
Pr=0.04

Determine the MET multiple (M)

M for walking/intensity level 2=4 (see Appendix 10.19)
M=3

Calculate the activity factor (AF_{a2}) for this activity

AF_{a2}=Pr2 x M2
$AF_{a2=}$0.04 x 4
$AF_{a2=}$0.16

To calculate the daily activity factor (AF) for participant 1234

AF=AF_{a1} + AF_{a2}
AF=0.14 + 0.16
AF=0.30

3.7.2 Wellness records

Daily wellness record - swimmers

Daily records of health problems were self-recorded in the training and wellness diary (see Appendix 10.5). The format for the wellness diary was based on a log used for self-recording of symptoms of URTI by athletes, including nasal symptoms, fever, sore throat, coughing [241].

A category for other symptoms was included for symptoms such as gastro-intestinal problems. However, as very few participants completed this category these results were not included in the analysis of wellness symptoms.

The swimmers were requested to place a tick against the health problem they experienced that day and rate it for severity (see Figure 10). The procedure followed to rate severity of symptoms was based on that used in a study of URTI and changes in salivary S-IgA levels in tennis players [242].

Symptom	Tick if present	Severity 1	2	3
Nil				
Nasal symptoms				
Fever				
Sore throat				
Coughing				
Other				

1=Very mild, no change to daily training.
2=Moderate, did not train today.
3=Severe, confined to bed.

Figure 10: Wellness record completed daily by participant

Daily wellness record - students and older adults

Daily records of health problems were self-recorded in the respective activity and wellness diaries (see Appendices 10.7 and 10.12). The format in figure 10 was used and the health problem was rated for severity following the guideline below:

Severity

1=Very mild, no change to daily activities

2=Moderate, was not active

3= Severe, confined to bed

For all cohorts the total number of days of URS reportage was calculated for each participant. Participants were encouraged to identify and rate symptoms where possible in order to only record those associated with URTI. No other means were used to verify reportage of URS as true URTI. To help control for mistaken recognition of URS as URTI, URS days were only included in the calculation if recorded on two or more consecutive days.

3.8 Estimation of daily energy expenditure (EDEE)

To assess the validity and reliability of the dietary information collected EDEE was calculated. This calculation was performed by converting the average mean weekly AF for each participant to a PAL using categories developed by Warwick, 1989, (see Table 16). The PAL multiplied by the estimated BMR (using Harris-Benedict and Schofield equations) provided a crude estimate of EDEE.

Table 16: Conversion of mean weekly AF (wAF) to PAL for M and F (Warwick, 1989 [231])

Activity	AF	PAL (M)	PAL (F)
Sedentary	0.04-0.15	1.4 (1.3-1.5)	1.4 (1.3-1.5)
Light	0.16-0.30	1.5 (1.4-1.6)	1.5 (1.4-1.6)
Light – moderate	0.31-0.60	1.7 (1.6-1.8)	1.6 (1.5-1.7)
Moderate	0.61-0.90	1.8 (1.7-1.9)	1.7 (1.6-1.8)
Heavy	0.91-4.00	2.1 (1.9-2.3)	1.8 (1.7-1.9)
Athletes in normal training*	0.91-4.00	1.8-2.2	1.8-2.2
Very heavy	4.01-7.00	2.3 (2.0-2.6)	2.0 (1.8-2.2)
Elite female athletes**	N/A	N/A	2.0-2.8
Extreme athletic activities*	N/A	2.5-4.0	2.5-4.0

* Black, 2001 [223]
**Haggarty *et al*., 1987 [236]

Validation of dietary intake

EDEE was determined using BMR calculated by Harris-Benedict and Schofield equations and was compared to RDEI to identify over- or under-reporting of dietary intake for each participant. The difference between RDEI and EDEE was calculated using each equation. The two results were averaged for each participant and this was used to develop the correction factor for estimating the macro and micronutrient intake from the dietary assessment records. It was recognised there were limitations to estimating EDEE by prediction equations. Through using an average of EDEE derived from both equations it was hoped some of the inter-individual variation would be minimised.

3.9 Assessment of reported daily dietary intake (RDDI)

Study 1: Swimmers and Students

Diet records

Participants were asked to complete two sets of 4-day food diaries. Instructions on how to complete the diaries were given in writing and verbally by the investigator. The first set was kept in the first week of the supplementation period to determine reported dietary intake at baseline; the second was kept in the final week of the supplementation period. The food records were analysed using FoodWorks Professional Edition, Xyris Software, version 4.00 with the NZ Food Files 2000, and NZ Vitamin and Mineral Supplements 1999 (Crop and Food Research, Palmerston North, New Zealand). Nutrient intake was determined from foods and supplements listed in the database or for which nutrient information could be obtained. Substitutions were made where necessary as not all brands of food are listed in the database for example 'Uncle Toby's Apricot and Honey Porridge'. When an appropriate substitution could not be made a new recipe was entered using information from the nutrition label: for example 'Nutralife protein bar'.

Study 2: Older Adults

24-hour diet recall interviews

Participants were asked to participate in two diet recall interviews with trained interviewers to record dietary intake for the previous 24 hours. The first interview was in the first week of the supplementation period and the second interview was in the last week of the supplementation period. The diet recalls were analysed as set out above.

Contribution to micronutrient intake from dietary supplements

A summary of the contribution to micronutrient intake from dietary supplements is included in Appendix 10.35 for the students, Appendix 10.36 for the swimmers and Appendix 10.37 for the older adults.

Accuracy of interpretation of dietary data

Results for reported energy, macronutrient and micronutrient intake were used for comparative analysis between groups within cohorts. This data is indicative of intake for individuals as the dietary data was collected for insufficient days [223].

The information collected in the food diaries was checked for accuracy by ranking the results for each nutrient and investigating outliers. A random selection of ten out of 103 food diaries and eight out of 90 diet recalls were double-entered; the mean difference in nutrient analysis was found to be 2.7% for the food diaries and 1.7% for the diet recalls.

A separate log of foods that were not appearing in the database was kept and included how these foods were substituted to ensure a standard approach during interpretation and analysis of the food records.

The reported daily dietary intakes (RDDI) were averaged for each participant and analysed for statistical differences between the BC and placebo groups. In addition changes in the RDDI between week one and week ten of the supplementation were also determined and analysed for statistical differences.

One swimmer did not complete the first food diary due to being away at an international competition and another swimmer did not complete the second food diary due to personal commitments. The results for the dietary analysis for these two swimmers were not included in the comparison between intake at baseline and post-supplementation. The contribution to daily energy and macronutrient intake of the 50g/day of dietary supplement was calculated. This was added to both sets of records for each participant. No adjustment was made for the actual intake of the BC supplement as determined by the calculated compliance (see Section 3.2).

3.10 Anthropometric measurements

Anthropometric measurements were performed at baseline and at the end of the supplementation period. This gave an indication of health status [243] and identified whether there were differences between the BC and placebo groups. Reported dietary intake of carbohydrate and protein was analysed by calculating it as the quantity consumed per kg body mass per day (g/kg BM/day). Therefore it was necessary to determine any change in body mass at the end of the supplementation period compared to baseline, and whether there were differences between the BC and placebo groups.

It was possible that gains in body mass could occur as a result of the increased energy intake from the supplement (two 25g sachets added 774kJ/day to the total energy from dietary intake) particularly in the non-exercising cohorts (students and older adults). Participants in these cohorts were advised to take the supplement instead of a snack. The effect of small to modest gains in body mass on immune function is unknown.

Body mass and height for all participants, and abdominal measurements for participants in study 2, were performed to determine differences between the BC and placebo groups at baseline within each cohort. The body mass and height measurements were converted to a BMI for comparative purposes in order to investigate whether there were physical differences between the BC and placebo groups. Due to the large percentage of lean body mass in athletes compared to non-exercising participants it was not meaningful to compare BMI between cohorts.

Studies 1 and 2

Body mass was recorded twice during the study, within one week of the diet diaries/recalls being completed. Body mass was measured using Wedderburn digital scales (0.1kg) that were checked with a 3.225kg and 0.300kg calibrated weights after every third participant was measured. Body mass was measured in the morning and post-prandially before training on one of the saliva sampling days.

Height was measured once using a portable stadiometer (Massey University, New Zealand).

Study 2

Abdominal (waist) circumferences were measured to the nearest millimetre using a flexible steel tape (Lufkin tape) with the subject standing. The waist circumference was measured at three sites; ileac crest (WIC), umbilicus (WU) and midway between ileac crest and the lower rib (WST) [244]-

Each measurement was performed twice. If there was more than 0.5cm difference, a third measurement was made.

Waist measurements were completed in week one and week ten of the supplementation period.

3.11 Statistical analysis

Summary data were presented as mean±SEM; non-normal data were presented as median with an interquartile range. The level of significance was set at *P*=0.05.

3.11.1 Investigation of differences in participant characteristics

Descriptive data

Differences in descriptive data were investigated with two sample tests using SAS software (SAS Release 8.02, SAS Institute, Inc, Cary, USA). Normally distributed data with equal variances were tested with student's t-tests. Non-normal data with equal variances were transformed and if the data normalised then student t-tests were used. For data that did not normalise with transformation non-parametric testing was used.

Haematological parameters and leucocyte subsets

Differences in results for mean haematological parameters between the BC and placebo groups were investigated using student t-test on normally distributed data. The mean difference between base and post-supplementation results was also tested using student t-test on normally distributed data.

Baseline differences between cohorts

Differences between the cohorts for salivary and plasma parameters were investigated at baseline using analysis of variance. Where a significant difference was found, post-hoc analysis was performed using the multiple comparison procedures (Tukey's honest significant difference [Tukey] and Duncan's New Mulitple Range test [Duncan]) to identify which cohorts were different to each other using SAS software. This procedure involves testing all possible pairs of cohort results. The cohort means were ranked from highest to lowest (A to C, A was the highest rank). Cohorts that had the same letter were not significantly different to each other. If the Tukey (being more conservative) multiple ranking procedure gave a different ranking to the Duncan procedure, the result for the Tukey procedure was used due to the very small sample size.

3.11.2 Effect of BC supplement on the primary outcome variables, and on plasma immunoglobulins and plasma biochemical parameters

Two-way repeat measures analysis of variance was used to identify the effects of BC, gender and time on salivary S-IgA, and on other salivary and plasma, immune and biochemical parameters using SAS software. The main effects are defined as:

Group=the effect of being in either the BC or placebo group, on the variable being investigated.

If there was a significant effect, and there was also a significant main effect of time, then it was assumed the variable could have been affected by consuming the BC supplement.

If there was a significant *Group* effect, *but the main effect of time was not significant*, then it was assumed there was no effect of consuming the BC supplement on the variable.

Gender=as both males and females were included in the cohorts the effect of gender was investigated.

Time=the effect of time on changes to the variable was investigated. That is, it was determined whether the variable changed at certain time points compared to baseline.

*Interaction of Group*Time*=if the variable did change with time, it was also determined whether there was an interaction of being in either the BC or placebo group. If the interaction was significant then the assumption was made that the variable changed over time and this was due to the consumption of either BC or placebo.

Post-hoc analysis using a multiple comparison procedure would have been used if there were significant differences between the BC and placebo groups that were time-related. As there were no significant differences in the primary outcome variables due to taking the BC supplement that were time-related, post-hoc analysis was not necessary.

Differences in URS reportage between BC and placebo groups

Differences in reportage of mean total URS days between the BC and placebo groups were investigated in numerical data by student t-test if the data were normal using Minitab software (Minitab Release 14, Statistical Software, Minitab Inc, US). If the data would not normalise then the non-parametric Moods median test was used.

Differences in reportage of mean total mild URS (URS1) days between the BC and placebo groups within the swimmers cohort were investigated in numerical data by student t-test.

Differences in the number of swimmers reporting URS was carried out in categorical data by chi-square analysis using Minitab software. The supplementation period was divided into two blocks (weeks one to four and weeks five to ten). This was because during the first four weeks of supplementation the researcher met with the swimmers twice per week. During the next six weeks of the supplementation period there were only three meetings. The regular meetings at baseline and during the first four weeks may have been sufficient to remind the swimmers to record any URS they were experiencing. More thorough record keeping occurred during this period by most participants.

3.11.3 Investigation of the mechanism by which BC may modulate mucosal immunity (analysis of cytokines, hormones and lymphocyte subsets in the subgroups)

Two-way repeat measures analysis of variance was used to identify the main effects of BC, gender and time on secondary variables using SAS software.

Study 1: Associations between immune and hormone parameters in the swimmers subgroup

Associations between immune and hormone parameters in the subgroup from the swimmers cohort were investigated using regression analysis with Minitab software.

Study 2: Effect of the influenza vaccination on URS reportage in the older adult subgroup

Because a large number of the older adults had an influenza vaccination during the fourth and sixth week of the supplementation, the possibility of a confounding effect on reportage of URS was investigated. Participants who reported URS in weeks seven to ten of the supplementation were categorised into those who did and did not have an influenza vaccination. Differences in the categorised data were investigated using Fishers exact test.

3.11.4 Control for dietary intake

Dietary assessment was performed twice with each cohort to identify any possible differences in intake between BC and placebo groups that may affect levels of salivary S-IgA. Results from the two reported daily dietary intakes (RDDIs) were averaged and analysed for statistical differences between the BC and placebo groups using student t-test if the data were normal.

In addition, changes in the RDDI between week one and week ten of the supplementation were investigated by determining the difference between base and post-supplementation results within each cohort and using student t-test if the data were normal. If the data would not normalise with transformation then a non-parametric test was used.

4.0 Results

The main aim of this study was to investigate the effects of BC supplementation on levels of S-IgA in a group of elite athletes, age-matched non-exercising control group, students (study 1) and older adults (study 2).

4.1 Characteristics of the participants

In Study 1, a total of 25 swimmers completed the study requirements (Pl=13, BC=12) except for the two swimmers who completed one food diary instead of two. In the age-matched control group a total of 28 students completed the study requirements (Pl=12, BC=16).

Participant characteristics are presented in Table 17. There were no significant differences at baseline for age and height (cm) between the BC and placebo groups within each cohort (see Table 17). There were also no significant differences in the mean body mass, BMI, weekly AF, grams of drink consumed per day (drink g/d) and changes in body mass between the BC and placebo groups within each cohort (see Table 17).

In Study 2, a total of 45 older adults completed the study requirements (Pl=23, BC=22).

There were no significant differences in age and height between the BC and placebo groups at baseline. There were also no significant differences in mean body mass, BMI, waist measurements weekly AF, mean drink g/day, and changes in body mass (see Table 17).

Table 17: Descriptive Statistics. Mean and standard deviation (SD) are reported for participants in BC or placebo (Pl) group within each cohort. Differences within cohort were determined by student t-test if the distribution was normal. If the data could not be corrected by transformation, non-parametric testing was used and median (Med) and inter quartile ranges (IQ range) are reported

	Students				Swimmers				Older adults			
Descriptor	BC (n=16)		Pl (n=12)		BC (n=12)		Pl (n=13)		BC (n=22)		Pl (n=23)	
Age (years)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
F	23.9	2.2	21.4	1.2	19.5	2.9	17.8	1.6	70.9	3.9	71.5	3.6
M	20.8	2.2	22.8	2.2	17.3	1.7	19.3	2.9	69.9	4.1	72.3	1.8
P	*0.205*				*0.956*				*0.188*			
wAF	Med	IQ range	Med	IQ range	Mean	SD	Mean	SD	Mean	SD	Mean	SD
F	0.46	0.33-0.83	0.63	0.44-0.76	3.31	0.75	2.96	0.91	0.87	0.32	0.79	0.61
M	0.28	0.16-0.59	0.33	0.11-0.38	3.09	1.76	3.38	1.19	1.05	0.71	2.23	2.19
P	*0.966*[a]				*0.969*				*0.685*			
Drink (g/d)	Med	IQ range	Med	IQ range	Mean	SD	Mean	SD	Med	IQ range	Med	IQ range
F	47.0	43.0-47.0	44.5	43.3-45.8	37.50	7.72	43.29	5.45	49.0	46.0-50.0	49.0	48.0-50.0
M	40.50	30.5-43.8	42.0	36.3-44.8	39.38	5.08	37.95	13.95	47.0	47.0-50.0	49.5	47.0-50.0
P	*0.622*[b]				*0.622*				*0.180*[c]			

wAF=weekly activity factor
g/d=grams per day
F=Female
M=Male
P=Significance level
IQ range=Interquartile range
[a]=Not normal, right-skewed variances equal. Differences between log transformed data were investigated using t-test
[b]=Not normal, left-skewed, variances equal. Differences between squared data were investigated using t-test
[c]=Not normal, left-skewed, variances unequal. Differences in data were investigated using Moods median test

Table 17 continued: Descriptive statistics. Mean and SD are reported for participants in BC or Pl group within each cohort. Differences within cohort were determined by student t-test if the distribution was normal. If the data could not be corrected by transformation, non-parametric testing was used and Med and IQ range are reported

	Students				Swimmers				Older adults			
	BC (n=16)		Pl (n=12)		BC (n=12)		Pl (n=13)		BC (n=22)		Pl(n=23)	
BM (kg)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
F	66.1	10.1	62.1	6.6	64.4	7.2	72.7	15.5	69.7	10.2	65.5	12.6
M	69.5	7.5	76.5	8.3	84.2	14.3	83.8	12.4	85.6	12.9	83.5	11.2
P	*0.932*				*0.136*				*0.407*			
H (cm)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
F	167.1	6.4	165.4	2.8	169.8	6.4	174.5	7.1	166.4	8.4	159.7	8.5
M	178.0	10.3	173.3	7.1	184.4	8.1	186.6	8.2	172.2	10.2	168.9	9.7
P	*0.422*				*0.082*				*0.084*			
BMI	Mean	SD	Mean	SD	Med	IQ range	Med	IQ range	Mean	SD	Mean	SD
F	23.2	2.2	23.2	2.7	22.0	21.0-23.5	21.9	20.9-27.6	25.2	2.9	25.8	5.1
M	21.9	1.1	25.5	2.4	23.6	21.0-26.6	24.5	21.9-25.9	28.7	3.5	29.4	4.3
P	*0.823*				*0.239*[c]				*0.596*			
Wst (cm)	NM	NM	NM	NM	NM	NM	NM	NM	Mean	SD	Mean	SD
F									83.76	9.94	92.57	12.62
M									101.18	12.74	98.28	8.73
P									*0.408*			

BM=Body mass
H=Height
Wst =Waist measurement. Midway between ileac crest and lower costal
F=Female
M=Male
P=Significance level
NM=Not measured
[a] =Not normal, right-skewed variances equal. Differences between log transformed data were investigated using t-test
[b] =Not normal, left-skewed, variances equal. Differences between squared data were investigated using t-test
[c] =Not normal, left-skewed, variances unequal. Differences in data were investigated using Moods median test

4.1.1 Haematological parameters

Study 1:

Swimmers cohort

There were no significant differences in the mean values (baseline plus post-supplementation) between the BC and placebo groups within the swimmers cohort for the following haematological parameters: ferritin (*P*=0.827), haemoglobin (*P*=0.312), red blood cell count (*P*=0.248), and mean cell volume (*P*=0.735) (see Appendix 10.21).

Student cohort

There were no significant differences in the mean values (baseline plus post-supplementation) between the BC and placebo groups within the swimmers cohort for the following haematological parameters: ferritin (*P*=0.869), haemoglobin (*P*=0.227), red blood cell count (*P*=0.227), and mean cell volume (*P*=0.303) (see Appendix 10.21).

Study 2:

Older adult cohort

In study 2 there were no significant differences in the mean values (baseline plus post-supplementation) for the following haematological parameters: ferritin (*P*=0.140), and mean cell volume (*P*=0.995).

There were significant differences for red blood cell count (*P*=0.026) and haemoglobin (*P*=0.027). Post-supplementation results compared to baseline increased in the BC group (but not the placebo group). The increases, although small, were significant *(all increases were still within typical physiological levels)* (see Appendix 10.21).

Plasma ferritin

While there were no significant differences in median ferritin levels for the participants in study 2 a few participants had ferritin results outside the typical range.

Ferritin results for two females were above the recommended upper value for women aged 65 to 75 years (upper value=380 µg/L) at baseline (range 553-967 µg/L). Results were still above the upper value post-supplementation (560, 1,009 µg/L).

Ferritin results for three males were also above the recommended upper value for men aged 65 to 75 years (upper value=450 µg/L) at baseline (range 498-843 µg/L). Results for two of the three were still above the upper value post-supplementation (676, 721 µg/L).

Liver function

In order to identify differences between BC and placebo groups for age-related decline of metabolic function, liver function analyses were chosen as an indicator of age-affected changes in metabolism [245]. There were no significant differences in mean plasma albumin (*P*=0.998) (see Appendix 10.24), gamma glutamyl transpeptidase (*P*=0.407), alkaline phosphatase (*P*=0.322), aspartate transaminase (*P*=0.288) and alanine transaminase (*P*=0.215) (see Appendix 10.21).

4.2 Main outcome variable

4.2.1 Salivary immunoglobulins

Salivary S-IgA

Swimmers cohort

Mean baseline levels for salivary S-IgA (see Table 18) were within the reference range 10-105 mg/L for non-fasting adults (see Table 19).

The reference range for 16 to 20 year olds is 20-80mg/L (see Table 19). Fourteen swimmers were aged 20 years or younger. Mean baseline levels of salivary S-IgA for the fourteen (see Table 18) were above the reference range for 16 to 20 year olds (8BC, 116.8±24.3, 6placebo, 97.7±10.4, mean±SEM) were above 20 mg/L.

There were no significant main effects of the supplement (*P*=0.914), gender (*P*=0.259), or of the time from baseline to the end of the supplementation (*P*=0.609) on levels of salivary S-IgA within the swimmers cohort.

Table 18: Baseline levels for salivary S-IgA, IgG and IgM for each cohort

		S-IgA mg/L	IgG mg/L	IgM mg/L
All swimmers (n=25)	BC	97.76 ± 10.51	18.92 ± 2.17	12.74 ± 1.78
	Placebo	96.65 ± 7.17	21.38 ± 4.43	9.89 ± 0.74
All students (n=28)	BC	82.7 ± 17.7	8.70 ± 0.85	9.97 ± 0.51
	Placebo	114.2 ± 11.6	16.22 ± 4.02	9.12 ± 0.39
Older adults (n=45)	BC	152.46 ± 14.37	28.66 ± 4.33	7.62 ± 1.33
	Placebo	123.35 ± 25.95	22.51 ± 4.26	8.94 ± 2.84

Table 19: Reference ranges for salivary immunoglobulins and albumin (taken from the Hunter Area Pathology Service Hand-Book [246])

	IgA mg/L	IgG mg/L	IgM mg/L	Albumin (mg/L)
16-20 years	20-80	0-20	0-10	10-110
Adult fasting	20-500	0-95	0-15	20-370
Adult non-fasting	10-105	0-20	0-10	10-110

Student cohort

Mean baseline levels for salivary S-IgA (see Table 18) were within the reference range for non-fasting adults (see Table 19).

Five students were aged 20years or younger. Results for baseline salivary S-IgA levels for all five students (BC n=3, 82.22±5.19, placebo n=2, 89.82±5.37 [mean±SEM]) were within the reference range (see Table 19).

There were no significant main effects of the supplement (*P*=0.154) or gender (*P*=0.601) on levels of S-IgA over the supplementation period within the students cohort. There was a significant time-related difference (*P*=0.050) which indicated salivary S-IgA levels changed with time irrespective of whether a BC supplement was consumed.

Older adult cohort

Mean baseline levels for salivary S-IgA (see Table 18) were higher than the reference range for non-fasting adults (see Table 19).

There were no significant main effects of the supplement (*P*=0.249) or gender (*P*=0.376) on levels of S-IgA over the supplementation period within the older adults cohort. There was a significant time-related difference (*P*=0.004) which indicated salivary S-IgA levels changed with time irrespective of whether a BC supplement was consumed (see Appendix 10.22).

Mean and SEM for salivary S-IgA levels, at baseline, week four, week eight, week ten and post-supplementation, are shown for the swimmers in Figure 11, for the students in Figure 12, and for the older adults in Figure 13.

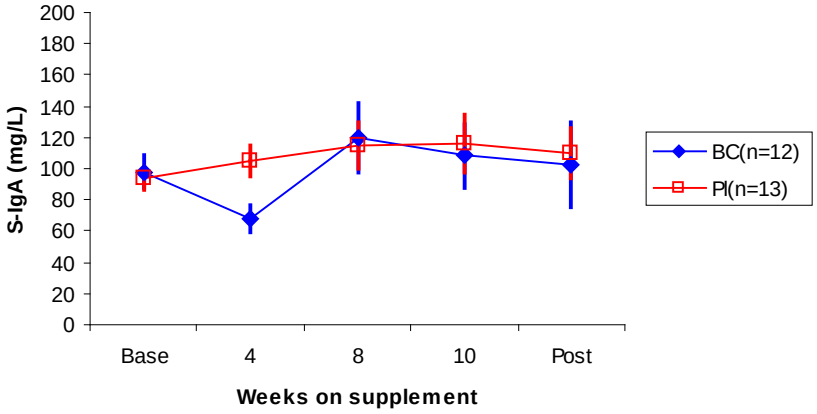


Figure 11: Salivary S-IgA levels (mg/L) in the swimmers cohort at baseline (Base), 4 weeks, 8 weeks, 10 weeks of supplementation and post-supplementation (Post), (mean and SEM). Group *P*=0.914, Gender *P*=0.259, Time *P*=0.609

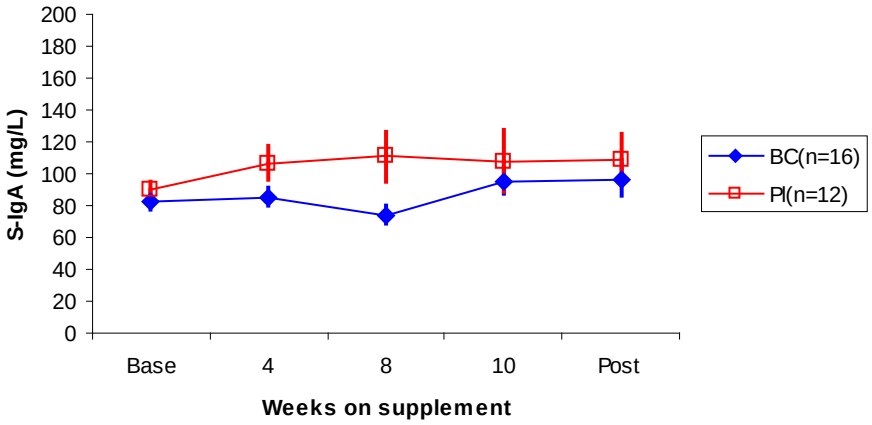


Figure 12: Salivary S-IgA levels (mg/L) in the student cohort at baseline (Base), 4 weeks, 8 weeks, 10 weeks of supplementation and post-supplementation (Post), (mean and SEM). Group *P*=0.154, Gender *P*=0.601, Time *P*=0.050

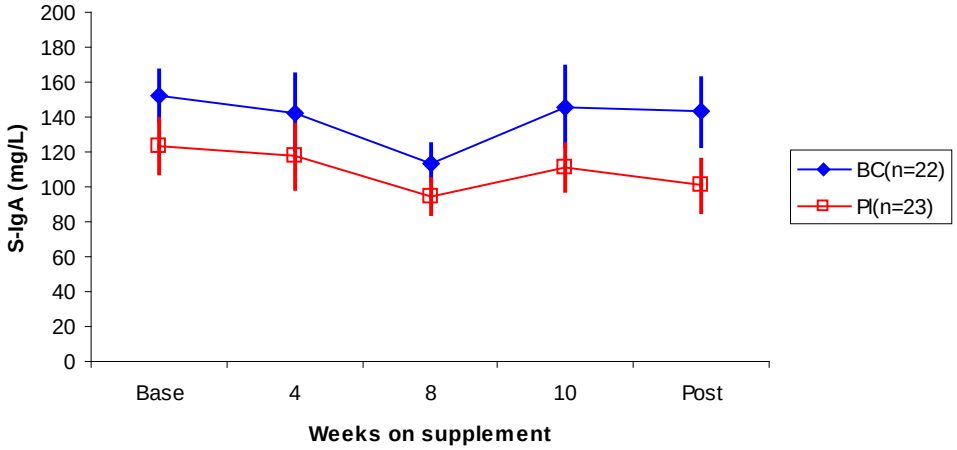


Figure 13: Salivary S-IgA levels (mg/L) in the older adult cohort at baseline (Base), 4 weeks, 8 weeks, 10 weeks of supplementation and post-supplementation (Post), (mean and SEM). Group *P*=0.249, Gender *P*=0.376, Time *P*=0.004

Salivary IgG

Swimmers cohort (BC n=13, placebo n=12)

Students cohort (BC n=16, placebo n=12)

Older adults cohort (BC n=22, placebo n=23)

Swimmers cohort

Mean baseline levels for salivary IgG for the BC group (18.92±2.17) (mean±SEM) (see Table 18) were within the reference range for non-fasting adults (0-20mg/L) (see Table 19), and above the upper range for the placebo group (21.38±4.43) (mean±SEM). The difference between the groups was not significant (*P*=0.410).

Student cohort

Mean baseline levels for salivary IgG (BC=8.70±0.85, placebo=16.22±4.02 [mean±SEM]) (see Table 18) were within the reference range for non-fasting adults (0-20mg/L) (see Table 19).

Older adult cohort

Mean baseline levels for salivary IgG (BC=28.66±4.33, placebo=22.51±4.23 [mean±SEM]) (see Table 18) were above the reference range for non-fasting adults (0-20mg/L) (see Table 19).

There were no significant main effects of the supplement (*P*=0.966, 0.262, 0.642), gender (*P*=0.191, 0.439, 0.520) or of time from baseline to the end of supplementation (*P*=0.189, 0.611, 0.168) on levels of salivary IgG within the swimmer, student or older adult cohorts respectively (*P*-values respectively) (see Appendix 10.22).

Salivary IgM

Swimmers cohort (BC n=13, placebo n=12)

Students cohort (BC n=16, placebo n=12)

Older adults cohort (BC n=22, placebo n=23)

Swimmers cohort

Mean baseline levels for salivary IgM for the BC group (12.74±1.78) (see Table 18) were above the reference range for non fasting adults (0-10mg/L) (see Table 19) but within the range for the placebo group (9.89±0.74) (mean±SEM). The difference between the groups was not significant (*P*=0.845).

Student cohort

Mean baseline levels for salivary IgM (BC=9.97±0.51, placebo=9.12±0.39) (mean±SEM) (see Table 18) were within the reference range for non-fasting adults (0-10mg/L) (see Table 19).

Older adult cohort

Mean baseline levels for salivary IgM (BC=7.62±1.33, placebo=8.94±2.84 (mean±SEM) (see Table 18) were within the reference range for non fasting adults (0-10mg/L) (see Table 19).

There were no significant main effects of the supplement (*P*=0.557, 0.890, 0.846), gender (*P*=0.797, 0.971, 0.524) or of time from baseline to the end of supplementation (*P*=0.121, 0.211, 0.524) on levels of salivary IgM within the swimmer, student or older adult cohorts respectively (*P*-values respectively) (see Appendix 10.22).

4.2.2 Measurement of hydration status

Dehydration can elevate levels of S-IgA in saliva. An indication of hydration status was determined by measuring levels of albumin and osmolality in saliva.

Swimmers cohort (BC n=13, placebo n=12)
Students cohort (BC n=16, placebo n=12)
Older adults cohort (BC n=22, placebo n=23)

Saliva osmolality

There were no significant main effects of the supplement (*P*=0.646, 0.446, 0.132), gender (*P*=0.932, 0.779, 0.101) or of time from baseline to the end of supplementation (*P*=0.535, 0.435, 0.407) on the osmolality of saliva within the swimmer, student, and older adult cohorts respectively (*P*-values respectively) (see Appendix 10.22).

Salivary albumin

There were also no significant main effects of the supplement (*P*=0.698, 0.213, 0.407), gender (*P*=0.285, 0.896, 0.908) or of time from baseline to the end of supplementation (*P*=0.293, 0.863, 0.396) on levels of salivary albumin within the swimmer, student, and older adult cohorts respectively (*P*-values respectively) (see Appendix 10.22).

To accommodate two swimmers who could not change their routine, consumption of a sport drink 30 minutes prior to sampling was allowed. It was of concern that the electrolyte content of the sport drink may have affected the osmolality of their saliva; there were no significant group or time-related differences in mean osmolality levels between the BC and placebo groups.

S-IgA/osmolality ratio

The salivary S-IgA/osmolality ratio may be used to assess the effect of hydration status on levels of salivary S-IgA. There were no significant changes in the salivary S-IgA/osmolality ratio from baseline to the end of supplementation between the BC and placebo groups within any of the cohorts: swimmers (*P*=0.670), students (*P*=0.838) and older adults (*P*=0.647). The results for baseline and end of supplementation S-IgA/osmolality ratio are presented in Table 20.

Table 20: Summary of the results for the salivary S-IgA/osmolality ratio at baseline and at the end of the supplementation for each cohort (normally distributed data is reported as mean±SEM), significance is for differences between groups within a cohort

		Base	End of supplementation	*P*
Swimmers	BC (n=12)	1.445±0.124	1.155±0.170	*0.670*
	Placebo (n=13)	1.693±0.318	1.343±0.220	
Students	BC (n=16)	1.311±0.150	1.426±0.092	*0.838*
	Placebo (n=12)	1.352±0.220	1.479±0.230	
Older Adults	BC (n=22)	*1.196 (0.534)	1.301±0.144	*0.647*
	Placebo (n=23)	*1.040 (0.667)	1.172±0.094	

*Not normally distributed. Reported as median and inter quartile range Q3-Q1

4.3 Secondary outcome variables

4.3.1 Plasma immunoglobulins, albumin, C-reactive protein

Plasma IgA

Swimmers cohort (BC n=12, placebo n=12)

Older adults cohort (BC n=22, placebo n=23)

There were no significant main effects of the supplement (*P*=0.430, 0.124), gender (*P*=0.076, 0.265) or of time from baseline to the end of supplementation (*P*=0.777, 0.150) on plasma IgA within the swimmers or older adult cohorts respectively (*P*-values respectively) (see Appendix 10.24).

Students cohort (BC n=16, placebo n=11)

There were significant differences in plasma IgA levels between the BC and placebo groups within the student cohort (*P*=0.012) (see Table 21). Participants in the BC group had significantly lower time-independent levels of plasma IgA compared to those in the placebo group. Mean plasma IgA levels for the student BC group ranged from 1.36 to 1.75 g/L. At all time-points mean results were within typical physiological levels (0.8 to 4.0 g/L [247]).

There were no significant main effects of gender (*P*=0.955) or of time from baseline to the end of supplementation (*P*=0.601) on plasma IgA levels (see Appendix 10.24).

Plasma IgG

Swimmers cohort (BC n=12, placebo n=12)

There were no significant main effects of the supplement (*P*=0.904), gender (*P*=0.105) or of time from baseline to the end of supplementation (*P*=0.645) on plasma IgG levels within the swimmers cohort (see Appendix 10.24).

Students cohort (BC n=16, placebo n=11)

There were significant differences in plasma IgG levels between the BC and placebo groups, within the student cohort (*P*=0.045). Participants in the BC group had significantly lower time-independent levels of plasma IgG compared to those in the placebo group (see Table 21). Mean plasma IgG levels for the student BC group ranged from 7.68 to 7.92 g/L. At all time-points mean results were within typical physiological levels for adults < 70 years (7.0 to 16.0 g/L [247]). There were no significant main effects of gender (*P*=0.319) or of the time from baseline to the end of supplementation (*P*=0.973) on plasma IgG levels (see Appendix 10.24).

Table 21: Students mean plasma IgA and IgG results (g/L), older adults mean plasma IgA (g/L) results at baseline, after 5 and 10 weeks supplementation and post-supplementation (mean±SEM). *P*=significant differences between BC and placebo (Pl) within each cohort.

	Students				Older adults	
	IgA		IgG		IgG	
	BC (n=16)	Pl (n=11)	BC (n=16)	Pl (n=11)	BC (n=22)	Pl (n=23)
Baseline	1.75±0.21	2.52±0.27	7.68±0.29	8.47±0.96	8.04±0.28	9.57±0.44
5 weeks	1.59±0.20	2.61± 0.33	7.72±0.30	9.38±1.01	7.94±0.35	10.00±0.69
10 weeks	1.39±0.12	2.53±0.30	7.92±0.28	9.55±1.12	8.20±0.38	10.02±0.71
Post	1.36±0.11	2.48±0.34	7.76±0.36	9.50±1.16	8.15±0.45	10.11±0.71
P (Group)	*0.012*		*0.045*		*0.024*	

Older adults cohort (BC n=22, placebo n=23)

There were significant differences in plasma IgG levels between the BC and placebo groups, within the older adults cohort (*P*=0.024). Participants in the BC group had significantly lower time-independent levels of plasma IgG compared to those in the placebo group (see Table 21). Mean plasma IgG levels for the older adults BC group ranged from 7.94 to 8.20 g/L. At all time points mean results were within typical physiological levels (adults < 70 years = 7.0 to 16.0 g/L and >70 years = 6.0 to 15.0 g/L [247]). There were no significant main effects of gender (*P*=0.670) or of the time from baseline to the end of supplementation (*P*=0.205) on plasma IgG levels (see Appendix 10.24).

Plasma IgM

There were no significant main effects of the supplement (*P*=0.319, 0.347, 0.602) or gender (*P*=0.200, 0.611, 0.837) on plasma IgM levels throughout the trial within the swimmers, students and older adult cohorts (*P*-values respectively). There was also no significant main effect of time (*P*=0.183, 0.816) in the swimmer and student cohorts (*P*-values respectively) (see Appendix 10.24). .

There was a significant effect of time (*P*=0.003) in the older adult cohort. This result indicated that plasma IgM levels changed irrespective of whether a supplement was consumed. Plasma IgM levels increased over the trial period for both the BC and placebo groups (see Appendix 10.24).

Plasma albumin

There were no main effects of the supplement (*P*=0.606, 0.746, 0.998) or of time from baseline to the end of supplementation (*P*=0.338, 0.325, 0.659) on plasma albumin levels within the swimmers, students and older adults cohorts (*P*-values respectively) (see Appendix 10.24).

In both the swimmers and students cohorts the results showed there was an effect of gender on plasma albumin levels at all time-points, *P*=0.037 and *P*=0.003 (*P*-values respectively). Females in both cohorts had lower plasma albumin levels compared to the males. However results for all participants were within the typical physiological range of 35-45 g/L [247]. As plasma levels of albumin can be affected by hydration status it seems likely, that despite the differences between the males and females, the participants were in a hydrated state when they provided a blood sample.

There was no significant main effect of gender (*P*=0.436) in the older adult cohort.

C-reactive protein

There were no significant main effects of the supplement (*P*=0.345, 0.832, 0.306), gender (*P*=0.083, 0.294, 0.839) or of time from baseline to the end of

supplementation (*P*=0.371, 0.839, 0.695) on plasma levels of C-reactive protein within the swimmers, students or older adults cohorts (*P*-values respectively).

4.3.2 URS reportage

The participants in both studies were asked to record URS in order to control for a possible confounding effect on levels of salivary S-IgA. In a previous study with swimmers levels of salivary S-IgA were found to increase prior to the first reportage of URS and remained elevated after URS ceased to be reported [48].

Study 1: Swimmers

URS reportage by the swimmers

URS was reported by both BC and placebo swimmers during the two-week period at baseline. There was no significant difference in the reportage between the groups (*P*=0.429) (see Figure 14 and Table 22).

Over the supplementation period the BC group tended to report fewer days of URS compared to the placebo group but this difference was not significant (*P*=0.080) (see Figure 14 and Table 22).

Table 22: Summary data for total URS days reported at baseline and over the supplementation period within each cohort. Data from study 1 (swimmers and students) reported as mean±SEM. Data from study 2 (older adults) reported as median and interquartile range (Q3-Q1)

		BC	Pl	*P*
Swimmers				
(BC n=12)	Base	1.58±0.72	2.83±1.10	*0.429*
(Pl n=13)	During supplementation	5.58±2.31	14.08±3.94	*0.080*
Students				
(BC n=16)	Base	3.19+1.10	1.08+0.51	*0.087*
(Pl n=12)	During supplementation	[a]10.60+2.88	[a]5.00+1.56	*0.198*
Older adults				
(BC n=22)	Base	[b]0 (1.25)	[b]0 (0)	*0.260*
(BC n=23)	During supplementation	[b]1.5 (14.3)	[b]0 (7.0)	*0.537*

[a]=Not normal, right-skewed. Significance from t-test on log-transformed data. Mean and SEM are on back-transformed data

[b]=Not normal and could not be normalised with transformation. Significance was by non-parametric testing, Moods Median test

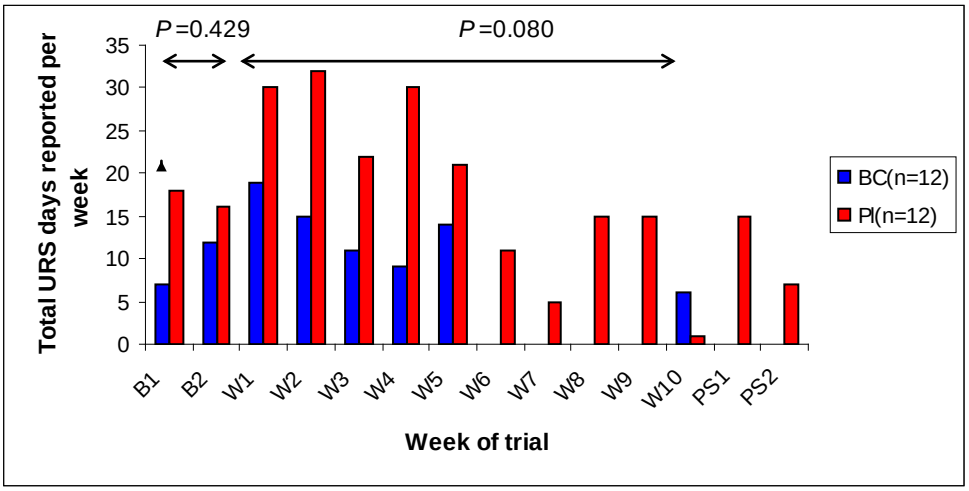


Figure 14: Total days of URS reported by the BC and placebo participants in the swimmers cohort in each week. B=Baseline, W=Week of supplementation, PS=Post supplementation

Numbers of swimmers reporting URS

Differences in the numbers of swimmers reporting and not reporting URS were investigated by categorising the results. Categorical analysis at baseline was not possible as there was insufficient data to test.

There were no significant differences in reportage of URS in weeks one to four (*P*=0.561). There was a trend (*P*=0.062) towards a difference during weeks five to ten between the BC and placebo groups (see Figure 15) with more participants consuming the placebo reported URS compared to those consuming BC.

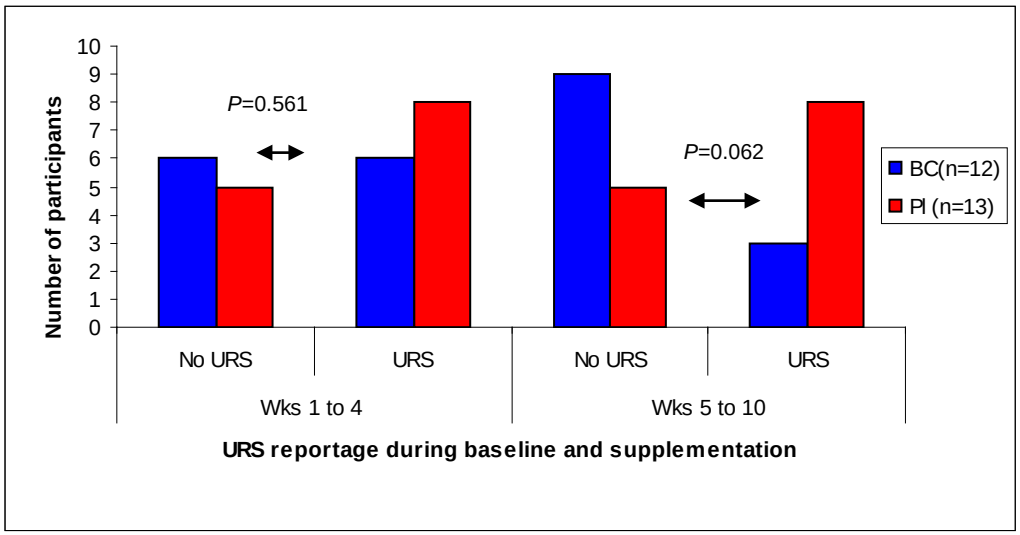


Figure 15: Number of swimmers reporting and not reporting URS. Significance was determined using chi square analysis. Wks=weeks

Reportage of total days of mild URS symptoms (URS1)

The reportage of mild symptoms (rated as severity 1 by the participants) were analysed for differences between the BC and placebo groups. The BC group tended to report less days of mild symptoms compared to the placebo group although this difference was not significant (*P*=0.075) (see Figure 16).

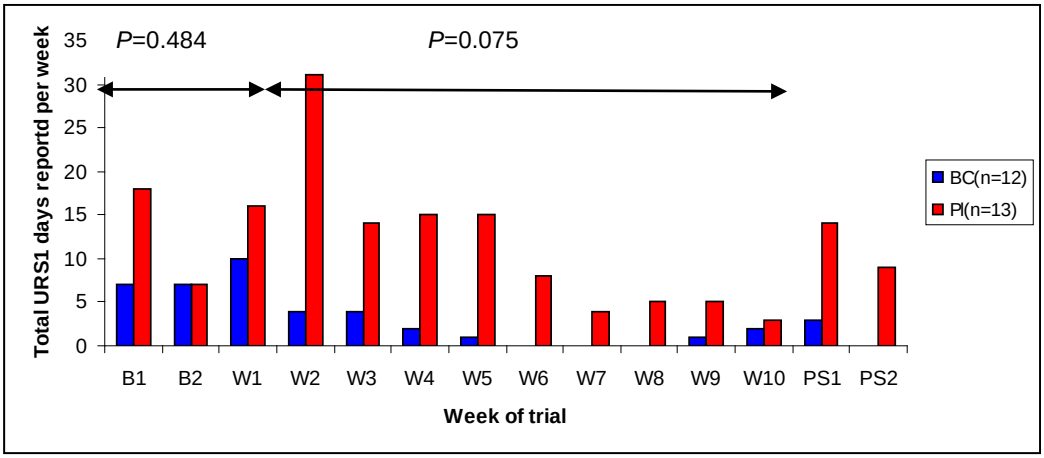


Figure 16: Total days of mild URS (URS1) reported by the BC and placebo participants in the swimmers cohort. B=Baseline, W=Week of supplementation, PS=Post supplementation. Significance was determined using student t-test on logged data

Study 1: Students

URS reportage by the students

More URS was reported by the BC compared to the placebo group at baseline (see Table 22), but this difference was not significant (*P*=0.087).

URS reportage over the supplementation period was also greater in the BC (10.6±2.88, mean±SEM) compared to the placebo group (5.0±1.56, mean±SEM) group. This difference was not significant (*P*=0.198) (see Table 22, Section 4.3.2 and Figure 17).

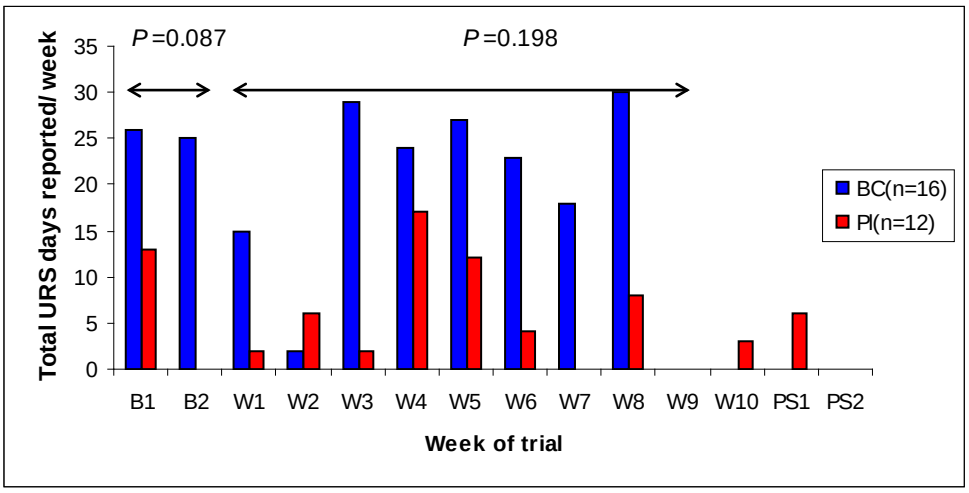


Figure 17: Total days of URS reported by the BC and placebo participants in the student cohort in each week**.** B=Baseline, W=Week of supplementation, PS=Post supplementation

Study 2: Older Adults

URS reportage by the older adults

There was no significant difference for URS reportage between the BC and placebo groups at baseline (*P*=0.260) or during the supplementation period (*P*=0.537) within the older adults cohort (see Table 22 and Figure 18).

Twenty-two of the forty-five participants were vaccinated against influenza during the fourth and sixth week of the supplementation period. The histogram shows an increase in reportage of URS in week seven (see Figure 18) but this increase was not significant (*P*=0.438).

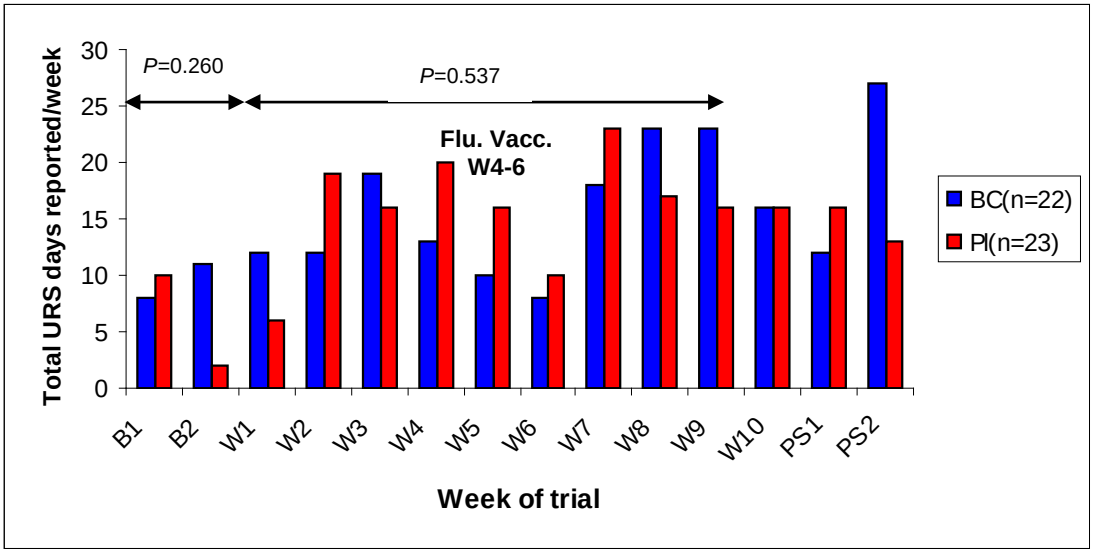


Figure 18: Total days of URS reported by the BC and placebo participants in the older adult cohort in each week. Flu Vacc=influenza vaccination, B=baseline, W=Week of supplementation, PS=Post supplementation

4.4 Investigation of the mechanism by which BC supplementation may modulate mucosal immunity.

Of the ten swimmers who volunteered for the subgroup (F=6, M=4), seven (BC=3 [2F,1M], Pl=4 [2F,2M]) completed the study requirements. One placebo male participant became sick with influenza-like symptoms after the first week of taking the supplement. He was unable to train for two weeks and as a consequence was unable to consume the required amount of trial drink (he consumed 8g/day, whereas the minimum for trial inclusion was16g/day). His data were excluded from the analysis leaving six in the swimmers subgroup (BC=3 [2F,1M], Pl=3 [2F,1M]).

All participants who volunteered for the students and older adults subgroups completed the study requirements.

4.4.1 Plasma cortisol

Swimmers subgroup (BC=3, placebo=3)

After four weeks of supplementation there was a significant main effect of consuming the supplement (*P*=0.004) in the swimmers subgroup which was time related (*P*=0.026). Plasma cortisol levels for the BC group were lower than the placebo group (see Table 23, Appendix 10.25). Because the sample size was small, post-hoc analysis was not performed on the means despite the significant interaction effect of group and time (*P*=0.040).

Table 23: Plasma cortisol (nmol/L) levels for the swimmers after four weeks of supplementation (mean±SEM). Significance differences (*P*) after repeat measures ANOVA.

	Pl (n=3)	BC (n=3)
Base	863.45±37.77	749.84±65.56
5 weeks	1111.39±105.5	816.7±5.43
P (Group)	*0.004*	
P (Time)	*0.026*	
*P (Group*time)*	*0.040*	

Student subgroup (BC=5, placebo=5)

Older adult subgroup (BC=5, placebo=5)

After four weeks of supplementation there were no significant main effects of the supplement (*P*=0.996, 0.269), gender (*P*=0.365, 0.259) or of time from baseline (*P*=0.102, 0.323) for plasma cortisol levels within the students and older adult subgroups (see Appendix 10.25).

4.4.2 Plasma cytokines

Swimmers (BC=3, placebo=3)

Students (BC=5, placebo=5)

Older adults (BC=5, placebo=5)

IL-6

After four weeks supplementation there were no significant main effects of the supplement (*P*=0.576, 0.551, 0.450), gender (*P*=0.419, 0.534, 0.296) or of time (*P*=0.620, 0.999, 0.708) for post-exercise plasma levels of IL-6 within the swimmers, student and older adult subgroups (*P*-values respectively) (see Figures 19, 20 and 21 and Appendix 10.25).

IL-1ra

After four weeks of supplementation there were no significant main effects of the supplement (*P*=0.760, 0.559, 0.251), gender (*P*=0.274, 0.643, 0.471) or of time from baseline (*P*=0.277, 0.677, 0.827) for post-exercise plasma levels of IL-1ra within the swimmers, student and older adult subgroups (*P*-values respectively) (see Figures 19, 20 and 21 and Appendix 10.25).

Interferon-alpha (IFN-α)

After four weeks of supplementation there were no significant main effects of the supplement (*P*=0.183, 0.353, 0.940), gender (*P*=0.399, 0.398, 0.411) or of time from baseline (*P*=0.835, 0.804, 0.277) for post-exercise plasma levels of IFN-α within the swimmers, student and older adult subgroups (*P*-values respectively) (see Figures 19, 20 and 21 and Appendix 10.25).

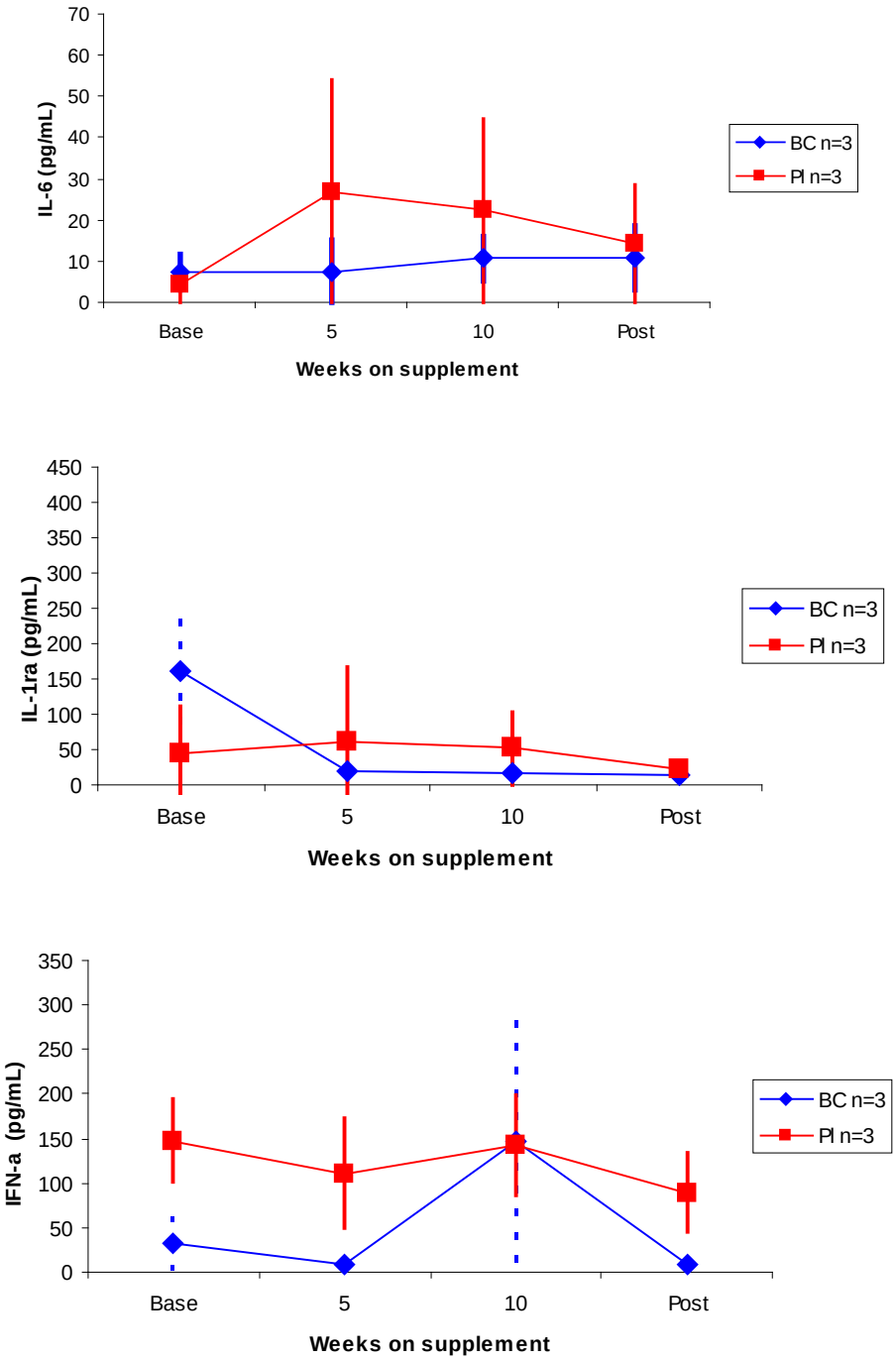


Figure 19. Cytokine (IL-6, IL-1ra, IFN-α) trends (pg/mL) for the swimmers subgroup at baseline (Base), 5 weeks, 10 weeks and post-supplementation (Post) (mean±SEM)

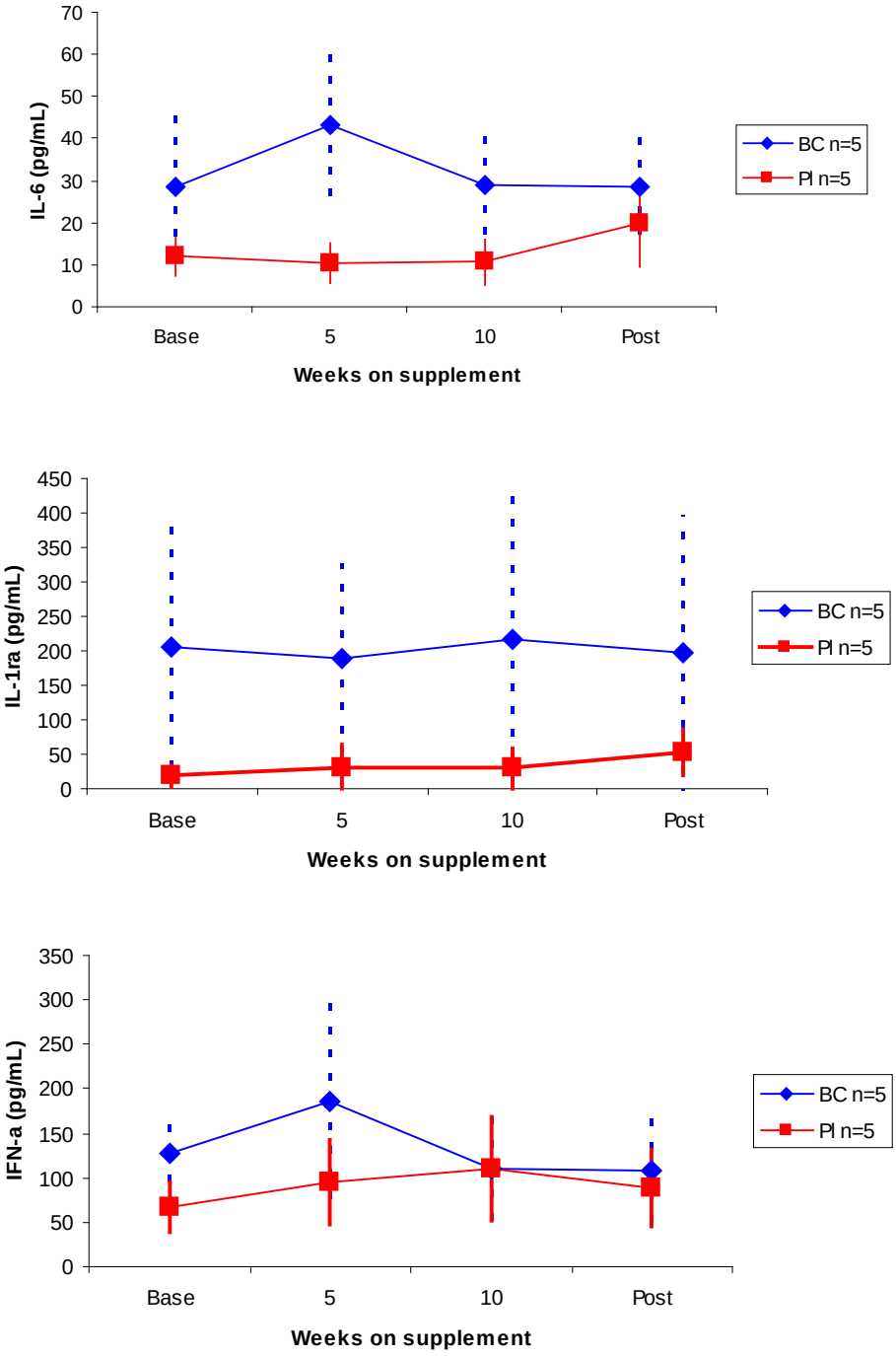


Figure 20: Cytokine (IL-6, IL-1ra, IFN-α) trends (pg/mL) for the students subgroup at baseline (Base), 5 weeks, 10 weeks and post-supplementation (Post) (mean±SEM)

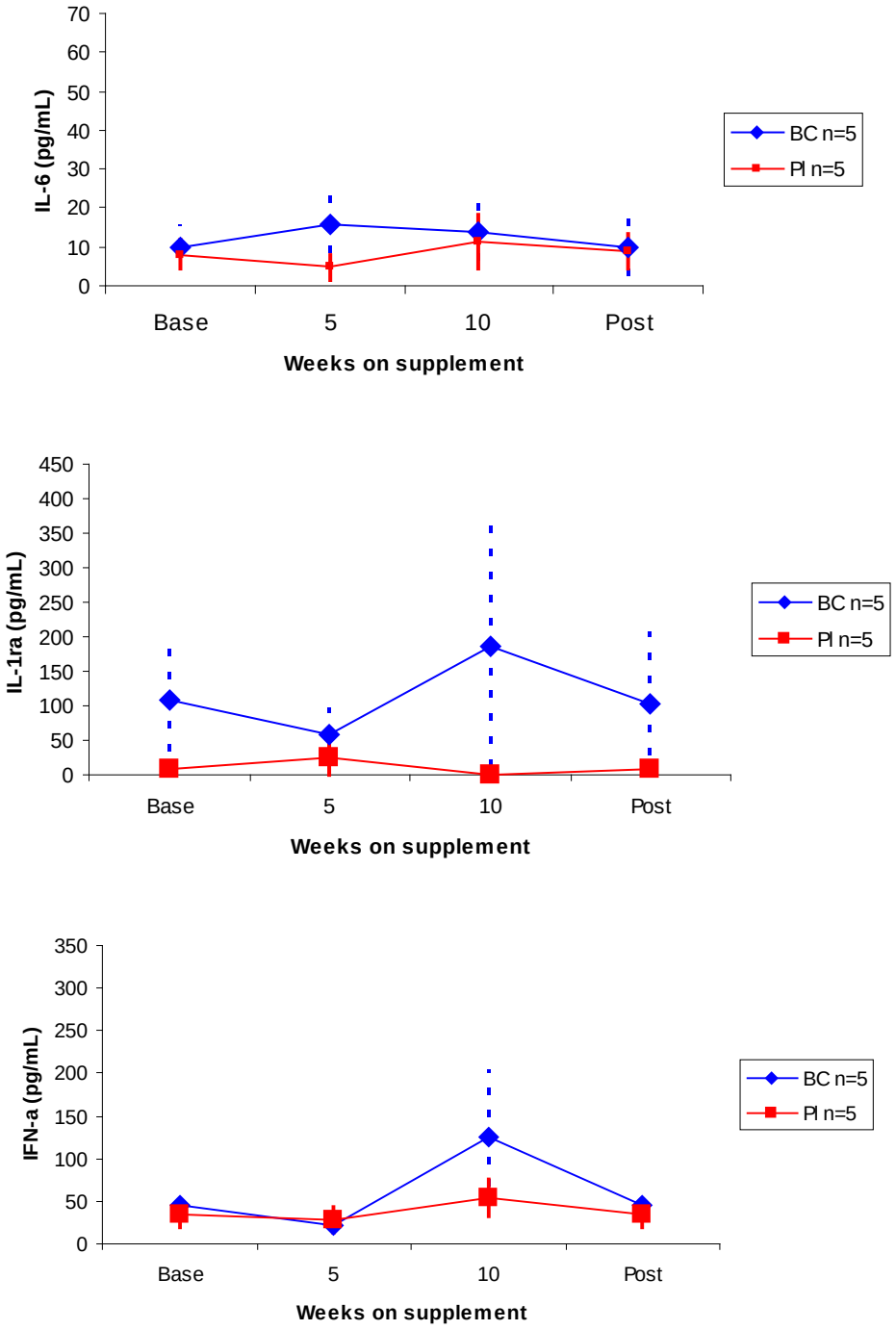


Figure 21: Cytokine (IL-6, IL-1ra, IFN-α) trends (pg/mL) for the older adult subgroup at baseline (Base), 5 weeks, 10 weeks and post-supplementation (Post) (mean±SEM)

4.4.3 Plasma insulin-like growth factor (IGF-1)

There were no significant main effects of the supplement (*P*=0.391), gender (*P*=0.184) or of time from baseline to the end of supplementation (*P*=0.177) on plasma IGF-1 within the swimmers cohort (BC n=12, placebo n=13). Plasma IGF-1 was measured in all swimmers to determine whether there were any time related increases due to consuming a BC supplement. This was important for those swimmers who had qualified for the 2004 Athens Olympics. To comply with the World Anti-Doping Agency's requirements, levels of insulin-like growth factors found in an athletes blood should not deviate from the range normally found in humans that is likely to be inconsistent with endogenous production [248]. Cost constraints meant plasma IGF-1 was not measured in the students or older adult subgroups (see Appendix 10.25).

4.4.4 Lymphocyte subsets

Swimmers subgroup (BC n=3, placebo n=3)
Students subgroup (BC n=5, placebo n=5)
Older adults subgroup (BC n=5, placebo n=5)

After four weeks of supplementation there were no significant differences between the BC and placebo groups for the following (swimmers, students, older adults (*P*-values respectively):
Absolute numbers of cell phenotypes (see Appendix 10.26):
Lymphocytes: (*P*=0.587, 0.662, 0.954)
T-cells: (*P*=0.587, 0.550, 0.246)
Helper T-cells: (*P*=0.583, 0.429, 0.140)
Cytotoxic T-cells: (*P*=0.914, 0.820, 0.663)
B-cells: (*P*=0.785, 0.082, 0.460)

Percentage of cell phenotypes (see Appendix 10.27):
T-cells: (*P*=0.150, 0.329) (swimmers, students [*P*-values respectively])
Helper T-cells: (*P*=0.387, 0.329, 0.816)
Helper T-cells also expressing CD49d: (*P*=0.726, 0.061, 0.903)
Cytotoxic T-cells: (*P*=0.212, 0.966, 0.973)
Cytotoxic T-cells also expressing CD49d: (*P*=0.450, 0.966, 0.363)
B-cells: (*P*=0.653, 0.363) (swimmers, older adults [*P*-values respectively])
B-cells also expressing CD49d: older adults (*P*=0.960). All B-cells in the samples from the swimmers and students were also expressing CD49d.

Significant differences between the BC and placebo groups were noted for:
Percentage of B-cells in the student subgroup (*P*=0.042)
BC=10$\pm$0.84% and placebo=14.8$\pm$1.28%, mean$\pm$SEM
This difference was not time related (*P*=0.082)

Percentage of T-cells in the older adult subgroup (*P*=0.015),
BC=64.28$\pm$1.50% and placebo=76.08$\pm$2.99%, mean$\pm$SEM
This difference was not time related (*P*=0.691)

The change in the numbers of B-cells in the older adult cohort after four weeks supplementation neared significance (*P*=0.052). B-cell numbers increased in the BC group and decreased in the placebo group.
BC= +0.0399 $\pm$ 0.020 x 10^9/L, placebo= -0.0247 $\pm$ 0.019 x 10^9/L, mean$\pm$SEM

After controlling for the effect of the influenza vaccination the difference between the groups for the change in B-cell numbers was still close to significant (*P*=0.057).

Mean fluorescence intensity (MFI) of lymphocyte subsets expressing the alpha integrin CD49d

Swimmers subgroup (BC n=3, placebo n=3)
Students subgroup (BC n=5, placebo n=5)
Older adults subgroup (BC n=5, placebo n=5)

After four weeks of supplementation there were no significant differences between the BC and placebo groups for the following mean fluorescent intensity (MFI) values for lymphocyte subsets also expressing the alpha integrin (CD49d) (see Appendix 10.28):
Helper T-cells: Students (*P*=0.210), Older Adults (*P*=0.402)
Cytotoxic T-cells: Swimmers (*P*=0.505), Students (*P*=0.575), Older Adults (*P*=0.721)
B-cells: Swimmers (*P*=0.806), Students (*P*=0.963), Older Adults (*P*=0.767)

Significant differences between the BC and placebo groups were noted for the MFI of helper T-cells also expressing CD49d (*P*=0.041) in the swimmers subgroup. This difference was not time related (*P*=0.248).

4.4.5 Associations between baseline levels of plasma IGF-1 and %CD8 cells and the CD4/CD8 ratio in the swimmers subgroup

Blood was sampled from the swimmers immediately following their early morning training session. At baseline, prior to the supplementation commencing, subgroup (n=6) results for plasma levels of IGF-1 were negatively associated with %CD8 cells (*P*=0.013) and positively associated with the CD4/CD8 ratio (*P*=0.029) (see Table 24). There were no significant associations with absolute numbers of CD8 (*P*=0.163) percentage CD4 (*P*=0.547) and absolute numbers of CD4 cells (*P*=0.887).

Table 24: Baseline associations between immune parameters and plasma hormones in the swimmers subgroup (n=6)

Dependent variable	Independent variable	*P*	Association	r^2 (Adj)
%CD8	IGF-1 ng/L	0.013	Negative	77.8%
CD4/CD8 ratio	IGF-1 ng/L	0.029	Positive	66.7%

Adj=Adjusted

4.4.6 Baseline comparisons between the cohorts for salivary and plasma parameters

Several salivary parameters were significantly different between the cohorts (students n=28, swimmers n=25 and older adults n=45) at baseline (see Appendix 10.29). The parameters listed here are those that were significantly different between the cohorts and had the most relevance to mucosal immune protection (mean±SEM):

Salivary S-IgA

Baseline levels for salivary S-IgA were significantly different (*P*=0.007) between the cohorts. Testing by the Tukey honest significant difference (Tukey) multiple comparison procedure indicated that mean salivary S-IgA levels were significantly higher for the older adult cohort (137.58±10.82mg/L) compared to the other two cohorts (swimmers [95.84±5.73mg/L] and the students [83.71±3.52mg/L]). The Tukey multiple comparison procedure indicated there

were no significant differences for salivary S-IgA levels between the swimmers and students.

Salivary IgG

Baseline levels for salivary IgG were significantly different (*P*=0.010) between the cohorts. Testing by the Tukey and Duncan's new multiple range test (Duncan) multiple comparison procedures indicated that all cohorts were significantly different to each other. Highest mean salivary IgG levels were identified in samples from the older adult cohort (25.17±3.01mg/L), followed by the swimmers (19.62±2.10) then the students (11.97±1.74mg/L).

Osmolality of saliva

Baseline levels for osmolality of saliva were significantly different (*P*=0.010) between the cohorts. Testing by the Tukey and Duncan multiple comparison procedures indicated that mean saliva osmolality for the two cohorts (swimmers [99.29±11mOsmol/kg] and older adults [96.38±3.21mOsmol/kg]) were not different from each other but were significantly higher compared to the student cohort (68.64±2.51mOsmol/kg).

Plasma IgA

Plasma IgA levels were significantly different (*P*=0.002) between the cohorts (students n=26, swimmers n=24 and older adults n=45) at baseline (mean±SEM).

Testing by the Tukey and Duncan multiple comparison procedures indicated that the cohorts were all different to each other. Mean levels of plasma IgA were highest for the older adults (2.464±0.10g/L), followed by the student cohort (2.036±1.17g/L) then the swimmers (1.616±0.10g/L).

4.4.7 Baseline comparisons between the cohorts for lymphocyte subsets

Several lymphocyte subsets were significantly different between the younger participants in the student (n=10) and swimmers (n=9) cohorts and the older adult cohort (n=10) at baseline (see Appendix 10.29). The parameters listed

here are those that were significantly different between the cohorts (mean±SEM):

Percentage of helper T-cells

Baseline levels for percentage of helper T-cells were significantly different (*P*=0.011) between the cohorts. Testing by the Tukey and Duncan multiple comparison procedures indicated that the mean percentage of T-cells identified in blood sampled from the older adult cohort (51.83±2.99%) was significantly higher compared to the swimmer (42.87±1.58%) and student (42.95±1.37%) cohorts. Mean percentage of helper T-cells for the swimmer and student cohorts were not significantly different to each other.

Percentage helper T-cells co-expressing CD49d

Baseline levels for percentage of helper T-cells co-expressing CD49d were significantly different (*P*=0.001) between the cohorts. Testing by the Tukey multiple comparison procedure indicated that the mean percentage of T helper cells co-expressing CD49d for the swimmer (86.37±1.28%) and student (89.54±1.75%) cohorts were not significantly different to each other but were significantly higher compared to the older adult cohort (70.56±3.51%).

Percentage of cytotoxic T-cells

Baseline levels for percentage of cytotoxic T-cells were significantly different (*P*=0.026) between the cohorts. Testing by the Tukey and Duncan multiple comparison procedures indicated that the mean percentage of cytotoxic cells identified in blood sampled from the swimmers (23.20±2.41%) and students (23.70±1.53%) were not significantly different to each other but were significantly higher compared to the older adult cohort (16.40±2.03%).

Percentage cytotoxic T-cells co-expressing CD49d

Baseline levels for percentage of cytotoxic T-cells co-expressing CD49d were significantly different (*P*=0.009) between the cohorts. Testing by the Tukey and Duncan multiple comparison procedures indicated that the mean percentage of cytotoxic cells co-expressing CD49d in blood sampled from the swimmers (99.14±0.76%) and students (97.79±1.01%) were not significantly different to

each other but were significantly higher compared to the older adult cohort (95.13±1.18%).

Percentage cytotoxic B-cells co-expressing CD49d

Baseline levels for percentage of B-cells co-expressing CD49d were significantly different (*P*=0.001) between the cohorts. All of the B-cells examined in the blood sampled from the participants in the student and swimmer cohorts were co-expressing CD49d.

Testing by the Tukey and Duncan multiple comparison procedures indicated that the mean percentage of B-cells that were co-expressing CD49d identified in blood sampled from swimmer and student cohorts was significantly higher compared to the older adult cohort (97.2±0.68%).

4.5 Reported daily dietary intake (RDDI)

Dietary analysis was performed at the beginning and end of the supplementation period using diet records for the swimmers and students and 24-hour diet recall for the older adults. The results in this study are indicative of differences in reported energy and macronutrient intake and micronutrient comparisons between groups as dietary data were collected for an insufficient number of days to provide the precision required [223] (see Section 5.6).

4.5.1 Reported daily energy intake (RDEI)

A summary of the results for RDEI along with estimated daily energy expenditure (EDEE) (determined using Harris Benedict and Schofield equations for estimating basal metabolic rate [BMR]) are in Appendix 10.32. Physical activity levels (PALs) were estimated for each participant from the mean weekly activity factors (wAFs) (see Appendix 10.20).

The assumption made was that:

EDEE=BMR x PAL=RDEI (approximately)

There were no differences between baseline and end of supplementation for RDEI between the BC and placebo groups within the student (P=0.290), swimmer (P=0.263) and older adult (P=0.806) cohorts (see Appendix 10.32).

Anthropometric measurements

Changes in body mass

Study 1: Swimmers

Mean body mass decreased for both females and males from baseline to week ten of the supplementation period; there was however no significant difference in body mass loss between the BC and placebo groups (P=0.459) (see Table 25). From the reported dietary intake records a reduction in body mass was not unexpected, as the RDEI was less than the EDEE.

Mean BMI for the swimmers cohort was within the healthy weight range for Europeans of 18.5-25.0 kg/m^2 range (Ministry of Health nutrition guidelines for healthy adults [249]), and there were no significant differences between the BC and placebo groups (*P*=0.239) (see Table 17).

Study 1: Students

Mean body mass increased for the BC and placebo females and decreased for the BC and placebo males from baseline to week ten of the supplementation. There were no significant differences for mean body mass at baseline compared to week ten between the BC and placebo groups (*P*=0.778). The loss of body mass in the males was not unexpected as the RDEI was less than the EDEE. As mean body mass increased in the female students it is possible either the RDEI was under-reported or the EDEE was over-estimated (see Table 25).

Mean body mass index (BMI) for the student cohort was also within the guidelines of 18.5-25.0 kg/m^2 range and there were no significant differences between the BC and placebo groups *(P*=0.823) (see Table 17).

Study 2: Older adults

The mean body mass increased for both females and males from baseline to week ten of the supplementation period; there were no significant differences in mean body mass between the BC and placebo groups (*P*=0.912). As mean body mass increased it seems either the RDEI was under-reported or the EDEE was over-estimated (see Table 25).

The mean BMI for the older adult cohort was above the healthy guideline of 18.5-25.0 kg/m^{2}range. The mean BMI for both the BC and placebo females groups was within the extended BMI range 20-29 kg/m^2 recommended by the Ministry of Health [162]. The mean BMI for the BC males was 28.7 kg/m^2 and for the placebo males 29.4 kg/m^2. There were no significant differences between the BC and placebo groups (*P*=0.596), (see Table 17).

Table 25: Changes in mean BM between baseline and week 10 of the supplementation period. Mean differences and SEM (median [Med] and interquartile range [IQ range] for data that would not normalise) between EDEE using Harris-Benedict (HB) and Schofield equations to calculate BMR compared to RDEI**.** Significant differences (*P*) between BC and placebo (Pl) groups within each cohort.

	Students				Swimmers				Older adults			
	BC (n=15)		Pl (n=13)		BC (n=12)		Pl (n=13)		BC (n=22)		Pl (n=23)	
BM change (kg)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	0.49	0.44	0.78	0.37	-0.91	0.76	-0.70	0.41	0.36	0.42	0.04	452
M	-0.72	0.34	0.52	1.01	-1.10	0.86	-0.59	0.38	0.52	0.55	0.83	388
P(Group)	*0.778*				*0.459*				*0.912*			
%BM change	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	1.07	0.72	0.88	0.61	-1.54	1.11	-0.98	0.58	0.50	0.61	0.27	0.69
M	-1.01	0.50	-1.18	1.64	-1.76	0.80	-0.52	0.48	0.61	0.70	1.07	0.42
P	*0.880*				*0.288*				*0.459*			
Reported RDEI (kJ)	Med	IQrange	Med	IQrange	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	7,575	6,939–8,460	7,686	7,120–11,329	10,171	1,085	12,153	870	7,148	500	8,174	463
M	10,700	9,027–13,747	12,651	8,008–14,780	13,020	1,543	15,348	1,738	9,160	1,041	10,274	824
P(Group)	*0.705*				*0.055*				*0.181*			
% Difference (HB)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	-29.0	8.0	-19.9	9.8	-34.1	12.6	-14.0	8.4	-37.6	9.2	-11.9	8.7
M	-8.0	8.7	-7.1	14.6	-43.9	23.5	-23.5	16.2	-63.3	15.1	-29.9	10.6
P(Group)	*0.562*				*0.431*				*0.015*			
% Difference (Schofield)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	-28.6	8.33	-18.8	10.3	-31.5	12.7	-13.8	9.47	-39.3	9.4	-14.7	7.9
M	-7.4	7.80	-9.74	15.3	-31.0	22.1	-13.9	13.6	-39.9	13.9	-19.9	9.01
P(Group)	*0.603*				*0.150*				*0.027*			
Mean% difference (HB and Schofield)												
F	*-28.8%*		*-19.3*		*-32.8*		*-13.9*		*-38.5*		*-13.3*	
M	*-7.7*		*-8.4*		*-37.45*		*-18.7*		*-51.6*		*-24.9*	

4.5.2 Reported daily macronutrient intake

Reported daily carbohydrate intake

After adjusting for EDEE there were no significant differences from baseline to the end of supplementation for the mean reported dietary carbohydrate intake (g/kg BM) between the BC and placebo groups within the student (*P*=0.647), swimmer (*P*=0.906) and older adult (*P*=0.266) cohorts (see Appendix 10.33).

The mean reported dietary carbohydrate intake for the swimmers (females=6.1 and 6.1 g/kg BM, males=6.9 and 6.3 g/kg BM, for BC and placebo respectively) nearly met the recommended guidelines for athletes exercising more than 2 hours per day (see Table 26, Appendix 10.33).

Table 26: Macronutrient requirements for athletes exercising more than 2 hours per day and 4–7 days per week [158]

Macronutrient	g/kg BM
Carbohydrate	7-8
Protein (endurance training)	1.2-1.4
Protein (adolescent athlete)	2
Fat (g/day)	80-100

Reported daily protein intake

After adjusting for EDEE, there were no significant differences from baseline to the end of supplementation between the BC and placebo groups for mean reported protein (g/kg BM) within the student (*P*=0.726) and older adult (*P*=0.266) cohorts (see Appendix 10.33).

There was however a significant difference in reported protein intake between week one and week ten within the swimmers cohort (*P*=0.025). The mean reported daily protein intake increased in the BC females (+0.037 g/kg BM), placebo females (+0.306 g/kg BM) and the placebo males (+0.333 g/kg BM), but decreased in the BC males (-0.282 g/kg BM).

This result indicated that the mean dietary intake of protein increased in the placebo group compared to the BC group.

Reported daily intake of percentage fat contributing to total energy intake (Fat%E)

After adjusting for EDEE, there was no significant difference for the mean reported daily Fat%E intake from baseline to the end of the supplementation period between the BC and placebo groups in the swimmer (*P*=0.998) and older adult (*P*=0.410) cohorts (see Appendix 10.33).

Student cohort

There was however a significant difference in reported intake between baseline and the end of the supplementation period within the student cohort (*P*=0.007). Mean reported daily Fat%E decreased in the BC group (female=-6.11%, male=-3.23%) and increased in the placebo group (female= +2.82%, male= +4.88%).

Older adult cohort

There was a significant difference (*P*=0.023) in mean reported daily Fat%E between the BC and placebo groups. Mean reported daily Fat%E was higher in the BC group compared to the placebo group. Mean reported intake was above the dietary guidelines for healthy older adults (30-33% contribution to total energy intake, see Appendix 10.40) for the BC females=39.5%, BC males= 41.5%, placebo males=37.1% but below the guideline for the placebo females= 28.5%.

4.5.3 Reported daily micronutrient intake

Differences in intake between the BC and placebo groups for the following micronutrients were determined: iron, zinc, selenium, vitamin A, vitamin C and vitamin E. These micronutrients were considered important to immune function and deficiency or excess could affect levels of salivary S-IgA. As dietary data was collected for insufficient day's results are suitable for comparing differences between groups but are not suitable for investigating individual intake. Reported daily micronutrient intake for data which were below the estimated average requirement are summarised for each group in Table 27.

Swimmers cohort

After adjusting for EDEE there were no significant differences for the mean reported daily dietary intake of iron (*P*=0.116), zinc (*P*=0.181), selenium (*P*=0.643), vitamin A (*P*=0.866), vitamin C (*P*=0.251) and vitamin E (*P*=0.127) between the BC and placebo groups in the swimmers cohort (see Appendix 10.34).

Mean daily dietary intake of iron, selenium, vitamins A and C for both the BC and placebo groups (see Appendix 10.34) were above the estimated average requirement for young adults (see Appendix 10.39).

Zinc

Mean reported daily dietary intake of zinc for the placebo males (see Table 27 and Appendix 10.34) was marginal (12.7% below the estimated average requirement for 14 to 18 year olds and 20% below the average requirement for 19 to 30 year olds [see Table 28 and Appendix 10.39]).

Table 27: Summary of reported daily dietary intake of micronutrients that were below the estimated average requirement (mean±SEM or median[IQrange])

		Swimmers		Students		Older Adults	
Micronutrient	Gender	BC	Placebo	BC	Placebo	BC	Placebo
Zinc	M	12.3 (3.9)	*9.6 (4.0)	N/A	N/A	N/A	N/A
	F	13.1 (4.0)	*6.4 (3.4)	N/A	N/A	N/A	N/A
Vitamin E	M	29.4 (20.4)	*6.5 (5.8)	*8.1 (5.7-14.4)	*9.9 (8.1-0.8)	N/A	N/A
	F	31.3 (22.4)	*6.2 (3.3)	10.7 (9.0-13.4)	13.0 (9.5-27.3)	N/A	N/A
Selenium	M	N/A	N/A	N/A	N/A	55.3 (11.0)	74.0 (18.9)
	F	N/A	N/A	N/A	N/A	44.3 (9.3)	43.0 (11.7)

* mean or median reported daily dietary intakes were below the estimated average requirement
Yrs=years
N/A=mean or median results were above the estimated average requirement and are not summarised in this table

Table 28: Estimated average requirement for Zinc, Vitamin E (14 to 18yrs and 19 to 30 yrs) and Selenium (51 to 70yrs and >70yrs) (from NHMRC, 2006 [160])

Micronutrient	Gender	Estimated average requirement			
		14-18 yrs	19-30 yrs	51-70 yrs	>70 yrs
Zinc (mg)	M	11	12	N/A	N/A
	F	6	6.5	N/A	N/A
Vitamin E (mg)	M	10	10	N/A	N/A
	F	8	7	N/A	N/A
Selenium (µg)	M	N/A	N/A	60	50
	F	N/A	N/A	50	50

Yrs=years
N/A=mean or median results were above the estimated average requirement and are not summarised in this table

Vitamin E

Mean reported daily dietary intake of vitamin E for the placebo males (see Table 27 and Appendix 10.34) was marginal (35% below the estimated average requirement for 14 to 18 year olds and the 19 to 30 year olds [see Table 28 and Appendix 10.39]).

Mean reported daily dietary intake of vitamin E for the placebo females (see Table 27 and Appendix 10.34) was marginal (22% below the estimated average

requirement for 14 to 18 year olds and 11.4% below the estimated average requirement for 19 to 30 year olds [see 28 and Appendix 10.39]).

Student cohort

After adjusting for EDEE there were no significant differences for the mean reported daily dietary intake of iron (*P*=0.722), zinc (*P*=0.946), selenium (*P*=0.285), vitamin A (*P*=0.886), vitamin C (*P*=0.377) and median reported daily dietary intake for vitamin E (*P*=0.705) between the BC and placebo groups in the students cohort (see Appendix 10.34).

Median reported daily dietary intake of iron and mean reported daily dietary intake of zinc, selenium and vitamins A and C for both the BC and placebo groups were above the estimated average requirement for young adults (see Appendix 10.39).

Vitamin E

Median reported daily dietary intake of Vitamin E for the BC male students (see Table 27 and Appendix 10.34) was marginal (19% below the estimated average requirement [see Table 28 and Appendix 10.39]).

Older adult cohort

After adjusting for EDEE there were no significant differences for the median reported intake of iron (*P*=0.884), zinc (*P*=0.529), selenium (*P*=0.795), vitamin A (*P*=0.884), vitamin C (*P*=0.295) and vitamin E (*P*=0.295) between the BC and placebo groups within the older adults cohort (see Appendix 10.34).

Mean daily dietary intake of zinc and median daily dietary intake of iron, vitamins A,C and E for both the BC and placebo groups was at or above the estimated average requirement for older adults (see Appendix 10.40).

Vitamin B6 and B12 are also considered to be important to immune protection in older adults [162]. After adjusting for EDEE there were no significant differences for the median reported daily intake of vitamin B6 (*P*=0.652) and vitamin B12 (*P*=0.435) between the BC and placebo groups within the older

adult cohort (see Appendix 10.34). Median daily dietary intake for vitamin B6 and B12 was above the estimated average requirement for both the BC and placebo groups within the older adult cohort (see Appendix 10.40).

Selenium

Mean reported daily selenium intake from the two diet recalls (see Table 27 and Appendix 10.34) was marginal (11.4% below the estimated average requirement for females aged 51 to 70 years and 14% below for females >70 years [see Table 28 and Appendix 10.40]).

5.0 Discussion

5.1 Overview

The main aim of this study was to investigate whether the effect of BC supplementation on levels of salivary S-IgA in marathon runners observed in a previous study (Appendix 10.1), could be identified in other groups with potentially depressed immune systems, such as elite athletes and older adults. An additional aim was to investigate the mechanisms by which a nutritional supplement such as BC might modulate levels of S-IgA in saliva. There is also a need for clinical trials in humans seeking to demonstrate the effect of functional foods on immune protection in the context of the whole diet.

5.2 Methodological considerations

This study investigating the effects of BC on levels of salivary S-IgA was performed with participants in their 'real-life' of daily training or exercising and/or work or study environment. In these surroundings there is less control over variables that may affect immunity, such as dietary intake, amount of exercise, psychological stress, and the amount of sleep. This approach is more rigorous and has advantages over laboratory based trials performed under controlled conditions. Any identified benefits to health attributable to the BC supplement will more likely be experienced by consumers in their everyday environment.

The effect of BC supplements on immune protection has been investigated in participants from several sports: cross country skiers [216], sprinters [32], cyclists [217] and recreational distance runners [1]. Because of the varying study designs it has been difficult to compare results. These include different periods of supplementation, different amounts of supplement and the use of different placebos. In addition it is still not known what effect BC supplementation would have in the context of the whole diet.

It is also recognised that there are many potential confounding variables which impact on salivary S-IgA levels, including age, gender, training level of the

athlete, adequacy of dietary intake, seasonal effects (e.g. exposure to URTI viruses), other stressors (e.g. pressure of exams in young athletes) and an influenza vaccination [88]. The type and amount of bio-active component in the nutritional supplement can also affect immune function [4, 25]. In this current study an attempt was made to control for the variables known to have an effect on levels of S-IgA in saliva in order to elucidate any immunostimulatory effect of BC. This included using selection criteria during recruitment of participants, and standardising all methodological steps (time of sampling, saliva collection practices, sample storage practices and analytical methods). In addition the placebo (skim milk powder) used previously in a trial with middle aged distance runners [1] was chosen for these current investigations. Dietary intake was assessed in order to identify an immunomodulatory effect of BC in the context of the whole diet. Levels of physical activity and wellness symptoms were recorded, along with the measurement of various biochemical, immunological and hormone parameters in saliva and plasma.

Previously the consumption of a BC supplement was associated with a significant increase in levels of salivary S-IgA in middle-aged distance runners [1]. The effect on non-exercising age-matched controls was not investigated, which was a limitation [1]. For the first part of this current study a cohort of age-matched non-exercising or (lightly exercising) students was included. The intervention trial for this cohort started three weeks after the swimmers. Logistically it was not possible to run the trials simultaneously. Running the two trials at similar times (three weeks apart) meant that influences on immune modulation from seasonal differences (e.g. exposure to URTI pathogens) would be minimised for these two cohorts.

Characteristics of the participants

Swimmers

Prolonged intensive training by swimmers preparing for the Sydney Olympics was found to have a negative effect on pre-exercise levels of salivary S-IgA over a seven-month season which could put the athletes at increased risk for URTI [82]. Therefore, because it was thought that elite swimmers may have reduced levels of S-IgA during periods of intensive training, and because of the

comparatively controlled training environment compared to other sporting codes, young swimmers that trained for the Auckland swimming championships were asked to volunteer for Study 1.

Variable training practices by the distance runners were considered to be a limitation that may have affected the change in levels of salivary S-IgA [1]. In this current study the aim was to standardise training practices. There were many advantages in choosing swimmers to investigate the effects of a nutritional supplement on levels of salivary S-IgA. This included having the same coach, following similar training programmes, training in the same environmental conditions, and participants meeting at a central location which facilitated the standardisation of sample collection practices.

However, in this study it was not possible to recruit sufficient participants from a single club which was a confounder for this study. Volunteers were therefore sought from three clubs, at each club there was a different coach and training was carried out at a different swimming pool. At two of the clubs volunteers tended to be those athletes who were already experiencing a high frequency of URTI. This may have meant they were experiencing altered immune function that may not have been representative of young elite swimmers. At one of these two clubs only two swimmers volunteered for the study; they both felt they were experiencing colds frequently. In the double-blind randomisation process both of these swimmers were assigned to the placebo group; they also reported the highest level of URS in the swimmers cohort (see Section 5.7). At the third club most of the senior squad volunteered, at the recommendation of the coach. Despite this, there were no differences between the BC and placebo groups for participant characteristics, haematological or immunological parameters at baseline and at the end of the study. This indicated that participants from the different swimming clubs were similar for these characteristics and it was unlikely that there was a confounding effect of the swimming pool environment or training programme on immune modulation by BC.

Elite athletes may also experience more variability in immune parameters such as salivary IgA and IgG than active and sedentary individuals [250]. Research

published by Francis *et al*., (2005) suggested that it was difficult to compare mucosal immune parameters between populations with varying levels of activity [250]. Mucosal parameters for populations exercising at different levels were found to vary significantly which affects the determination of sample size [250]. For the current studies, deriving a sample size based on a power calculation from the results for salivary S-IgA measured in the recreational middle-aged distance runners may not have been appropriate for a group of younger athletes training at an elite level. A further confounder was that it was not possible to recruit the number of participants required (as determined from the power calculation) for each cohort (fifty in each cohort).

Students

For the student cohort it was not logistically possible to collect saliva samples at the same time as the swimmers (at 5.30am). A later time was established to fit in with laboratory sessions and lectures. There were no differences between the BC and placebo groups for participant characteristics and haematological parameters within the student's cohort at baseline and at the end of the study. However, there were significant differences in levels of plasma immunoglobulins between the BC and placebo groups that may have affected their immune status. This makes it difficult to determine whether there has been an effect of the BC supplement (see Section 5.4).

Older adults

In Study 2, healthy older adults with no known respiratory problems were recruited and were randomly assigned to either BC or placebo. There were no significant changes from baseline to the end of the supplementation period between the BC and placebo groups for participant characteristics. Levels of salivary S-IgA, IgM and IgG, plasma levels of IgA and IgM and levels of leucocytes all appeared to be within ranges typical for healthy adults and do not indicate that the participants were experiencing immune suppression. Mean plasma IgG levels in the BC group were at the lower end of the typical range and were significantly different to the placebo group. This difference may not have been significant in a larger sample of healthy older-adults but in this cohort may have confounded any protective effect of the BC supplement on salivary S-

IgA levels (see Section 5.4). While mucosal immunity is important in the first line defence against pathogenic organisms invading the respiratory tract, there is still much to be learned about the effect of ageing on mucosal immunity [251].

Dehydration may occur more frequently in older adults compared to younger adults, due to a reduced fluid intake, or an increased fluid loss, or a combination of both [252]. Saliva flow rate slows with dehydration which has the effect of concentrating immunoglobulins; additional monitoring of hydration status (i.e. measuring specific gravity of urine) was therefore performed for this group. It is thought that as a consequence of dehydration there is an increased risk for respiratory infections in older adults [252]. In Study 2 the majority of urine samples had a specific gravity less than 1.029 g/L which indicated that the participants were adequately hydrated. A few participants had specific gravity results greater than 1.029 g/L, which indicated a degree of dehydration and was related to the participant forgetting to drink that morning. To help ensure saliva flow rate wasn't affected, these participants were asked to have a drink and to provide a saliva sample after a 30 minute wait. As the older adults in Study 2 had urine specific gravities largely within the euhydrated range the effect of dehydration on saliva flow rates and salivary S-IgA levels was considered to be insignificant.

Hydration status did not appear to be affected for the cohort in this study of older adults which was a similar finding to a previous study where specific gravity of urine was used to determine hydration status. Older adults aged 65 to 93 years who lived independently were found to be in a euhydrated state (defined as a specific gravity between 1.013 and 1.029 g/L) even after an overnight fast. Together this suggests that dehydration may not be a problem for independently living older adults. Dehydration may be more of a problem for those with medical conditions or in dependent living situations rather than ageing itself [252].

Anthropometric measurements

Various anthropometric measurements were taken to help determine that the participants were healthy [243], and that there were no significant differences

between the BC and placebo groups before and after the intervention. BMI was calculated from the body mass and height measurements, and used to indicate health status (obesity contributes to coronary heart disease due to increased risk for hypertension, diabetes and hypocholesterolaemia [162]). The World Health Organisation (WHO) states a healthy weight range is indicated by a BMI of 18.5-24.9 kg/m^2. While there were no significant changes in BMI between the BC and placebo groups within any cohort, there are limitations to using this anthropometric measure as a guide to health in both athletes and older adults as discussed below.

Athletes

The WHO BMI range may not be appropriate for athletes due to their high percentage of lean body mass. Individuals with high muscle mass have been classified as being overweight using the WHO BMI range [253]. No other anthropometric measures were performed (e.g. skin folds) due to time constraints and availability of the swimmers.

Older Adults

In adults aged 65 years and older the consequences to immune health of being mildly overweight are not known. The WHO guideline may not be as reliable a predictor of morbidity and mortality in this age group as in younger adults [162]. An extended BMI range 20–29 kg/m^2 may be a more appropriate guideline to indicate health status for older adults [162].

Supplement

To help improve compliance of the consumption requirements of the supplement a flavour choice (all chocolate or half berry/half chocolate or one third malt/one third chocolate/one third berry) was offered to the participants. In the current study a number of problems were encountered with the different flavoured supplements. Firstly, logistically it was difficult to accommodate flavour preferences, particularly as several participants changed their minds about their choice of flavour mix after seeing what others had. This complicated the process of setting up the trial, when the main focus should have been to train participants in the study protocol e.g. record keeping. Secondly there were

noticeable unexplained differences in bio-active levels between the BC flavour variants (e.g. TGF-β1 and TGF-β2). Levels of bio-actives in the placebo flavour variants were also similar to the BC flavour variant e.g. levels of TGF-β1 in the BC and placebo malt variant.

A possible explanation for the variable bio-active levels could be that reliable analytical methods for analysis of bio-actives in milk-products (a complex mixture of biologically active components) have yet to be established [254, 255]. For example various quantitative techniques have been utilised in the analysis of IgG levels in BC including: separation based techniques, electrophoresis, immuno-based, and physical techniques [254]. The choice of an analytical method depends on the purpose of the analysis and as yet there are no internationally accepted reference methods [254]. For this study bovine IgG levels were identified by two methods: high performance liquid chromatography (HPLC) and radial immunodiffusion (RID). Consistently higher IgG levels were determined by RID compared to HPLC. The IgG levels in the different BC flavour variants were similar by both methods and levels of IgG in the placebo were negligible (see Appendix 10.15).

Quantitative analysis of the remaining bio-actives was determined using enzyme-linked immunosorbent assay (ELISA). However TGF-β exists in a latent complex that can't be detected by ELISA. Activation of the complex is required for analysis and various protocols have been established [255]. The activation procedure used was not specified for the results supplied for this study. Additionally it is not known if the latent TGF-β complex present in BC would be activated in the gut of humans [255].

There were differences in the formulation of the flavour variants that may have affected either the analysis or the bio-active level, e.g. variable levels of whole milk powder and malt powder in the BC flavour variants and calcium caseinate in the chocolate placebo blend (see Appendix 10.14). In this current study bio-active levels were not known prior to the commencement of the intervention trials which has limited interpretation of results and comparison with other BC research.

5.3 Optimising levels of S-IgA in saliva during sample collection

There are many advantages in measuring changes to immune function in saliva, including the ease of sampling; providing a saliva sample is non-invasive and therefore more acceptable to participants, particularly in nutrition intervention studies where healthy volunteers are often sought. Saliva contains factors that reflect the state of the mucosal immune system; for example changes in levels of salivary S-IgA may indicate changes at other mucosal surfaces [4]. In addition, salivary levels of IgG may also provide a picture of serum levels [256]. However, saliva analysis can also be problematic when investigating immunity; it contains proteases that can degrade the salivary immunoglobulins. Immunoglobulin levels might also be affected by diurnal and monthly variations [256] although more research is needed to determine this.

Collection of saliva samples

When investigating the effects of a nutrition supplement such as BC on levels of salivary S-IgA in participants in their real-life environment, it is necessary to understand the constraints when interpreting the results. There is a lack of agreement on how to control for hydration status in athletes. Blannin *et al.,* 1998 determined that salivary S-IgA secretion rate or S-IgA/osmolality ratio provided the best means to control for the effects of dehydration on saliva flow in males with mixed fitness levels [238]. Fahlman and Engels, 2005 found that the absolute concentration of S-IgA or IgA secretion rate are the best predictors for URTI risk in American footballers [21]. While Blannin *et al.*, 1998 were not investigating the relationship of salivary S-IgA levels with URTI risk; both groups favoured measuring the secretion rate of salivary S-IgA as a means for controlling for the effect of hydration status.

In practice it is difficult to measure flow rate accurately when collecting whole mixed unstimulated saliva samples. The method used by many researchers [21, 48, 85, 153, 241] requires the participant to drool for a set time e.g. one to four minutes, into a standard container and the volume of saliva produced is measured. It is difficult to time the flow rate accurately if the participant and researcher are not in an area that is private and without interruption. This is a

problem when sampling participants in their normal training or work environment. In addition the imposition of time on the participant may encourage them to drool more than they would normally. The measurement of the degree of change in absolute levels of salivary S-IgA, which does not rely on saliva flow rate, could provide the best indication that there has been an effect of an intervention. Given the variability that can occur in design methodology it is very difficult to compare absolute values of salivary S-IgA levels between studies. It is more appropriate to compare the degree of change in levels in response to an intervention.

The time taken to freeze saliva may be one of the most important factors to optimise IgA concentrations in saliva during sample collection procedures [256]. In a review of various saliva sampling and storage methods, inactivation of the proteolytic enzymes appeared to be key in order to optimise salivary S-IgA levels [256]. It appeared that it was the immediate freezing to -70ºC rather than the collection method itself that had the greatest effect on immunoglobulin levels [256]. In addition, saliva samples were supplied from participants who were asked not to eat or drink in the two hours prior to sampling; the reason for this was not specified. The optimal time to collect a saliva sample after eating and drinking has still not been determined and thirty minutes was chosen in this current study to accommodate the participants.

To help minimise time to freezing, saliva samples were immediately placed on dry ice after collection. Production of saliva varied between participants with some producing the required volume within a minute while others took up to five minutes. The frozen samples were held on dry ice until transported to a -80ºC freezer where they were stored until analysis. This was found to be the simplest and most practical method to use when collecting saliva from participants outside the laboratory environment.

It is also not known if there is a diurnal effect on levels of salivary IgA [256]. Previously, to fit in with the distance runners, saliva samples were collected between 6.15am and 7.00am. In this current study saliva samples were collected from the swimmers prior to their morning training session, between

5.15am and 5.30am. At one of the clubs there were only two volunteers, and it was not possible to collect samples for these two before the morning training session. These athletes provided saliva and blood samples on the nominated days at 4pm prior to their afternoon session. The aim of the study was to investigate changes in levels of salivary S-IgA due to BC supplementation; it was assumed that any diurnal effect would not have impacted on changes in absolute salivary S-IgA values if samples were collected from the participant at the same time of day.

There may be a seasonal effect on saliva flow rate [257], lower saliva flow rates have been observed in the summer perhaps due to a more dehydrated state which would have a concentrating effect on levels of salivary S-IgA [257]. In the previous study, the marathon runners were sampled from January to April, 2002 (summer to autumn), and an increase in salivary S-IgA levels was measured in the BC group. It is unlikely that this was due to a seasonal effect on hydration status. In this current investigation saliva samples were collected from the swimmers and age-matched controls between September and December 2003 (spring to summer). In Study 2 saliva samples were collected from the older adults between April and July 2005 (autumn to winter). There were no increases in levels of salivary S-IgA levels, within any of these cohorts, at the end of the supplementation period compared to baseline. There were also no time-related changes in salivary albumin, plasma albumin or saliva osmolality within any of the cohorts; it seems unlikely therefore that there has been an effect of a change in season on saliva flow rate.

In summary in order to determine whether changes to levels of S-IgA in saliva occurred after BC supplementation the same study protocol was applied to each participant throughout the trial. This included standardising the time of day when the saliva samples were collected, the post-prandial sampling time and the time to freeze the saliva samples.

5.4 Haematological parameters

Various haematological parameters were measured at baseline and at the end of the supplementation period, to determine if there were differences between the BC and placebo groups that may have affected levels of salivary S-IgA and confounded the effect of the supplement.

Iron status may impact on immune function, since iron deficiencies can affect the functioning of many immune cells [147]. Therefore it was important to determine whether there had been a change in iron status in the swimmers due to the increased volume of training (given that they were preparing for the Auckland swimming championships). In this study there were no significant changes within this cohort or the age-matched control cohort (students). There were also no changes for either the swimmers or the student cohorts for any of the other haematological parameters.

Within the older adult cohort there were also no significant changes in iron status. However five of the forty five participants (11%) had plasma ferritin levels above the typical physiological level for this age group. Elevated ferritin levels may be indicative of an impaired iron metabolic condition known as haemochromatosis, which results in excessive iron absorption and can lead to parenchymal damage in the liver, heart and pancreas [258]. Haemochromatosis in most individuals is a genetically predetermined condition, although genetic disposition does not explain all incidences [258]. In this study, while there were no differences in median ferritin levels between the BC and placebo groups; four of the five older adults who had elevated ferritin levels were in the BC group. It is known that iron deficiencies affect lymphocyte proliferation in older adults [147] which could impact on salivary S-IgA levels; however it is not known whether elevated iron stores could also impact on levels of salivary S-IgA. Elevated ferritin levels can be indicative of infection or an inflammatory condition. The C-reactive protein results (a marker of inflammatory conditions) indicated that all participants had levels within normal physiological ranges. It seems the elevated ferritin levels were due to some non-infectious cause. As there were no differences between the BC and

placebo groups for the results for liver function tests it was assumed there were no differences in age-related decline of liver function between the groups; despite some of the participants in the BC group having higher ferritin levels. In future studies of nutrition interventions and mucosal immune function in older adults it would be useful to monitor iron status along with liver function tests at baseline and at the end of the intervention. Alternatively, those with ferritin levels outside the physiological typical levels could be excluded.

In the older adult cohort there were also no significant differences and no changes with time for most of the haematological parameters. This suggested that within this cohort there was a degree of homogeneity between the BC and placebo groups. There was an exception however; there were small but significant increases in the red blood cell count and haemoglobin levels in the BC group but not the placebo group after ten weeks of supplementation compared to levels at baseline (see Section 4.1.1 and Appendix 10.21). All results were within the normal physiological range for this age group. It was not possible to determine whether the increase was due to an effect of BC or if it was simply due to normal physiological variation that may not be significant in a larger sample.

In summary measurement of the various haematological parameters indicated that the BC and placebo groups were similar within each cohort and that the parameters did not change between baseline and the end of the supplementation period. It was important to know that there was no adverse effect of training on iron status which may affect immune function in the swimmers cohort over this period. The small significant increases in some of the parameters in the older adult BC group suggest the safety of BC on immune function should be investigated further in this age group.

5.5 The effect of BC supplementation on salivary S-IgA (the main outcome variable), IgG, IgM and albumin

The main outcome variable for this study was the effect of BC on levels of S-IgA in saliva of potentially immune-depressed groups that is, young elite athletes, their age-matched controls and healthy older adults. In this study the results showed there was no effect of BC on levels of salivary S-IgA within any of the cohorts. This indicated that the effect of BC supplementation on levels of salivary S-IgA was not identifiable in these potentially immune-depressed groups.

There are a number of possible explanations for this result. The mean baseline levels of salivary S-IgA measured in this study were within the reference range for the swimmers and students and above the reference range for the older adults. This indicated that salivary S-IgA levels were not depressed for any of these cohorts. It may be appropriate to include mucosal immune status as part of the selection criteria in future investigations of the effect of BC on levels of salivary S-IgA.

In addition the mean salivary S-IgA levels for the participants aged 16 to 20 years from both the swimmer and student cohorts were above the reference range for these age groups. The reason for this was unknown. Possible explanations include: an effect of hydration status (however there were no differences between the BC and placebo groups for the markers of hydration, salivary albumin, osmolality and S-IgA/osmolality ratio); exposure to infectious micro-organisms (the first saliva samples were collected during September 2003 when there would have been exposure to winter URTI viruses), and the upper limit for the reference range for salivary S-IgA levels may be too low for those aged 16 to 20 years.

Baseline levels of salivary S-IgA were not significantly different between the swimmers and student cohorts. Mean salivary S-IgA and IgG levels for the student placebo group were higher than the BC group throughout the trial although this difference was not significant (see Figure 12 Section 4.2). This

suggested that consuming a BC supplement did not enhance mucosal immune protection in this cohort of students.

A comparison between the three cohorts for baseline levels of salivary S-IgA indicated that levels were significantly higher in the older adults compared to the swimmer and student cohorts (see Section 4.4 and Appendix 10.29). This result suggested that local production of S-IgA was not impaired in this cohort of healthy older adults. Levels of albumin and IgG in the saliva of the older adult cohort were significantly higher compared to the other two cohorts and may be indicative of increased crevicular transudation in this cohort. In addition saliva secretion rates can reduce with ageing. It is possible that the higher concentration of salivary IgG observed in the older adult cohort may have a role in compensating for any loss in immune protection associated with reduced saliva flow rate. This could be an example of immune remodelling with ageing.

Prolonged intensive exercise and ageing affect several physiological processes including both immune function and gut function. Changes in levels of salivary S-IgA that have been observed in athletes following training regimes involving intensive bouts of exercise may reflect temporary alterations to immune function and are not directly related to an increased risk for URTI (e.g. disturbed T-cell control mechanisms [94]). The risk for URTI will also be affected by the type of exercise (intensity and duration) and the age of the athlete. Greater variability in levels of salivary S-IgA has been observed in elite athletes compared to recreational athletes [250]. For this current study a sample of fifty athletes was required to observe a significant change in salivary S-IgA levels based on the power calculations of the results from the study with recreational distance runners [1]. As only thirty athletes volunteered (and twenty-five completed the study requirements) this was unlikely to be sufficient numbers to observe an immunomodulatory effect of BC on levels of salivary S-IgA. In addition because of the greater within subject variability in elite athletes [250], more frequent saliva sampling compared to recreational distance runners may have been necessary. Daily sampling could identify changes in salivary S-IgA that occur as a result of exercise and or environmentally induced changes. In this study it was not possible to sample on a daily basis due to cost constraints.

Minimising the effect of exercise-induced changes to immune system homeostasis during or following prolonged bouts of intensive exercise may enhance an athlete's ability to resist infection. It is possible that the mechanism affecting this is through maintaining gut homeostasis. One way of maintaining gut homeostasis could be through the enhancement of intestinal repair processes known to be affected by the physiological stress of exercise [91] as has been seen in heat-stressed rats fed a BC supplement [116].

A nutrition supplement such as BC (which contains many biologically active components) may modulate immune function through various mechanisms, the extent of which may also be affected by the age of the athlete and the type of exercise. In a previous study a significant increase in salivary S-IgA levels occurred in middle-aged distance runners consuming 12g/day of a BC supplement [1]. In the middle-aged distance runners levels of salivary S-IgA in the BC group may have been restored to levels more typical for this age group. In this current study there were no time-related changes in salivary S-IgA but there was a trend towards a difference in the reportage of URS by the young elite swimmers (see Section 5.7). However it is difficult to compare results between the studies when the bio-active levels were not known in the former study. If there has been an effect of BC on URS reportage this may have been to exercise-induced non-infectious inflammatory processes.

5.6 The effect of BC supplementation on plasma immunoglobulins (secondary outcome variables)

Plasma immunoglobulins

There were no differences for any of the plasma immunoglobulins at baseline, at weeks five and ten and at the end of the supplementation period between the BC and placebo groups within the swimmers cohort. The plasma immunoglobulin results were within typical physiological ranges at all time points. This result together with the salivary immunoglobulin results is evidence that the swimmers cohort was not immune-depressed during this study.

There were however significant differences within the age-matched controls (the students) between the BC and placebo groups for plasma IgG and IgA at baseline, weeks five and ten of the supplementation and post-supplementation. At all time points the BC group had significantly lower levels of IgA and IgG than the placebo group. This was indicative of a difference between these groups for plasma IgG and IgA due to normal physiological differences or to some unidentified factor. The mean results for plasma IgG levels (7.68–7.92g/L) for the BC group were also at the lower end of the typical range for this age group (7.0–16.0g/L [247]). Interestingly, the histogram of the weekly URS reportage by the student cohort showed that the BC group reported more URS over the ten weeks of the supplementation period compared to the placebo group although the difference was not significant (see Figure 17 and Section 4.3). The cytokine trend graphs (see Figure 20 and Section 4.4) also showed that the student BC subgroup had higher levels of IL-6 and IL-1ra compared to the placebo subgroup at all time points, but these differences were not significant. As well levels of IFN-α were non-significantly higher in the BC group at baseline and after five weeks of supplementation, which possibly indicated there was a response to infection in the BC group that may have confounded immunomodulation by the BC supplement.

The significantly lower plasma IgG levels observed in the student BC group may explain some of their higher reportage of URS. Experiencing URS is known to have an effect on levels of salivary S-IgA [48]. Therefore for those students in

the BC group reporting increased days of URS, there may have been a confounding effect on the results for salivary S-IgA. In 55 children aged 4–14 years who had recurrent respiratory infections (more than five infections in a one year period), 27 (49%) had either an IgA or an IgG subclass deficiency [259]. This was significantly higher than in the healthy control group of 43 children, where 6 (14%) were found to be deficient [259]. Together the plasma IgG, IgA results and the non-significant results for URS reportage suggest the BC group may have been immunologically different to the placebo group. These differences may not be apparent in a larger cohort. This highlights a problem for nutrition intervention studies of immune function with small numbers of participants.

There were also significant differences in plasma IgG at baseline, weeks five and ten and post-supplementation between the BC and placebo groups within the older adult cohort. The BC group had lower results for plasma IgG levels than the placebo (7.94 to 8.20 g/L). The IgG results were at the lower end of the typical range (adult=7.0 to16.0g/L and over 70 year olds=6.0 to 15.0 g/L [247]). As for the student cohort this was indicative of a difference between these groups for plasma IgG due to normal physiological differences or some unidentified factor. There were no apparent differences in reportage of URS between the BC and placebo groups. It is also possible that the differences in plasma immunoglobulins in the older adult cohorts may not be present in a larger sample.

In summary there were no significant differences in plasma immunoglobulins within the swimmers cohort. There were significant differences between the BC and placebo groups for plasma levels of IgG and IgA within the student cohort and for IgG within the older adult cohort. This difference may have confounded the effect of the BC supplement but may also have been insignificant in a larger cohort.

5.7 URS reportage (secondary outcome variable)

Reportage of URS was a secondary outcome variable for the current study. Upper respiratory symptoms were monitored because levels of salivary S-IgA have been found to increase several days prior to URS being reported, and to remain elevated for several days after symptoms ceased to be reported [48]. As the reportage of URS may have a confounding effect on levels of salivary S-IgA in a nutritional intervention trial it was important to determine if there were differences in URS reportage between the BC and placebo groups.

5.7.1 Self-reported URS by the swimmers cohort

There was a trend towards a significant difference (P=0.080) for the frequency of URS reportage between the BC and placebo groups within the swimmers cohort. While the reportage of URS for all swimmers tended to decrease over the supplementation period, the BC group reported fewer days (see Figure 14 and Section 4.3). In addition there was also a non-significant (P=0.075) trend for the swimmers consuming the BC supplement to report fewer days of mild symptoms compared to the placebo group (see Figure 16 and Section 4.3). There was also a non-significant (P=0.062) trend for fewer swimmers to report URS after four weeks of consuming the BC supplement (see Figure 15 and Section 4.3). These observations are in agreement with a reduction in reportage of URS in two other studies investigating the effect of a BC supplement on immune health in athletes [33, 217]. The levels of bio-actives in the BC supplement were not reported in these studies, therefore it is difficult to compare results and speculate on a mechanism. By comparison there were no trends towards or significant differences for URS reportage in the age-matched controls and older adults.

There are many difficulties with the interpretation of URS reportage by athletes. A major problem is the accuracy of self-recording of URS and the correct identification of URS as being due to viral infection and not some other irritation [89, 260]. Additionally, athletes may over-report URS compared to the non-exercising population because of an increased awareness of health and the

impact on performance. Athletes may therefore recognise symptoms more quickly than the non-exercising person [13].

In this study reportage of URS was determined from the analysis of the daily self-recorded wellness diaries. The quality of record-keeping by the participants was variable; some athletes were very diligent and may have over-recorded their symptoms. A number kept poor records (did not complete the diary on a daily basis and relied on memory) that required following up by the researcher. The design of the diary was based on an upper respiratory symptom survey that had previously been validated [261]. The self-reported symptoms were not verified by an independent source and were not verified by laboratory analysis such as serological examination of plasma samples. Therefore it was not possible to determine whether the reported symptoms were due to viral infection of the respiratory tract or to some other irritation. Possible explanations for URS reportage by athletes are discussed below.

Reportage of URS in athletes may be partly explained by viral infection

A range of symptoms with varying degrees of severity are experienced when upper respiratory infections take hold. In order to help identify whether a viral infection was being experienced, when symptoms were reported in the wellness diaries, levels of the cytokine IFN-α were measured in the subgroups. Leucocytes produce IFN-α in response to viral infection in order to prevent further viral replication [262] (IFN-α levels are also elevated in response to other URTI pathogens). The IFN-α trend graph (see Figure 19 and Section 4.4) indicated that the three swimmers in the BC subgroup tended to have lower levels than the three swimmers in the placebo subgroup. However it is very difficult to interpret these results which are limited by the small numbers of participants (BC n=3, placebo n=3). Due to cost constraints, IFN-α analysis was not performed on plasma samples from the remaining participants in the cohorts.

Infectious causes of URS in symptomatic elite athletes may only partially account for the reportage of URS [89]. In a study of elite swimmers at the Australian Institute of Sport less than a third of URS episodes reported were

due to an upper respiratory tract pathogen [89]. If this rate is applied to the swimmers in the current study it is unlikely that there has been a significant effect of infection on salivary S-IgA levels in those reporting URS. It is also difficult to identify an effect of BC on immune protection when only a small percentage of symptoms may be a result of an infectious episode. It is therefore important to also consider the non-infectious causes of URS reported by elite athletes when investigating the immunomodulatory effects of BC.

Reportage of URS in athletes may be partly explained by exercise-induced increase in circulation of cytokines

There were no significant differences for average levels of IL-6 and IL-1ra at baseline compared to the other two cohorts (see Appendix 10.31). In adults IL-6 has an important role as a mediator of anti-inflammatory processes and metabolic functions [16, 105]. A recent study found that there were smaller changes in levels of IL-6 during and following exercise in child athletes, thought to be indicative of their resistance to the inflammatory response of exercise [16]. It is important that the effect of the inflammatory response on anabolic mediators necessary for growth such as IGF-1 is minimised in young athletes [16]. This could partly explain why post-exercise levels of IL-6 measured in the swimmers were similar to the age matched controls and older adults.

After four weeks of supplementation post-exercise levels of IL-6 in the swimmers cohort were significantly associated with IL-1ra (P=0.006). In a previous study of 265 primary health care patients (91 men and 174 women) it was found that feelings of self-rated health were associated with levels of circulating cytokines that are involved in inflammation and sickness behaviour [106]. This relationship was significant only for the women patients; those that reported poorer health also had higher levels of IL-1β, IL-1ra and TNF-α [106]. Exercise-induced increases in levels of circulating cytokines may be similarly associated with feelings of sickness rather than being a result of increased URTI symptoms [263]. It is possible that some of the URS reported by the swimmers might be explained by an exercise-induced circulation of cytokines, however interpretation of the cytokine results is limited by the small number of participants in the subgroup.

Reportage of URS in athletes may be partly explained by irritated airways

Inhalation of cold air [263] or chloramines in the swimming pool environment [264] are both thought to be associated with an increase in URS reportage. Irritation of mucosal surfaces in the respiratory tract may partly explain some of the URS reported by the swimmers in the current investigation. A recent study in Quebec found that monochloramines formed in the swimming pool environment (from the reaction of chlorine with organic matter) were associated with increased reportage of URS in young elite swimmers compared to similarly aged soccer players [264]. Monochloramines are known to interfere with DNA repair processes [265], and there has been some suggestion that there may be a link of chloramine inhalation with occupational asthma in swimming pool life guards [264]. There was no association of reported URS with airway hyper-responsiveness (AHR) in the study by Levesque *et al.*, 2006 and no indication as to whether the reported URS was due to viral infection [264]. The swimmers did train at a higher intensity, compared to the soccer players and training sessions were of longer duration which may explain some of the difference noted.

The nature of swim training is that it involves a large percentage of interval-work where exercise is performed at greater than 90% VO_2 max compared to other sports [266] (Table 29).

Table 29: Intensity levels (VO_2 max) at which exercise is performed at for different sports

Sport	Intensity level
Marathon runners	70-80% VO_2 max [267]
Soccer players	75% VO_2 max [268]
Swimmers	Interval training has been measured as being at about 92% VO_2 max [266]

It is thought that there is increased risk for respiratory tract damage when exercising above 77% VO_2 max [267]. The human lung has a surface area of about 50-100m^2 and is only 0.2-0.3 µm thick, as it is designed to provide efficient gas exchange, but at extreme levels of exercise there is an association with increased susceptibility to mechanical damage of pulmonary capillaries [267]. Therefore some of the URS reportage by the swimmers in this current

study may have been due to both the intensity of training in these athletes and to the presence of environmental chloramines. However no measures of exercise intensity were performed in the current study which limits the interpretation of the URS results.

There are different types of physiological stress experienced by swimmers during exercise, compared to other sporting codes such as distance running, which could affect mucosal surfaces differently. The swimmer may be more at risk for damage to mucosal tissue in the respiratory tract, as a result of the high training intensity and the training environment, compared to the physiological effects experienced by the distance runner (see Section 5.11). Some of the URS reported by the swimmers in the current study may have been as a result of irritated airways. Two of the placebo swimmers were training at a different swimming pool to all of the other swimmers and under a different coach, which is likely to have further confounded these results particularly as they also reported the highest number of days of URS.

5.7.2 The effect of the influenza vaccination on URS reportage in the older adult cohort

There were no significant differences in URS reportage between the BC and placebo groups in the older adult cohort. There was a non-significant increase in URS reportage after the vaccination period in both the BC and placebo groups which may have confounded any effect of the BC supplement (see Figure 18 and section 4.3.2). This highlights a problem with longitudinal studies of this nature. The aim was to determine the effect of BC on levels of salivary S-IgA in older adults prior to winter as this period was a similar time of the year compared to the previous study with middle-aged distance runners [1]. Influenza vaccination is encouraged in older adults because of age-impaired immunity and in this study 22 of the 45 volunteers reported that they were vaccinated. In longitudinal studies investigating nutrition interventions on immune function in older adults, those intending to have an influenza vaccination could be excluded. Alternatively the study could be run earlier in the year ahead of the vaccination period.

5.8 Plasma cortisol levels in the swimmers subgroup (investigation of the mechanism by which BC supplementation may modulate mucosal immunity)

In this current study the post-exercise levels of plasma cortisol in the swimmers subgroup were significantly lower in those who consumed the BC supplement (n=3) compared to those who consumed the placebo (n=3). This time-related difference was observed after four weeks of supplementation. It was not possible to compare plasma cortisol results at the end of supplementation, as blood sampling was performed outside of the specified sampling times, and some swimmers were not available until the afternoon, plasma cortisol levels are affected by diurnal variation.

For the swimmers in this study it is possible there have been beneficial effects of consuming the BC supplement on the physiological stress of an intensive training session. Elevated levels of plasma cortisol may negatively affect immune function. For example the transport of S-IgA across the epithelium [103] and TGF-β enhanced expression of secretory component, which is also involved in the transport of S-IgA, are affected by high levels of cortisol [185]. Therefore there may be an overall depressive effect of high circulating levels of cortisol on levels of salivary S-IgA.

Supplementation with nucleotides in endurance athletes was shown to have an effect on post-exercise salivary levels of cortisol (attenuated) and salivary S-IgA (enhanced) [118]. BC contains significant levels of the nucleotides adenosine, cytidine and uridine (see Table 6 and Section 1.7.6), although levels of these in the BC supplement and placebo used in this study were not known. This study, however, this study was not designed to investigate the effect of nucleotides on cortisol. It is also difficult to interpret the differences in plasma cortisol observed in due to the small number of athletes in the subgroup (BC n=3, placebo n=3). Measurements of plasma levels of cortisol may be a useful marker of the physiological stress of exercise in future studies of the effect of BC supplementation on immune protection in athletes.

5.9 Lymphocyte subsets (investigation of the mechanism by which BC supplementation may modulate mucosal immunity)

There were no significant time-related changes for the percentages and absolute numbers of lymphocyte subsets, within any of the cohort subgroups. There was a significant association of the CD4/CD8 ratio and of the %CD8 cells with plasma IGF-1 levels in the swimmers subgroup at baseline (see Table 24 and section 4.4). There were also age related differences between the cohorts at baseline. This was seen in: the number and percentage of CD3/CD4 cells, the percentage of CD4, CD8 and CD19 cells expressing the alpha integrin marker and in the CD4/CD8 ratio (see Section 4.4 and Appendix 10.30).

Association between CD4/CD8 ratio and plasma IGF-1, and %CD8 and plasma IGF-1 in the swimmers subgroup at baseline prior to commencement of supplementation (n=6)

There was no significant effect of consuming the BC supplement on numbers or percentage of lymphocyte cells. There was a significant (*P*=0.029) positive association (r^2=66.7%) between plasma levels of IGF-1 and the CD4/CD8 ratio. There was also a significant (*P*=0.013) negative association (r^2=77.8%) between plasma IGF-1 and %CD8 cells at baseline (see Table 24 and Section 4.4). This suggests there is a relationship between plasma levels of IGF-1 levels and %CD8 cells in these swimmers.

A possible explanation for the negative association of plasma IGF-1 with %CD8 cells is that there has been an effect of growth hormones. In aged rats and monkeys growth hormones stimulated T-cells and their function, and may actually have countered the immunosuppressive effects of cortisol [221]. Daily administration of growth hormones to dexamethasone-treated rats had the effect of increasing the CD4/CD8 ratio, and it was proposed this was mediated by IGF-1, as increased IGF-1 levels occurred with the administration of the growth hormone [221]. Exercise may prime circulating leucocytes to express cytokines and growth factors, possibly to prepare for the physiological stress of exercise [16, 99, 269]. However interpretation of the associations observed in

this study is limited by the small numbers of swimmers in the subgroup (BC n=3 and placebo n=3).

BC supplementation prevented post-exercise induced reduction in the percentage of CD8 cells compared to the placebo group in a study with endurance cyclists [217]. The cyclists who consumed BC also tended to report less URS compared to the placebo group. Cytotoxic T-cells secrete a Th1 cytokine profile which can down-regulate the binding site for rhinoviruses. It was proposed that this was a mechanism by which the cyclists reported less URS [217]. In this current study the mean post-exercise %CD8 cells were higher for those consuming the BC supplement compared to the placebo, however this difference was not significant or time related. However as there were only a small number of athletes in the subgroup it is not possible to determine if there has been an effect of the BC supplement on the %CD8 cells.

Association between plasma IGF-1 and IL-6 levels in the swimmers subgroup (n=6)

There was a significant (*P*=0.040) positive association (r^2 = 61.7%) of plasma IGF-1 and IL-6 levels in the swimmers subgroup after four weeks participation in the BC intervention trial. IL-6 stimulates the release of IGF-1 from its insulin-like growth factor binding protein-1, in order to make it available to support transport of glucose to the muscle post-exercise [204]. As blood was sampled from the swimmers after their morning training session, this association may be partly explained by the need to supply glucose to fatigued muscles.

Therefore together the associations observed in this study between plasma IGF-1 and IL-6 levels, and plasma IGF- 1 levels and %CD8 cells support previous observations that the relationship between immune cells, expression of growth hormones and cytokines, glucose metabolism and cortisol production in the athlete is complex. The effect on potential immune depression of disturbing this relationship in athletes requires further investigation. In this study there was no effect of consuming the BC supplement on plasma IGF-1, IL-6, the CD4/CD8 ratio and the %CD8 cells. However it is difficult to interpret these

results because of the small numbers of swimmers in the subgroup (BC n=3, placebo n=3).

Age-related difference in percentage of lymphocyte subsets expressing the integrin marker

A comparison between the cohort subgroups results at baseline (prior to the commencement of supplementation) for percentage of CD4, CD8 and CD19 lymphocyte subsets, which also expressed CD49d, indicated that results for the older adult cohort were significantly lower compared to the swimmer and student cohorts. The most noticeable difference was in the expression of CD49d on the CD4 cells; the mean percentages of cells expressing CD49d were: for the older adult group (70.56%); swimmers (89.54%); and students (86.37%). There could be a negative effect of ageing on the expression of the integrin marker on lymphocytes which may affect their homing ability. This finding is in agreement with studies measuring α4β7 integrin expression on T-cells and B-cells in the peripheral blood of aged rats [135]. No reports of this age-related difference in expression of alpha integrin markers on human lymphocytes could be found in the literature. Age-related differential expression of integrin markers may affect the homing ability of lymphocytes to distal mucosal sites and impact on the immune health of older adults (see Section 5.10).

Age-related differences in the CD4/CD8 ratio in peripheral blood

Results for the subgroups indicated that the CD4/CD8 ratio was significantly higher in the older adults compared to the swimmers and students at baseline. The mean ratio was 3.61 compared to 1.90 and 2.10 respectively. This was in agreement with a previous study by Meyer and Soergel,1999 which also found differences between the ratios for nineteen 19-36 year olds and fifteen 63-80 year olds (mean ratios were 1.4 and 2.0 respectively) [129]. There were more participants in the Meyer and Soergel, 1999 study [129], the smaller sample size in this current study may explain some of the larger CD4/CD8 ratios obtained. Meyer and Soergel [129] also found the ratio of CD4/CD8 cells was significantly increased in the bronchoalveolar lavage fluid of the older adults compared to the younger participants, a change mostly due to an increase in

numbers of CD4 cells [129]. This suggests that there is a degree of immune system remodelling in older adults whereby CD4 cells accumulate in the air spaces of the lower respiratory tract of healthy older adults, possibly to provide improved immune protection [129]. In this current study the CD4 cells measured in blood sampled from the older adults expressed significantly less homing integrin. This could indicate that there are fewer CD4 cells able to migrate and accumulate in the air space of the lower respiratory tract where they may participate in the regulation of antibody production.

Difference in absolute numbers of B-cells

There were no time related differences for the absolute numbers of B-cells within the swimmer and student cohort subgroups. However there was an almost significant difference (*P*=0.052) for the change in absolute numbers of B-cells after four weeks of supplementation in the older adult subgroup. The change in absolute number of B-cells in the BC group was greater than the placebo group after four weeks supplementation. After controlling for those who had an influenza vaccination the difference was still nearly significant (*P*=0.057). Unexplained increases in B-cells require further investigation in older adults, an increased incidence of autoimmune diseases occurs with ageing partly due to an enhanced ability of B-cells to produce auto antibodies [123].

Future studies investigating the use of a BC supplement and its influence on immune function in older adults should include the effect to B-cell numbers in the peripheral blood and assess the risk for autoimmune diseases. Those participants intending to have an influenza vaccination during the course of the study could be excluded or alternatively the time of year that the study would run could be changed

In summary, there was no effect of BC supplementation on any of the lymphocyte subsets measured in the peripheral blood between the BC and placebo groups within any of the cohorts. The association between the %CD8 cells, CD4/CD8 ratio and plasma IGF-1, and IGF-1 and IL-6 in the swimmers indicates the effect of exercise on immune function is complex. However,

interpretation of the results is limited by the small sample size (BC n=3, placebo n=3). The age-related differences observed for the percentage of lymphocytes expressing alpha integrin have not been reported previously and require further investigation to determine if this is an effect on the homing ability of CD4 cells to the respiratory tract. Further research is also required to determine if there is any effect of BC on autoimmunity in older adults.

5.10 The effect of ageing on the homing ability of lymphocytes

For all of the lymphocyte subsets measured the percentage of cells also expressing alpha integrin was significantly lower in the older adult subgroup (n=10) when compared to both the students (n=10) and the swimmers (n=9) (see Appendix 10.31). The expression of CD49d on cells also expressing CD3/CD4 appeared to be the most affected in the older adults. While there is still much to be learned about the effect of ageing on mucosal immune function, ageing may have an effect on the homing ability of lymphocytes. A 30% reduction in expression of α4β7 on T-cells and B-cells in the peripheral blood of aged rats compared to young rats has been observed [135], which may affect mucosal immunity at various sites. The most striking change in intestinal mucosal immunity with ageing in rats was found to be a reduction in the ability of immunoblasts to home back to the intestine, due to reduced expression of integrins [135], (see Table 30).

It is thought that ageing affects gut motility and bowel transit times [136], which could impact on intestinal tissue renewal processes. Intestinal mucosal immunity may be further affected because of the reduced ability of lymphocytes to home to distal sites [84]. Successful ageing in healthy older adults is associated with accumulation of CD4 cells in the BALT [128]. It may be possible that homing of lymphocytes to this site is also affected by the age-associated down-regulation of alpha integrin expression. In this study the percentage of CD4 T-cells expressing the alpha integrin in the older adult cohort ranged from 64 to 71%, which was significantly (*P*=0.011) less than the students (83 to 86%) and the swimmers (86 to 90%). Interestingly however, there was no effect of ageing on levels of salivary S-IgA. Mean levels for the older adult cohort at baseline were significantly higher than in the student or swimmers cohort. This suggests that the ability of older adults to produce antibody is not affected.

Table 30: Comparative effects of ageing on the intestinal mucosal immune response in aged rodent models and with various markers of changes in mucosal immune function measured in older adults in this study

	Intestinal immunity in the rat-model (Schmucker *et al*., [84])	Older adults who participated in this study.
Uptake of antigens by M-cells overlying PPs	Lack of research	Not investigated in this study
Antigen presentation by dendritic cells and lymphocyte isotype switching	Unclear	Not investigated in this study
Maturation of IgA immunoblasts and migration from PPs to intestinal mucosa	Compromised in old animals. L-selectin and α4β7 expression is diminished	Reduced expression of α integrin on CD4 and CD8 cells in peripheral blood
Local antibody production by mature plasma cells	Unchanged	In this current study, levels of salivary S-IgA were significantly higher in the older adults compared to the young adults suggesting that there is no adverse effect on the ability to produce antibody with ageing
Transport of IgA across epithelial cells via receptor-mediated vesicular translocation	Unchanged	As levels of salivary S-IgA were not reduced in the older adults there is no reason to suspect transport across the epithelial cells is affected

5.11 The possible effect of prolonged intensive exercise on intestinal permeability and the homing ability of lymphocytes

There was no effect of consuming a BC supplement on expression of the alpha integrin marker (CD49d) on lymphocytes expressing CD3, CD3/CD4, CD3/CD8 and CD19 within the swimmers or students cohorts in this study. In the previous study investigating the effect of BC supplementation on levels of salivary S-IgA in middle aged distance runners, the levels of lymphocytes were not measured (Appendix 10.1). Immune remodelling begins in middle age; it is possible that expression of alpha integrin on lymphocytes in the distance runners would also have been affected. This could have an effect on cells involved in antibody production homing to distal mucosal sites.

There is an acute negative effect of prolonged intensive exercise on salivary S-IgA levels after a bout of exercise and this may reflect levels of S-IgA at other mucosal surfaces [4]. A key role for mucosal IgA in the gut is to regulate the microbiota [51]. Exercise-induced reduction in IgA levels after prolonged periods of intensive exercise could result in the expansion of populations of bacterial species that produce LPS. In addition, disruption to intestinal surfaces can further expose the mucosal immune system to bacterial products that may affect systemic immune function [51, 114]. Recently it has been shown that LPS affects expression of integrins on IgA producing cells [76]. Therefore increased levels of LPS in circulation could have consequences for IgA-producing cells involved in homing to the oropharyngeal region and could subsequently affect S-IgA production at those sites.

In this current study it was not possible to determine whether the self-reported URS symptoms were due to infectious episodes or to some other non-infectious exercise-induced inflammatory response. It may be that some of the URS could be explained by an inflammatory response induced as a result of an effect of exercise on intestinal levels of S-IgA. The BC supplement may have benefited by assisting with intestinal repair which would help with the restoration of immune homeostasis in the gut. It was not possible to determine from this study if there was an exercise-induced effect on intestinal mucosal integrity and

whether this is affected by BC. Future studies investigating the effect of BC on the immune system in athletes should determine how gut permeability is affected by different intensity and duration of exercise, and whether BC supplementation has an effect. It would be appropriate to measure levels of circulating LPS as well as to determine intestinal permeability e.g. by administering non-metabolisable sugars and measuring levels appearing in urine.

5.12 Measurement of dietary intake

As this study was an investigation of a nutritional supplement on levels of salivary S-IgA, it is important to interpret the results in the context of the whole diet. The aim of measuring dietary intake was to determine if there were significant differences to group data for energy, macronutrient and micronutrient intake within the cohorts, which may impact on mucosal immune function.

BC is a very complex mixture of nutrients and bio-actives that benefit the development of the bovine neonate. It is recognised that determining the effect of a single nutrient on immune function in humans is a difficult task [25]. Therefore the identification of any effect of BC would be more complicated unless a nutrient is present in sufficient quantity to be immunomodulatory. Further the pre-blended supplement used in this study contained levels of protein that may have been sufficient (15g/day) to impact on their daily protein intake, and therefore affect immune status, especially in the older adult cohort.

Two previous studies investigating the effect of a BC supplement on immune function in athletes have measured dietary intake, to establish that there were no confounding differences in nutrient intake between the intervention and placebo groups. The effect of 10g/day BC supplement was investigated on various immune parameters in cyclists [217] and of 20g/day BC supplement on various immune parameters including salivary S-IgA in endurance athletes [216]. In the latter study it was determined that labelled IGF-1 in the BC supplement was not absorbed by the athlete [216]. In several other studies investigating the effect of BC on sport performance, only some have determined the effect of BC in the context of the whole diet. For example, dietary intake was not measured in studies determining the effect of BC on tissue composition in resistance trained and untrained young men [270], but was recorded in a study of protein metabolism and strength performance in physically active men [271]. In this current investigation, EDEE was calculated for comparison with RDEI. This would provide an indication of the accuracy of the recording of dietary intake. Dietary intake was estimated from records (swimmers and students) and diet recalls (older adults).

5.12.1 Estimated daily energy expenditure (EDEE)

EDEE was determined using prediction equations to calculate basal metabolic rate (BMR). Both the Harris-Benedict and Schofield equations were used for each participant and the difference between EDEE and reported daily energy intake (RDEI) indicated the percentage of under- or over-reporting. An average difference was calculated for each participant because there was inconsistent variation in estimating energy expenditure by the two different prediction equations (see Appendix 10.32). For example a greater EDEE was found when Schofield compared to Harris-Benedict equations were used to estimate basal metabolic rate for the placebo male swimmers whereas a greater EDEE was found by Harris-Benedict compared to Schofield equation to estimate basal metabolic rate for the BC male older adults. The average difference became the correction factor that was applied to the reported results for macronutrients and micronutrients to estimate actual intake. Various other approaches have been tried previously to adjust for the error in results for estimating energy expenditure by prediction equations. This has included averaging results for basal metabolic rate determined using indirect calorimetry and prediction equations [272].

One of the best prediction equations to estimate basal metabolic rate for athletes has been the Cunningham equation [233]. However this equation requires lean body mass to be known. As lean body mass was not measured in this study the Cunningham equation was not suitable to use. The next best predictor of basal metabolic rate for active males and females is the Harris-Benedict equation which does not require lean body mass [234]. This is simplest to use when taking measurements from participants in their real-life environment, and was used in this study to estimate energy expenditure in all cohorts. The Harris-Benedict equation was also found to be a suitable predictor equation for adult participants involved in the Dietary Approaches to Stop Hypertension study during periods of weight stabilisation [235]. In this study there were no significant differences in mean body mass or mean body mass index between the BC and placebo groups within any of the cohorts, therefore the Harris-Benedict equation was considered appropriate to use for all cohorts.

The Schofield equations were also used to estimate basal metabolic rates for participants from all three cohorts. These equations were used by the National Health and Medical Research Council to estimate nutrient reference values for Australia and New Zealand [160]. Schofield equations were considered to be the best for predicting basal metabolic rate of healthy people by the WHO/FAO/UNU but may be limited in the upper and lower age ranges [231]. Recently it has been suggested that the WHO derived energy requirements over-estimate the energy needs for older adults due to the wide variety of activities performed [273]. In a study of older people, without major disability, a CV of 40.9% was found for activity-associated energy expenditure using the WHO equations [273].

Equations for estimating resting metabolic rate (BMR)

Harris-Benedict (1919) [233]

	Equation
Females	655.1 + 9.56(BM kg) + 1.85 (ht cm) – 4.68 (age yrs)
Males	66.47 + 13.75 (BM kg) + 5 (ht cm) – 6.76 (age yrs)

Schofield (1985) [231]

	Age (years)	**Equation**
Females	**10 - 18**	(0.056 x BM kg) + 2.898
	18 - 30	(0.062 x BM kg) + 2.036
	30 - 60	(0.034 x BM kg) + 3.538
	> 60	(0.038 x BM kg) + 2.755
Males	**10 - 18**	(0.074 x BM kg) + 2.754
	18 - 30	(0.063 x BM kg) + 2.896
	30 - 60	(0.048 x BM kg) + 3.653
	>60	(0.049 x BM kg) + 2.459

The activity levels of the healthy older adults in the current study also varied considerably, with activity factors ranging from 0.24 to 6.58. In part this may have been due to an over-estimation of energy expenditure by some participants who were very diligent in recording their activity. The highest level of activity was recorded by a 75 year old male who was employed as a caretaker (which involved a high level of physical activity for eight hours per day) (see Table 31). In addition he also worked out at a gymnasium two to three times per week. Because of the length of time he spent in physical activity during the day, his calculated activity factor was as high as that of the swimmers.

Table 31: Differences in RDEI compared to EDEE in an active older male and inactive older female

	PAL value	Equation used to estimate BMR	DR1	DR2
Active older male				
RDEI			10,789	8,359
Est. PAL	2.20	HB	8,565	8,574
		% difference	- 26	+ 3
		Schofield	13,666	13,677
		% difference	+ 21	+ 64
WHO PAL	1.56	HB	6,073	5,847
			- 77	- 43
		Schofield	9,318	9,326
			_ 15.8	+ 11.6
Inactive older female				
RDEI			6,390	4,984
Est. PAL	1.50	HB	8,492	8,511
		% difference	+ 24.7	+ 41.4
		Schofield	8,475	8,498
		% difference	+ 24.6	+ 41.4
WHO PAL	1.51			

PAL=Physical activity level
DR1=24 hour diet recall 1
DR2=24 hour diet recall 2
RDEI=Reported daily energy intake
Est. PAL=Estimated physical activity level
WHO=World Health Organisation

In this study physical activity level (PAL) was estimated from the calculated activity factor for each participant. This enabled all of the recorded activity to be accounted for in the estimation of energy expenditure. For the older adults this was important due to their participation in a large range of activities. WHO derived PALs underestimate activity [272], can be highly variable [274] and were not used in this study. The WHO derived PAL values for over 65 year olds (1.56 and 1.51 for males and females respectively) also did not appear suitable for the very active older participants. For the active older male (see Table 31), when the basal metabolic rate was estimated from the Harris-Benedict equation and used in conjunction with the estimated PAL, estimated energy expenditure was similar to the reported energy intake. By comparison when the basal metabolic rate was estimated by the Harris-Benedict equation and used in conjunction with the WHO derived PAL value, the energy expenditure estimated was notably lower than the reported energy intake (see Table 31). As there were no changes to body mass (BM1=76.6 kg, BM2=76.7 kg) for this participant during the supplementation period this supports using the estimated PAL values. There were no differences in estimated energy expenditure when using either the estimated or WHO PAL values for inactive older females (see Table 31).

Differences between reported dietary intake and estimated energy expenditure

The percentage difference between EDEE and RDEI was determined for each participant. It was found that both the BC and placebo groups within each cohort under-reported their dietary intake, but by variable amounts. The difference was not significant within the swimmer and student cohorts. Within the older adult cohort significant differences were found when both the Harris-Benedict and Schofield equations were used to estimate basal metabolic rates Both males and females in the BC group under-reported more compared to the placebo male and females (see Table 32) by both equations. The reason for this is not known.

Table 32: Summary of EDEE for the older adult cohort when HB and Schofield equations are used to estimate BMR. Significant differences *(P)* between BC and placebo (Pl).

Harris-Benedict				
	BC		Pl	
	Mean	SEM	Mean	SEM
F	-37.6	9.16	-11.9	8.7
M	-63.3	15.1	-29.9	10.6
P(group)		*0.015*		
Schofield				
	BC		Pl	
	Mean	SEM	Mean	SEM
F	-39.9	9.4	-14.7	7.89
M	-29.9	13.9	-19.9	9.01
P(group)		*0.027*		

Misreporting of dietary intake is characteristic of free-living subjects in nutritional studies. Some individuals either over- or under- report for various reasons irrespective of the dietary assessment method used [230]. In previous community-based studies a figure of 20% under-reporting of dietary intake has often been observed [230, 275, 276]. Under-recording and under-eating have been found to be particularly problematic for participants keeping food diaries [223, 275, 276]. When subjects are asked to record what they eat, changes in eating behaviour have been noticed, for example reduced consumption of foods that are sweet or contribute to dietary fat intake [224, 275]. In this study it is possible that some subjects changed their diet during the recording period, to appear to eat more healthily. There was no apparent reason for the significant difference in under-reporting between the BC and placebo groups in the older adult cohort. After correcting for under-reporting there were no significant differences in the dietary intake for macro and micronutrients that may have impacted on the effect of the BC supplement.

5.12.2 Comparison of reported daily dietary intake to the dietary guidelines

It was important to know whether the estimated dietary intake of macronutrients differed between the BC and placebo groups within each cohort. Increased physiological stress can be experienced by athletes with insufficient dietary

carbohydrate intake. High levels of pro-inflammatory hormones and cytokines have been measured in blood samples from athletes post-exercise when dietary carbohydrate intake is insufficient [153], and by this mechanism could contribute to a temporarily depressed immune system in athletes [12].

Reported macronutrient intake

There was no significant difference within the student cohort in the results for mean dietary intake over the supplementation period for energy and macronutrients between the BC and placebo groups. Due to the significant difference in under-reporting between the BC and placebo groups in the older adults, there could be a difference in macronutrient intake.

There was a significant difference in the results for mean reported protein intake between the BC and placebo groups within the swimmers cohort. In three previous studies [32, 216, 217] investigating the effect of BC on immune function in athletes, no differences were found between the BC and placebo groups for reported daily energy and macronutrients from dietary intake. However all these studies compared the contribution of macronutrients to the percentage of total energy intake. In this current study reported carbohydrate and protein intake were investigated as g/kg BM /day in order to assess the differences in the adequacy of nutrient intake for the individual's BM. The reported dietary intake for protein increased for the females (BC by 0.037 and placebo by 0.306 g/kgBM/day). For the males reported dietary protein intake decreased for the BC group, -0.282 g/kgBM/day and increased for the placebo group, 0.333 g/kg BM/day. These results indicated that the reported dietary intake for protein increased in the placebo group for the second period of dietary records. The reason for this is unknown, as reported intakes for both groups were within the guidelines it is unlikely that this difference impacted on immune function.

The male swimmers in the BC group reported a higher level of consumption of dietary carbohydrate (6.9g/kgBM/day) compared to the placebo group, (6.3g/kgBM/day), although the difference was not significant. A diet high in carbohydrate intake (12.0g/kgBM/day) was associated with less post-exercise

physiological stress in competitively trained male triathletes [153]. It is possible that there was some benefit to the males in the BC group of a higher dietary carbohydrate intake. The exercise-induced physiological stress experienced by these athletes may have been less than the placebo group. This may have contributed to the lower level of URS reportage by the whole BC group.

Reported micronutrient intake

There were no significant differences between the BC and placebo groups within any of the cohorts for dietary iron, Zinc, selenium, vitamin A, vitamin C, and vitamin E. It seems unlikely there has been any effect of intake of these micronutrients on immune function that may have confounded the results of consuming the BC supplement. This may not be the case for the older adult cohort.

The average reported dietary intake of selenium estimated from the two diet recalls for the older adult females indicated that their intake was below the EAR. The reported intake for the older females was below the EAR by 17%. In a community-based study of older women within the urban area of Dunedin, New Zealand, mean plasma selenium levels were found to be below that recommended for full expression of glutathione peroxidase [178]. Low levels of plasma selenium are thought to place the individual at increased risk for infection and other health-related issues [178]. Therefore despite the limitations of the dietary assessment method used in this current study (two days of diet recalls are too short in duration for sufficient information to be collected about the true dietary intake of selenium), these results indicate that further research is required in dietary intake of selenium in this age group throughout New Zealand.

In addition it is thought that moderate exercise diminishes lymphocyte apoptosis induced by oxidative stress possibly through the maintenance of glutathione peroxidase content in the lymphocytes [93]. In future studies of selenium status in New Zealanders it would be relevant to determine the effect of exercise and ageing on the expression of glutathione peroxidase and whether these levels could be affected by dietary intervention.

6.0 Conclusion

The aim of this study was to investigate the effects of a nutritional supplement, BC (in the context of the whole diet) on levels of salivary S-IgA in potentially immune-depressed groups. Previously, levels of salivary S-IgA were significantly enhanced in middle-aged distance runners consuming a BC supplement. The results in this current study demonstrated that the effect of BC supplementation is not repeatable in young swimmers, age-matched non-exercising controls and healthy older adults. However it was not possible in this study to recruit the required number of participants (fifty in each cohort). This sample size was estimated from the power calculation performed with the results from salivary S-IgA levels measured in the recreational marathon runners.

The results for the analysis of bio-active levels in the BC supplement used in these trials indicated there were large differences between the BC flavour variants (including TGFβ-1, TGFβ-2 and IgA levels). In addition, for some bio-actives (e.g. TGFβ-1) there were no noticeable differences to the levels in the placebo. As BC is a natural product, levels of bio-actives vary between breeds of cows, are affected by stage of lactation, and feeding. In addition there are no recognised reference test methods for analysing levels of bio-actives in BC [254].

Therefore interpretation of results for this study are limited as is the comparison of results for immune parameters measured to those measured in other BC intervention studies. This highlights the difficulty for identifying the immune modulating effects of a food or food component. Future research should focus on identifying immunomoduatory bio-active(s) with approved reliable analytical methods. Bio-active levels should be different to the placebo and at a concentration that will be immunomodulatory.

The swimmers BC group reported fewer days of URS compared to the placebo group although this difference was not significant. Within all cohorts additional

analyses were performed on a subgroup. Within the subgroup of swimmers, those athletes consuming a BC supplement experienced significantly lower exercise-associated increase in cortisol levels compared to the placebo. The interpretation of this result is limited due to the small subgroup sample size (BC n=3, placebo n=3).

A large proportion of URS reported by elite athletes appears to be due to non-infectious causes (approximately 70%) [89]. Therefore it seems likely any benefit experienced by the swimmers consuming a BC supplement in this study was to exercise-induced non-infectious responses. The causes of non-infectious URS in elite athletes require further investigation to identify inflammatory conditions that may impact on immunity or performance.

A non-infectious inflammatory response may result from impaired intestinal mucosal protection during bouts of intensive exercise. In athletes reduced gastric blood flow has been observed after prolonged intensive periods of exercise and increased levels of LPS have been measured in circulation. This suggests that the intestinal mucosal barrier has been disrupted facilitating the permeation of luminal LPS. It is also possible that intestinal levels of IgA have been affected resulting in an increase in the presence of LPS producing gram negative bacteria. Levels of S-IgA in saliva reduce immediately after exercise and this may reflect changes at the gastrointestinal surface. It is now thought that along with its antimicrobial role in saliva that decreases in salivary S-IgA may be a proxy measure of disturbance to the systemic immune response.

BC may therefore play an important role in ameliorating the effect of intensive exercise on the inflammatory response. This may occur through protection of the gastrointestinal tract in two ways. Firstly BC contains a number of growth factors (e.g. TGFβ, EGF) and nucleotides all of which may have a role in stimulating intestinal repair. Regulatory peptides are normally expressed in the mucosa. If expression is affected by intensive exercise peptide growth factors in BC may be of assistance. Secondly there are a number of humoral anti-microbial factors in BC that could potentially assist with restoring immune homeostasis if levels of IgA are affected. IgA in the lumen regulates the

microbial community, neutralises LPS located inside epithelial cells and therefore prevents over stimulation of the non-mucosal immune system.

These results indicate the effects of a BC supplement on immune function are not the same across different age groups or different sporting codes. It is known that intestinal permeability is affected by the physiological stress experienced by runners. An explanation for the effect of the BC supplement, seen previously in the distance runners may be that it enhanced intestinal repair. As a result levels of intestinal S-IgA may have been restored and changes to these levels could possibly have been reflected in salivary S-IgA levels.

Swim training may place greater physiological stress on the respiratory tract compared to distance running; it is not known if swimmers experience the same levels of gastrointestinal disturbances as distance runners or whether there is an age-related effect. BC may have enhanced intestinal mucosal repair processes in the swimmers and as a result minimised local and systemic immune responses. This would partly explain the trend towards lower level of URS reportage in the swimmers taking the BC supplement compared to the placebo. This is in agreement with the results from a study that found more URS was self reported by elite swimmers as a result of non-infectious compared to infectious causes [89]. As it was not possible to determine the cause of the URS reported by the swimmers in this study, this is speculative.

The results from this study suggest there has been no direct effect of BC on levels of salivary S-IgA. BC contains many bio-active factors that have a role in the development of the bovine neonate's gastrointestinal tract such as TGFβ, IGF-1, EGF and nucleotides. Some of these molecules are also similar in structure with the human form (e.g. TGFβ) [187]. There is an indication that some BC Igs remain intact in the intestine after passage through the human stomach [186], so it may be possible that other bio-active components survive transit. During intestinal repair there is a shift of receptors from basolateral to apical membranes; this is to assist with the binding of molecules involved in repair such as EGF [65]. Clinical studies in patients administered NSAIDs

indicate reduced intestinal damage when consumed with BC [214]. Together this suggests it is possible bio-actives in BC could have a physiological effect in the repair processes of the adult human intestine.

In this study there was no significant effect of BC supplementation on levels of salivary S-IgA or URS in the older adults. A significant increase in B-cell numbers occurred after four weeks of BC supplementation in the older adult cohort. The ability of these B-cells to produce auto-antibodies requires investigation as autoimmune diseases occur more frequently with ageing.

Reduced expression of alpha integrins on lymphocytes was observed in the older adults compared to the students and swimmers. This did not appear to affect salivary S-IgA levels but may have had an effect on the ability to produce specific S-IgA in response to infection. This finding is in agreement with the reduction in expression of homing integrins on lymphocytes observed in aged rats. It is possible that reduced lymphocyte homing-ability may impact on resistance to respiratory tract infections in older adults and this requires further investigation.

There may have been a confounding effect of significantly lower plasma IgG levels observed in the BC group from the student cohort compared to the placebo group. The mean IgG level in the BC group was at the low end of the typical physiological range. This may have impacted on the higher URS reportage in the students consuming the BC supplement. Reportage of URS in the older adult's cohort appeared to be increased in those who had the influenza vaccination during the study. In future longitudinal studies of a nutrition intervention on immune function it may be advantageous to include these as exclusion criteria.

The effect of BC on immune function in the older adult cohort may also have been confounded by their dietary intake. Selenium intake from the diet appeared to be below the recommended guidelines for the female older adults. It is difficult to confirm this as there were limitations in the method used to estimate energy expenditure with prediction equations. This was largely due to

the wide variation in activity performed by this cohort. In future studies alternative means of determining BMR could be considered. Applying existing PAL values to very active older adults was also problematic.

While it is difficult to interpret the results from this study due to the small number of participants and the variable bio-active levels, it may be the benefits of a BC supplement are limited to athletic groups (or possibly to clinical patients). If the temporarily altered immune function experienced by athletes can be partly explained by impaired intestinal repair processes resulting in local and systemic immune responses, there may be a role for BC supplements in post-exercise recovery. Future investigations should focus on identifying the immune-modulating bio-active component(s) in BC, and any synergistic effect of the different bio-actives. Additonally future research should consider the complex relationship of intensity and duration of exercise and age on immune protection.

7.0 Limitations of the study

There were a number of limitations to this study investigating the effect of a BC supplement on levels of salivary S-IgA. This included the small number of participants within each cohort, the variability in the measured bio-active levels between flavour variants in the BC supplement, the small differences in bio-active levels between the placebo and BC supplement, the unavailability of the students and swimmers towards the end of the intervention period, the influenza vaccination in many of the older adult participants, and the variability in level and amount of exercise of the older adult participants.

The small numbers of participants recruited for the two studies made it difficult to determine the effect of a nutrition intervention separate from physiological variation. For example there may have been no differences between the BC and placebo groups for plasma IgG levels (within the student and older adult cohorts) with more participants. The differences observed for the haematological parameters between the BC and placebo groups in the older adult cohort may not be present in a larger group.

BC contains many bio-active factors that may affect immune function and gastrointestinal repair processes. Therefore it is important to identify which bio-active is likely to have the most effect, and or whether there is a synergistic effect with other bio-active(s) on the immune parameter being monitored. Levels of the identified bio-active(s) should be known prior to the commencement of an intervention trial and should be at levels sufficiently different to the placebo. For example in this study the bio-active results indicated there were no differences in TGF-β1 levels between the BC and placebo.

Due to time limitations it was not possible to run the supplementation trials for the swimmer and the student cohorts at the same time of year as the distance runners (the previous study was run from February to May, summer to autumn). The delayed start of the trials (September to January, spring to summer) meant

that the end of the trial overlapped with final examinations and the end of year when the participants were starting employment or travelling out of Auckland. Many participants were not available for the final sample days and alternative arrangements needed to be made.

Differences in the fitness levels of the swimmers in this study were not controlled for as baseline fitness parameters were not measured. It was hoped all swimmers would come from one swimming club and be coached by the same swimming coach. It was not possible to recruit sufficient numbers from one club. While there were no differences in the calculated activity factors between the BC and placebo groups within the swimmers cohort their baseline fitness may have differed. There may have been differences in adaptations of the respiratory tract to the physiological demands of previous training cycles [267] that could have impacted on immune function. Measures of aerobic capacity such as VO_2 max at baseline would have helped to determine any variability in fitness level between the groups.

There was a large range in the amount and type of physical activity performed by participants in the older adult cohort. Converting the recorded physical activity into an activity factor meant it was possible to determine whether there were differences between the BC and placebo groups. While no significant differences were detected, those older adults who were exercising the most may have experienced more benefit to immune function than those exercising less [277].

The second part of the study, investigating BC supplementation on immune protection in older adults, was carried out from April to July (Autumn to Winter) and included the period when influenza vaccination occurs. It was not anticipated that large numbers of the cohort would receive the vaccination. It seems reportage of URS in the older adult subgroup may have been affected after the vaccination and would likely have confounded any effect of BC. Exclusion criteria for future trials should take this into consideration. It may be appropriate to only include those who have been vaccinated.

In this longitudinal study the URS were self-reported, and the symptoms were not verified medically or serologically. Therefore it was not possible to determine whether the URS were due to viral infection or to some other irritation or inflammation of the respiratory tract.

A mix of males and females were included in this study (most other studies of BC on immune function have studied both genders together) and there was no effect of gender on levels of salivary S-IgA. However for younger age groups, it is difficult to interpret the effect of a nutrition intervention on immunological status in athletes [16].

8.0 Recommendations for future research

Before further investigations of a BC supplement on immune function in athletes or other cohorts is undertaken, the bio-active(s) under investigation should be determined and the levels in the supplement established. It is possible that the effect is not the result of a single bio-active and a synergistic relationship should also be investigated. This could involve the study of the regenerative effects of BC on human cell-lines. After identification of a potential bio-active(s), it must be possible to measure levels in the supplement and to ensure that it is present in sufficient quantities and significantly different to the placebo to prove efficacy.

The safety of the effect of a BC supplement on immune function in older adults and other populations at increased risk for autoimmune diseases should be investigated.

In longitudinal studies of this nature a greater number of participants should be recruited. This would help to ensure normal physiological levels of the parameter under investigation do not confound the effect of the supplement.

In investigating the effect of BC on levels of salivary S-IgA it is important to determine if there is an effect of URS. However in athletes a large proportion of URS may be a result of non-infectious causes. Therefore it is essential that the cause of the URS is established. This may be achieved by serological examination of blood samples in addition to the use of a validated wellness questionnaire.

Including or excluding only those who intend to have influenza vaccinations should also be considered. Alternatively the trial could be run from summer to autumn prior to the period of vaccination.

The effect of disturbing the relationship between immune cells, expression of growth hormones and cytokines, glucose metabolism and cortisol levels on potential immune depression in athletes requires further investigation. In this study there was a difference in post-exercise plasma cortisol levels after four

weeks of BC supplementation in the swimmers subgroup (BC n=3, placebo n=3), the role of BC in post-exercise recovery could be investigated further. As there may be an effect of gender in younger cohorts, males and females should be studied separately.

In future studies of a nutrition intervention on immune function in New Zealanders, selenium (a co-factor for the antioxidant enzyme glutathione peroxidase) status should be established as a baseline measure. It is important to identify whether dietary intake of selenium meets the recommended guidelines. Moderate exercise enhances the expression of glutathione peroxidase in sedentary individuals, therefore activity levels should be identified in future studies investigating a supplement on immune protection.

Due to the small numbers of athletes in the subgroup (BC n=3, placebo n=3) it was not possible to determine whether the BC supplement had an effect on plasma cortisol levels, circulating levels of neutrophils and cytokines (IL-6, IL-1ra). Future investigations of the effect of BC on immune protection (in larger numbers of athletes) could include these parameters as markers of the effect of the physiological stress of exercise on immune function. A comparison of pre-exercise with post-exercise levels should be made.

It is possible that there is an effect of prolonged intensive exercise on intestinal repair and permeability that could have an inflammatory effect. This may affect local and systemic immune function. Production of salivary S-IgA could be diminished which would affect the gut microflora further contributing to inflammation and permeability. The effect of a BC supplement on intestinal permeability in athletes from different sporting codes should be investigated in future studies. Markers such as circulating levels of LPS could be measured. The degree of intestinal permeability could be monitored by administering large indigestible sugars such as raffinose, and measure levels that appear in urine.

The expression of CD49d on cells also expressing CD3/CD4 could be monitored in older athletes. Ageing appears to have an effect on expression of the alpha integrin; it is not known what would be the combined effect of ageing and prolonged intensive exercise.

Adequately recovering from bouts of intensive exercise is the key to improving an athlete's performance. While the interpretation of the URS result from this study is limited, because of the small number of participants and the unknown cause of URS, the results suggest there was a trend towards a lower reportage of URS when consuming a BC supplement. Therefore further investigation is required to determine whether BC may be a useful aid in post-exercise recovery. The role of BC in ameliorating non-infectious symptoms of URS experienced by athletes has not been studied.

9.0 References

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10.0 Appendices

Appendix 10.1
The effect of bovine colostrum supplementation on salivary IgA in distance runners

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The Effect of Bovine Colostrum Supplementation on Salivary IgA in Distance Runners

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Secretory IgA in saliva (s-IgA) is a potential mucosal immune correlate of upper respiratory tract infection (URTI) status. Nutritional supplements may improve mucosal immunity, and could be beneficial to athletes who are at increased risk of URTI. In this study, 35 distance runners (15 female, 20 male, age 35 to 58 y) consumed a supplement of either bovine colostrum or placebo for 12 wk. Saliva samples were taken prior to training at baseline, monthly during supplementation, and 2 wk post supplementation. Median levels of s-IgA increased by 79% in the colostrum group after 12 wk intervention, and the time-dependent change from baseline value was significant ($P = 0.0291$). This significance was still apparent after adjusting for training volume and self-reporting of upper respiratory symptoms. This study has demonstrated increased s-IgA levels among a cohort of athletes following colostrum supplementation. While this result is statistically significant, its physiological interpretation must be viewed with caution due to the small numbers in this study and the large variability in s-IgA levels.

Key Words: dietary supplementation, upper respiratory tract infection, upper respiratory symptoms, mucosal immunity.

Exercise when performed at strenuous levels can act as a stressor that may temporarily alter the immune system. The athlete experiences a transient period of depressed immunity that is usually reversed with rest. This stress response is exacerbated if the athlete is under psychological stress, has poor nutrition, is lacking sleep, or is in an unfamiliar environment during competition. Moderate levels of exercise appear to improve the immune response, but when exercise is performed at intensive levels a higher incidence of upper respiratory tract infection (URTI) has been observed (28) which is possibly as a consequence of the transient reduction in immune defense. For the athlete, this is of concern if the illness occurs close to a competition as performance can be affected.

While many immune parameters are altered by strenuous exercise, the association with an increased incidence of URTI has not been conclusive. The exception

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has been the effect of exercise on mucosal immunity. Some mucosal immune parameters are depressed after exercise, including reduced salivary immunoglobulin A (s-IgA) concentrations and a lower overall secretion rate, and it has been speculated that temporary deficiency of antibodies at the mucosal surfaces could make individuals susceptible to infection from pathogenic viral and bacterial organisms (15-17, 24).

Upper respiratory tract infections in athletes are usually of viral origin and the presentation of symptoms occurs within a few days of a viral infection. The symptoms experienced are a result of disruption to the normal function of infected cells and the body's immune response to rid itself of the infection (17). Upper respiratory symptoms (URS) are typically sore throat, cough, runny nose, fever, and headache (17, 30). In athletes, URS can last from a few days to 1 to 2 wk and many studies of URTI in athletes have generally relied on self recording of URS which have not been verified by a physician. In longitudinal field studies such as this preliminary one it may not always be possible to involve a physician, and so establishing true URS depends on the reliability and accuracy of the records kept by the athlete.

Various nutritional supplements have been studied for their potential to enhance immune function in athletes. Carbohydrate supplementation immediately before, during, and after exercise has shown the most promising results by attenuating the stress response (29). In distance runners, levels of cortisol, interleukin (IL)-6, and IL-1ra were attenuated after 2.5 h running in those runners who were supplemented with a drink containing 6% carbohydrate (27). Modulation of the cortisol response could improve immune function as it is known to have a depressive effect. In addition, adequate dietary carbohydrate intake in the days leading up to a strenuous bout of exercise is also important in attenuating the stress response (1, 3). However, no effect is seen on levels of s-IgA with carbohydrate supplementation in distance runners after completing a marathon (31).

Recently the effect of bovine colostrum supplementation on immune function in athletes has been investigated (5, 25, 26). Anecdotal reports suggest that many athletes take bovine colostrum to help their immune system. Two reports also suggest that bovine colostrum improves the health status in female endurance athletes (25) and in active males (5) although the physiological reason for this was not elucidated. Bovine colostrum has been shown to improve performance in 30 male endurance runners after a second bout of exercise. It was thought that this was due to improved recovery mechanisms (6), which possibly could include improvement of immune function due to less physiological disturbances.

Another study has shown that supplementation with bovine colostrum in a group of athletes for 3 wk resulted in a 33% increase in mean levels of s-IgA (25). However, there were a number of limitations to this study; there was no discussion of how the quality of the saliva was controlled for (e.g., with respect to the effects of dehydration and URS symptoms), the athletes were from a range of sports, and the placebo used was maltodextrin and therefore did not have the same macronutrient profile as the intervention. Nevertheless, as s-IgA plays an important part in mucosal immunity, this is a significant finding and the effect of bovine colostrum on s-IgA levels needs further investigating in specific sports, in a normal training environment, in different age groups, and with various periods of intervention. Because concurrent URS may artificially elevate s-IgA levels and confound the

interpretation of diet-mediated effects, it is also important to establish a methodology to control for this variable.

Bovine colostrum is the first milk produced during lactation in cows and contains many bioactive factors and immunoglobulins which may be relevant to mucosal immunity, such as cytokines and transforming growth factor (TGF-β). Further, in vitro studies have shown that the addition of TGF-β as found in bovine colostrum can stimulate human lymphocyte cultures (38). To date the mechanism for how bovine colostrum could affect the immune system in humans has not been elucidated, although in animal model studies dietary bovine whey proteins have been shown to elevate secretory immunoglobulin levels in the gut mucosal environment (22, 37) suggesting the establishment of an enhanced mucosal immune state by bovine milk proteins. Additionally, enhancement of the specific IgA response to a bacterial pathogen was seen in 18 healthy humans (9 females and 9 males age 20 to 50 y) after supplementation with bovine colostrum (19). This response also suggests that there has been an immunomodulatory effect of orally administered bovine colostrum rather than an antimicrobial effect.

In this preliminary study investigating the effects of bovine colostrum on levels of s-IgA in distance runners in their normal training environment, the aim was to establish a valid saliva sampling and test methodology and to control for known variables that can affect levels of s-IgA. As in other studies (30) URS days have only be considered as true URS if two or more consecutive days of URS were reported. Because the immune response can result in local inflammation and systemic consequences (ache, fever, and fatigue) these latter symptoms may also be present days after the initial symptoms disappear (17), therefore it is appropriate to include the reporting of these systemic symptoms in a wellness record. However, if any of these symptoms reoccurred within a week after the end of the initial episode they were considered to be a complication of the first episode and not considered to be a second episode (18).

Methods

Subjects

Thirty-nine runners (22 men and 17 women) between the ages of 35 to 58 y volunteered for the study. These were recreational distance runners who were mostly members of the Auckland YMCA marathon club (New Zealand) or associated with it, working full time and were preparing for a marathon being held in April of 2002 (Rotorua Marathon, New Zealand). Inclusion criteria for the study stipulated that the participants must:

1) Meet weekly either at the marathon clubrooms on Sunday morning or at a specified site for Saturday morning pack runs
2) Be less than 60 y old
3) Be training for a marathon or marathon type events for the last 5 y
4) Not be lactose intolerant or have any other known allergy to cow's milk
5) Not taking whey protein supplements; and
6) Not be receiving treatment for any known medical condition (including asthma).

All subjects were fully informed verbally and in writing about the nature of the study, any known risks, and their right to terminate participation at will before signing a formal consent form. Ethical approval for this study was sought and given by the Auckland Ethics Committee (AKY 2001/305).

Study Design

The study was a randomized double blind, placebo-controlled design and required 14 wk participation (Figure 1). It was attempted to pair match subjects for gender but due to the small numbers it was not possible to assign equal numbers. Each subject was required to consume daily 250 mL of colostrum product or placebo and at four weekly intervals provide a saliva sample for IgA analysis. Training and wellness diaries were completed each day and a 7 d diet record at week 6 and 11.

Intervention

A chocolate drink powder containing colostrum was used as the intervention (Immulac; NZMP Ltd., Auckland, New Zealand). The athletes were given two tubs of chocolate drink powder each (2.2 kg of powder in total). The tubs were identified with a three-digit random number and this number became the identification code for the athlete. Each participant was supplied a scoop and was required to add two level scoops of powder (equivalent to 26 g) to 125 mL of cold water in the 250 mL drink shaker provided. The athletes began taking the supplement a day after the first saliva sample was supplied.

The placebo blend contained skim milk, matched to the test intervention for equivalent digestible protein content. Flavors and colors were added to achieve blends that were similar in taste and color. As colostrum powder has a strong distinctive flavor, these blends provided the best combination to minimize the difference in taste and color (Table 1).

Secretory IgA Assessment

Saliva Collection. Saliva samples were drawn, stored, and analyzed using handling procedures that had been suggested by standard diagnostic protocols

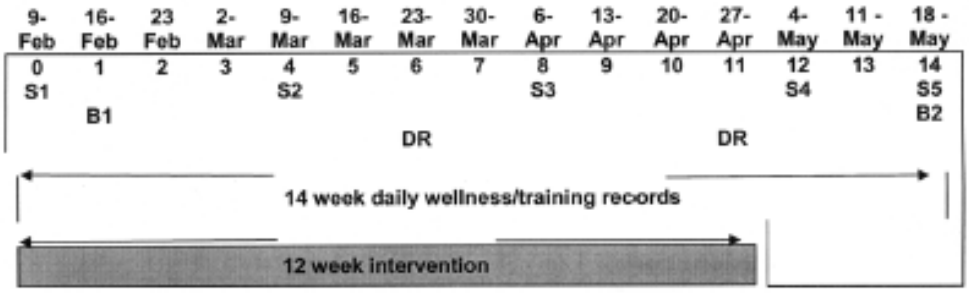

Figure 1—Schematic summary of the trial design and sampling time points. S, saliva sample taken on the Saturday or Sunday at the beginning of that week; B, blood sampled before breakfast on a day convenient to participant in nominated week; DR, 7-d diet record.

Table 1 Composition of Chocolate Powder Blends

	Placebo (Control)	Colostrum (intervention)
Immulac %w/w	–	40
Skim milk powder %w/w	70	30
Sucrose %w/w	30	30
Protein g/26 g	7.8	8.6
Carbohydrate g/26 g	14.3	13.6

developed at the Hunter Immunology Pathology Service, Newcastle, Australia [now the Hunter New England Pathology Service (13)].

Athletes were instructed to eat and drink at least 30 min before the sample was provided. They were also taught to produce a sample by gently spitting saliva collected underneath the tongue into a collection tube, to provide whole mixed unstimulated saliva. Approximately 1 mL of saliva was collected which took between a minute and 10 min to produce, depending on the individual.

Samples were collected before the start of the longest run of the nominated collection week. Five samples from each athlete were collected on separate occasions over a 14-wk period. The first sample was taken at baseline, the second, third, and fourth samples at monthly intervals (the fourth sample was also taken 1 wk after the New Zealand marathon which 21 athletes competed in). The athletes stopped taking the intervention the day after the fourth saliva sample. The fifth saliva sample was taken 2 wk later. Samples were placed immediately into dry ice until transported to a –70 °C freezer.

S-IgA Analysis. Saliva samples were excluded from analysis if there was insufficient sample and/or if the subject appeared to be dehydrated or fasted at the time of sampling. Dehydration and fasting can elevate levels of s-IgA and this was monitored by measuring salivary albumin and determining osmolality. Elevated levels of salivary albumin can indicate dehydration due to altered saliva flow rates or an increase in tissue permeability. Monitoring levels of salivary albumin is useful to identify differences between groups or time-points (10).

Salivary albumin was measured on the same day on a Roche Cobas Fara II by turbidimetry, using Dako human serum albumin standards (15 to 300 mg/L) and rabbit anti-human serum albumin. High osmolality can indicate dehydration as saliva flow rate is reduced or that there has been recent consumption of a drink high in electrolytes (e.g., a sports drink). Saliva osmolality was measured using a freezing point depression osmometer (Advanced Digimatic Osmometer 3D2, Advanced Instruments, Norwood, MA). The assay coefficients of variation for albumin were < 5% for values >30 mg/L (< 10% for values < 30 mg/L) and for osmolality were between 1.8 to 2.5%.

S-IgA was measured in diluted samples by ELISA, using unconjugated and biotin-conjugated polyclonal goat F(ab')2 antibody fragments, specific for human

IgA (Biosource International, Camarillo, CA). These reagents have no stated cross-reactivity against bovine immunoglobulins, and were further demonstrated in our laboratory to be non-reactive against samples of bovine colostrum containing bovine IgA. To assess levels of human IgA the assay used a two-step "sandwich" enzyme immunoassay, where unconjugated monoclonal antibody was coated onto a 96 well immunoplate (Nunc, Roskilde, Denmark) at a concentration of 0.5 µg/mL overnight. Plates were washed with PBS (pH 7.2, 0.05% Tween 20) and unbound sites were blocked with skim milk powder (5% in PBS). Pre-diluted standards (myeloma-derived human IgA; Dako X0908, Denmark, range 0 to 80 ng/mL) and test samples were added to each well and incubated for 2 h, followed by extensive washing and the addition of biotin-conjugated detection antibody at 1 µg/mL for a further 2 h. Streptavidin-conjugated horseradish peroxidase enzyme (Silenus 988210010 1/2000) was then added for a further 1 hour, before final washing and addition of reactive chromogenic substrate (ABTS - 2-2 Azino-bis-3 ethyl benzthiazoline 6 sulphuric acid). After acidification to stop the reaction the plates were read on a plate reader at 405 nm (CERES 900C Bio Tek microtitre plate reader). The ELISA used in this study to measure s-IgA detects both IgA_1 and IgA_2 subclasses.

Training and Wellness Records

Athletes kept a daily log of the distance run (kilometres) and time run (min). Time spent in other physical activities was also recorded.

Daily records of health problems were kept on the same log. The format for the wellness log was based on a log used for self-recording of health in rowers (30).

The athletes were required to record the absence or presence of any of the following symptoms:

Symptom	
No health problem	
"Cold" symptoms (runny and or stuffy nose, sore throat, coughing, sneezing, colored nasal and/or saliva discharge)	
"Flu" symptoms (fever, headache, general aches and pains, fatigue, weakness, chest discomfort, cough)	
Gastrointestinal (nausea, vomiting, diarrhea)	
Physical (muscle, joint or bone problems/injury)	
Other	

The total number of days with upper respiratory symptoms (URS) was calculated for each athlete. Days were only considered as true URS if two or more consecutive days of URS symptoms were reported. In this study no other attempt has been made to verify that a true URTI occurred.

Dietary Assessment

Participants were asked to complete two sets of 7-d food diaries. Instructions on how to complete the diaries were given in writing and verbally by the investigator. The first record was kept 6 wk into the marathon build-up (week 6) to determine reported dietary intake during heavier training weeks and the second was kept in the week prior to the marathon (week 11) to determine the reported dietary intake during the taper period. The food records were analyzed for intake of carbohydrate $g^{-1} \cdot kg^{-1}$ body mass $\cdot d^{-1}$, protein $g^{-1} \cdot kg^{-1}$ body mass $\cdot d^{-1}$, contribution of fat to total energy intake, and total energy intake using Foodworks version 2.10 with the NZ Food Files 2000, and NZ Vitamin and Mineral Supplements 1999 (Crop and Food Research, Palmerston North, New Zealand).

Assessment of Intake of Intervention

The daily consumption of the chocolate drink intervention (either placebo or colostrum powder) was recorded on the food records and included in the dietary analysis for each participant. Measuring the amount of remaining chocolate powder gave an indication of compliance.

Body Measurements

Body mass was recorded twice during the study within a week of the diet diaries being completed, using digital scales (Soehnle, accuracy 0.1 kg) that were checked with a calibrated weight after every third participant was measured. Body mass was measured in the morning and post-prandially before the long run on one of the saliva sampling days. Height was measured once using a portable stadiometer (Massey University, New Zealand).

Statistical Analysis

Two-way repeat measures analysis of variance was used to identify the effects of intervention and time on s-IgA levels; Dunnett's post hoc test was used to further identify specific time-points where any significant within-group time-dependent changes occurred (SAS version 8, SAS Institute, Inc., Cary, NC). Between-group analyses were undertaken using non-parametric testing. Multiple regression analysis was used to assess the effect that the independent variables (weekly mileage, gender, average self-reported URS, running the New Zealand marathon and colostrum) could have on levels of log s-IgA (dependent variable) (Minitab version 14, Mintab Inc., State College, PA). Those independent variables that were significant were investigated further in a general linear model. Any data that were non-normally distributed were log-transformed prior to analysis; where transformation failed to

normalize the distribution, non-parametric testing was applied (SAS). In all cases, the level of significance was set at $P = 0.05$.

Results

Characteristics of the Participants

The placebo and colostrum group had similar characteristics (Table 2). Thirty-nine athletes volunteered for the study, and data from 35 were used for analysis. One male participant withdrew 3 wk into the study because of an unrelated illness. Data from three athletes were excluded: one did not return training and wellness records (placebo group); one was breast-feeding (colostrum group); and one was participating in her first marathon (placebo group). Twenty-five athletes indicated they were training for the New Zealand marathon but due to injury, illness, or personal reasons, four did not compete in the event. While these four runners continued to be in the study, they reduced their weekly mileage. In addition, two athletes competed in marathon events early in April, 3 wk before the end of the study, and their weekly mileage was reduced after competition. There was a large range in average weekly distances run within the groups: placebo, 8 to 105 km/wk; colostrum, 18 to 114 km/wk. Non-parametric testing indicated there were no significant differences between the groups.

There was no significant difference between body mass at week 6 and week 11 for the placebo and colostrum groups.

Differences in s-IgA

Median levels of s-IgA increased in the colostrum group by 79% and in the placebo group by 16% after 12 wk intervention (Table 3). RMANOVA showed a main effect of group ($P = 0.0051$) and time ($P = 0.015$) on levels of s-IgA but no effect of gender ($P = 0.389$) or the interaction of group and time ($P = 0.3570$). Further investigation of the effect of time using post-hoc tests demonstrated that there was a significant increase in levels of s-IgA after 12 wk supplementation compared to baseline levels in the colostrum group ($P = 0.029$) but not the placebo group (Figure 2).

Multiple regression analysis was performed on the independent variables and the P values showed that there was no effect of weekly mileage, average URS days, or gender on levels of log of s-IgA (Table 4). After removing these variables from the multiple regression model the effect of being in the colostrum group on levels of log s-IgA was highly significant ($P = 0.004$) even after controlling for running the New Zealand marathon (M), which was just significant ($P = 0.045$). The general linear model showed the F-values (M = 4.37, colostrum = 9.60) and P values (M = 0.045, colostrum = 0.004) which were significant, this model accounting for about 29% of the variation in levels of log s-IgA.

URS

The mean number of URS days for the colostrum group was 5.3 d and for the placebo group 8 d. Non-parametric testing showed no significant difference between the groups. Mean URS episodes were 0.8 for the colostrum group and 1.1 for the placebo, and no significant difference was found.

Table 2 Descriptive Characteristics for the Subject Group

	Placebo		Colostrum		
	Male	Female	Male	Female	P*
Number	8	9	12	6	0.25
Age (y)	48 (36-56)	51 (41-58)	46 (35-57)	43 (30-53)	0.26
Height (cm)	180 (170-192)	160 (152-164)	179 (172-184)	168 (160-174)	0.11
Body mass (kg)	75.4 (70.0–83.8)	60.8 (52.4 – 71.2)	78.7 (66.6 – 83.1)	67.5 (47.7 -87.5)	N/A
% Weight change	-0.14 (-2.2 - 2.4)	-0.49 (-1.1 - 1.0)	0.8 ($P = 0.61$) (-3.0 – 4.5)	-0.38 ($P = 0.97$) (-2.7- 2.8)	N/A
Weekly mileage (km)	43 (19-105)	46 (8-57)	50 (18-114)	58 (26-70)	0.13
Total URS days	12 (0-16)	16 (0-25)	7 (0-23)	12 (0-15)	0.55
G drink/d	25 (20-27)	25 (20-28)	25 (16-30)	25 (16-26)	0.62

Note. * Values are medians and ranges. *P* refers to probability value. The *P*-value shows the statistical significance of the difference between the placebo and the colostrum groups.

Table 3 Saliva Analysis for Absolute Concentration of s-IgA mg/L, Osmolality, s-IgA/Osmolality Ratio, and Albumin

	Absolute IgA conc (mg/L)	Osmolality (mOsmol/kg)	IgA to osmolality ratio	Albumin (mg/L)
Base (sample 1)				
PL	50 ± 6	65 ± 5	1.07 ± 0.04	39 ± 5
BC	68 ± 3	72 ± 7	1.01 ± 0.08	60 ± 8
4 wk (sample 2)				
PL	57 ± 5	67 ± 10	0.86 ± 0.09	46 ± 9
BC	77 ± 7	76 ± 12	1.05 ± 0.09	84 ± 20
8 wk (sample 3)				
PL	55 ± 5	72 ± 7	0.92 ± 0.05	51 ± 9
BC	87 ± 14	77 ± 10	1.14 ± 0.10	70 ± 14
12 wk (sample 4)				
PL	65 ± 4	71 ± 11	1.07 ± 0.06	51 ± 8
BC	123 ± 20	81 ± 12	1.47 ± 0.14	89 ± 18
Post (sample 5)				
PL	58 ± 5	65 ± 9	1.02 ± 0.08	52 ± 15
BC	101 ± 12	74 ± 11	1.38 ± 0.04	91 ± 17

Note. Values are means ± standard error of the mean. s-IgA = salivary IgA; PL = placebo; BC = bovine colostrum

Salivary Albumin and Osmolality

RMANOVA of salivary albumin levels showed there was a marginally significant group effect ($P = 0.044$); however, this did not change with time ($P = 0.12$). There was no effect of gender ($P = 0.68$). RMANOVA of saliva osmolality showed there was no main effect of group ($P = 0.24$), gender ($P = 0.51$) or time ($P = 0.22$). The RMANOVA for the s-IgA/osmolality ratio also showed no main effect of group ($P = 0.11$), gender ($P = 0.16$), or time ($P = 0.37$) (Table 3).

Reported Dietary Intake

Analysis of reported dietary intake was carried out using two 7-d food records. The first record was kept 6 wk into the marathon build-up (week 6) to determine reported

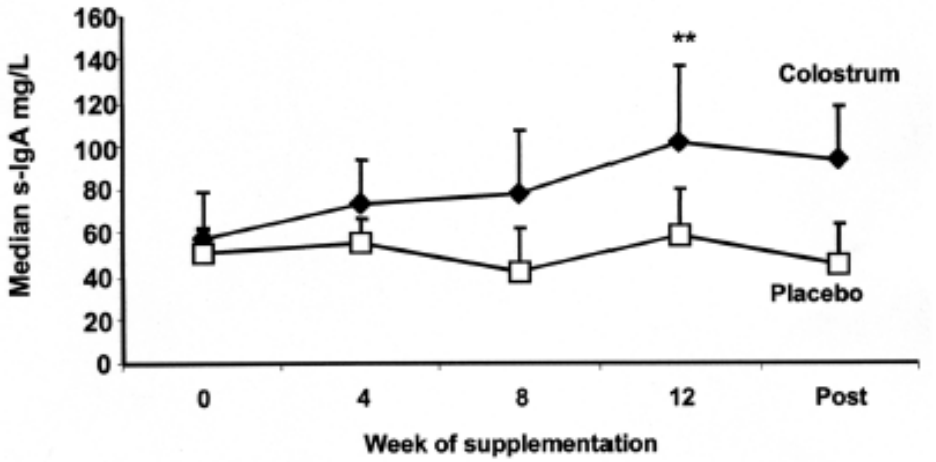

Figure 2—Time-dependent changes in the median absolute concentration of s-IgA and 95% confidence intervals. ** indicates significant increase in levels of s-IgA in colostrum group compared to baseline ($P = 0.0291$).

Table 4 Results from Multiple Regression Analysis Investigating the Effects of Independent Variables on Levels of Log S-Iga After 12 Weeks Intervention

Variable	Coefficient (SE coefficient)	*T*-statistic	*P*
Mean km/wk	–0.02 (0.14)	–0.15	0.88
Male	0.24 (0.23)	1.03	0.31
Mean URS	0.21 (0.20)	1.03	0.31
NZ marathon	0.42 (0.23)	1.81	0.08
Colostrum	0.65 (0.24)	2.76	0.01

Note. *Significance level set at $P < 0.05$

dietary intake during heavier training weeks and the second was kept in the week prior to the marathon (week 11) to determine the reported dietary intake during the taper period. Of the 35 participants who met the study criteria, 34 completed two sets of 7-d food records. Repeat measures ANOVA was used to determine differences in the reported dietary intake between the groups. There were no differences between the colostrum and placebo groups for reported daily intake of energy (kJ) ($P = 0.65$) carbohydrate g/kg body mass ($P = 0.27$), protein g/kg body mass ($P = 0.76$), or percent contribution of fat to energy (0.17). Means and standard errors of the mean at week 6 and week 11 are shown in Table 5. The RMANOVA

Table 5 Summary of Mean Daily Intake of Energy, Carbohydrate, Protein, and Fat Contribution to Energy Intake

		Week 6	Week 11
Colostrum	Energy (kJ)	10,058 ± 762	9,517 ± 619
	Carbohydrate (g/kg BM)	4.1 ± 0.33	4.0 ± 0.31
	Protein (g/kg BM)	1.4 ± 0.14	1.3 ± 0.09
	Fat (%)	31 ± 1.8	31 ± 1.3
Placebo	Energy (kJ)	10,162 ± 586	9,522 ± 539
	Carbohydrate (g/kg BM)	4.6 ± 0.24	4.3 ± – 0.27
	Protein (g/kg BM)	1.5 ± 0.07	1.4 ± 0.05
	Fat (%)	29 ± 1	30 ± 0.9

showed a time difference for reported energy intake. At week 11 intake was less than week 6 and this difference was significant for both groups ($P = 0.05$). There was no significant time change for the individual macronutrients.

Compliance and Side Effects

There was no significant difference between the groups in the consumption of drink powder (grams of drink per day). Two athletes started the treatment a week later than the others due to personal reasons.

Of the 17 women who participated in the study, six reported stomach problems after the commencement of the treatment. Of these six, two were on the colostrum treatment. All participants who reported stomach problems found the symptoms disappeared with time except for one of the women on the colostrum treatment. No adverse symptoms were reported by the men.

Discussion

The results in this study have shown that there was an increase of 79% in median s-IgA levels after 12 wk supplementation with bovine colostrum and that this increase was significantly different to the corresponding group's s-IgA levels at baseline. The differences in s-IgA could not be accounted for by other variables under investigation and were still significant after controlling for those athletes who ran the New Zealand marathon. A smaller increase in median s-IgA levels was seen in the placebo group (16%) but the difference after 12 wk supplementation was not significant compared to baseline levels. This study also demonstrated that the sampling and testing methodology established is sufficiently sensitive to detect changes occurring in s-IgA in the field among athletes undertaking marathon-type training.

Levels of s-IgA in athletes participating in other studies using similar laboratory procedures have demonstrated similar findings. These include a study on an elite group of 15 male and 11 female swimmers monitored monthly over a period of 7 months whose pretraining median s-IgA concentrations ranged from 42 to 84 mg/L. Additionally, the pre-training s-IgA levels for males was found to be higher on average than the females (15). In another group of elite swimmers (12 males and 10 females) pre-training s-IgA levels ranged from 41.5 to 71.1 mg/L during a 12 wk training cycle. Results were not separated by gender (14). Salivary IgA levels, from a group of 14 elite male swimmers, were monitored every second to third day over a 30-d period of intensive training. Levels of s-IgA varied significantly in response to training and the appearance of URS with medians ranging from 32.6 to 127.5 mg/L (17). This study also showed the benefit of frequent saliva sampling and verification of URS symptoms to identify the causes of the changes seen in s-IgA levels. Median s-IgA levels in the current study ranged between 41.5 to 54.7 mg/L for the placebo group and 56.8 to 101.5 mg/L for the colostrum group, which compares well with the above studies. This demonstrates that the sampling and testing methodology used were sensitive to changes in s-IgA levels, and commensurate with previous studies regarding exercise and s-IgA.

In a study of runners competing in the Los Angeles Marathon it was found that athletes who ran more than 97km/wk in training reported a greater incidence of URTI compared to those running less than 32km/wk (28). In comparison, the mean kilometers run per week by distance runners in this preliminary study was 44.5 km for the placebo group and 54 km for the colostrum group. The difference in training volumes was not significant between the placebo and colostrum groups ($P = 0.19$). Multiple regression showed that the volume of training was not sufficient to have an effect on levels of s-IgA during the study period ($P = 0.88$). The significant increase in levels of s-IgA however was partly due to participating in the New Zealand marathon ($P = 0.045$). It is possible, therefore, that the athletes who ran this marathon experienced moderately elevated levels of s-IgA via a mechanism not linked to dietary intervention. However, even after controlling for the runners (21/35 runners) who participated in the marathon, the increase in levels of log s-IgA was still very significantly associated with being in the colostrum group ($P = 0.004$).

The median incidence of self-reporting of URS over the study period was one episode per person (colostrum group range, 0 to 4; placebo group range, 0 to 3). It is interesting to note that this incidence is similar to that of a group of 26 elite swimmers who were monitored for 7 months (15). The median incidence in the elite swimmers was one episode (range 0 to 5) per male swimmer and three URS episodes (range 0 to 7) per female swimmer (15), the difference in incidence of URS observed between males and females was not significant. The rate of reporting of URS in the distance runners in the current study seems high in relation to the volume of training, as they were not elite athletes (except for two veteran elite males). However, it is possible the participants could have been experiencing combined stress from working full time, family pressures, poor dietary practices (as indicated by reported carbohydrate intake which was lower than that recommended for endurance athletes), and also participating in distance training. It is known that the risk for URTI in athletes is multifactorial in origin (2).

Viral infection is the most common cause of URTI in athletes, but is not the only cause of URS. It is possible that at least some of the symptoms self reported by the runners were due to local or systemic inflammation in response to exercise, rather than infection (33). The cytokine IL-6, for example, is produced during exercise by muscles in response to a lowering of muscle glycogen (34). IL-6 can co-stimulate the production of other anti-inflammatory cytokines (such as IL-1ra and IL-10) and can reduce levels of the pro-inflammatory cytokine TNF-α. Levels of IL-6 also increase as a result of a local response when infection occurs or tissue is damaged, where local inflammation results in a systemic acute-phase response associated with "feelings of sickness" (25), due to the in vivo pyrogenic and febrile nature of the cytokine. Further, high circulating levels of IL-1ra have been reported to be associated with a perception of poor health in 325 volunteers age 18 y and over visiting a primary health care unit (21). In the current study, it was interesting to note that the average total number of URS days reported by the colostrum group was lower than the placebo group; however the difference was not significant. As bovine colostrum contains growth factors that can help with tissue repair it is possible that the recovery process was improved in these runners. This supports the suggestion by Buckley et al. (2002) that colostrum supplementation improved recovery in distance runners as indicated by improved performance after a second bout of exercise (6).

Other methodological constraints that are known to affect levels of s-IgA were controlled for in the present study. This included standardizing the post-prandial sampling time in order to minimize the effects of dehydration. The post-prandial sampling time was set at 30 min to accommodate the athletes; however, several other studies have used 2 h (13, 35), therefore it may not be possible to compare absolute s-IgA concentrations between studies. Controlling for the quality of saliva is also important for cross-study comparisons. Recently the secretion rate of s-IgA and the absolute s-IgA concentration in American footballers have been found as the most useful clinical biomarkers for predicting the risk of contracting URTI (29). It has been suggested that the amount of s-IgA on mucosal surfaces is important in preventing infection (9). In this group of footballers the results for osmolality and s-IgA/osmolality ratio were not associated with increased incidence of URTI. The conclusion was that the absolute s-IgA concentration and s-IgA secretion rate were the most useful predictors when investigating the effects of exercise on URTI incidence in footballers. Therefore the use of osmolality in the preliminary study reported here may not have been the most appropriate method for controlling for saliva quality and may help to explain why no significant changes were seen in the s-IgA/osmolality ratio. However an increase in s-IgA concentration with no change in the s-IgA/osmolality ratio could indicate that at least part of the s-IgA increase was due to the subjects being less hydrated.

Prolonged intensive exercise may reduce resting salivary s-IgA levels and salivary s-IgA levels below 40 mg/L at the beginning of a training season have been associated with a higher risk for contracting URTI (15). While only one athlete showed a downward trend in s-IgA levels in the present study, 11 athletes (28.9% of all participants) had baseline concentrations below 40 mg/L. Of the 11 athletes who had s-IgA levels below 40 mg/L at baseline, seven (64%) reported URS later in the study. This observation is consistent with a previous study of the

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production, disturbances to the integrated mucosal network (such as alterations in T-cell cytokine control mechanisms) could lead to mucosal suppression. A number of cytokines are involved in the production of salivary s-IgA. Antigen-activated CD4+ T cells can stimulate production of IL-5, IL-10, and IL-6 which may all be involved in terminal differentiation of B cells into IgA secreting plasma cells (4). These potential markers of mucosal immunity were not measured in the present study. Other immune parameters that could help to identify the response of the mucosal immune system include full blood count, lymphocyte differentiation and phenotyping, and serum levels of IgA, IgM, and IgG. Further trials which aim specifically to measure those parameters linked to mucosal immunity following colostrum supplementation are currently being undertaken by our group.

In summary, this study has shown that the sampling and testing methodology established is sufficiently sensitive to detect changes occurring in salivary IgA (in the field) among athletes undertaking marathon-type training. It has also been shown that a change in salivary IgA levels occurred with athletes taking bovine colostrum compared to those taking a placebo. If this effect is real then colostrum could have potential benefits in terms of enhancing mucosal immunity and reducing recurrent infection thus allowing for a reduction in interrupted training schedules. However, due to the small sample size and the heterogenous mix of the participants, further research is needed to determine whether bovine colostrum truly has a physiologically relevant effect. Validating the results from this study with a more homogenous group of endurance athletes in terms of gender, fitness level and type of training program is necessary.

Acknowledgments

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Appendix 10.2
Information pamphlet: Study 1 (Swimmers and Students)

This study has received ethical approval from the Massey University Ethics Committee and the Auckland Ethics Committee.

If you have any questions?

..... about the study, either now or in the future, please feel free to call. If you need an interpreter, one can be provided.

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If you have any queries or concerns about your rights as a participant in this study, you may wish to contact:

Health Advocates Trust
(Northland to Franklin) 0800 555 050

Thank you for taking the time to read this information sheet.

Massey University

Food, Nutrition & Human Health

Massey University
Institute of Food, Nutrition and Human Health
Private Bag 102 904
North Shore Mail Centre
Auckland

Effects of bovine colostrum supplementation on the immune system

Does bovine colostrum enhance the immune system?

Information sheet for participants
6/8/2003
V2

You are invited to participate in the study 'Effects of bovine colstrum supplementation on the immune system of high performance athletes and non exercising controls". The study is part fulfilment of the completion of a PhD in Nutritional Science at Massey University and is sponsored by Fonterra Co-operative Ltd.

Why am I doing this study?

It appears that people doing prolonged intensive exercise are more susceptible to getting colds during a build up. To monitor the immune system an antibody in saliva can be measured. This antibody (called secretory immunoglobulin A or S-lgA) is important in the first line defence against viral infections such as colds. Levels of s-lgA are known to drop during periods of prolonged intensive exercise (Gleeson and Pyne, 2000).

The main aim of this study is to determine what the effect of bovine colostrum supplementation has on s-lgA levels during periods of intensive exercise.

By participating in this study you will help determine if an immune profile can be established that will indicate when an athlete is more at risk for contracting a cold. As well you will help determine whether bovine colostrum supplementation can improve the immune system in athletes.

Who is in this study?

① Senior squad members from swimming clubs in Auckland between 16 - 24 years.
② Massey University students who are not exercising regularly and are between 16 - 24 years old.
③ Must not be taking a colostrum supplement or be prepared to go through a washout period before the intervention starts.
④ Must not be lactose intolerant or have other allergies to dairy products
⑤ Must not be receiving treatment for any known medical condition.

What is involved?

Nutritional, biochemical and immunological information will be collected from all who participate in the study. The information gathered will be used to help protect the health of all swimmers.

Participants will be asked to participate in the following procedures:

- ▷ Consume daily either a bovine colostrum supplement or skim milk placebo.
- ▷ Record fluid and food intake for a period of 8 days. The first 4-day diary is to be recorded at the beginning of the intervention and the second at the end.
- ▷ Have 5 blood samples taken during the study period.
- ▷ Have saliva samples and temperature taken twice weekly for a month and then monthly.
- ▷ Complete a daily wellness and training diary.

Do you have to take part in this study?

Your participation is entirely voluntary (your choice). If you agree to take part in the study, you are free to withdraw at any time and have the right to refuse to answer any question at any time.

Compensation

The Auckland Ethics Committee has certified that this clinical trial is being conducted principally for the benefit of the manufacturer in respect of which this trial is being carried out. This means that if you suffer injury as a result of your participation, you will not be eligible for cover under accident compensation legislation. Compensation, however, will be provided by Fonterra Co-operative Group Ltd in accordance with the "New Zealand Researched Medicines Industry Guidelines on Clinical Trials - Compensation for injury resulting from participation in Industry Sponsored Clinical Trials."

These RMI Guidelines are only guidelines and until your claim is assessed by the insurers of Fonterra Co-operative Group Ltd it cannot be said with any certainty exactly what type or amount of compensation you will receive if you suffer injury as a result of your participation, or what sort of injury will be covered. The guidelines require that compensation must be provided by Fonterra Co-operative Group Ltd where the injury you suffer is serious and not just temporary and is one caused by the for dietary supplements or where you would not have suffered injury but for your inclusion in this trial.

The guidelines also require that the compensation you receive must be appropriate to the nature, severity and persistence of your injury. This means that you will be likely to receive some compensation from Fonterra Co-operative Group Ltd unless your injury is minor or temporary, however, you might not receive compensation from Fonterra Co-operative Group Ltd if your injury was caused by the investigators, if there is a deviation from the proposed plan of research, or if your injury was caused solely by you.

What will happen to the results?

Data collected from participants is completely confidential. No material, which could personally identify you, will be used in any reports in this study. The results will be stored by code number in a computer in a locked room throughout the study. Information will be stored for 10 years and then destroyed

Appendix 10.3
Consent form: Study 1 (Swimmers and Students)

Massey University
COLLEGE OF SCIENCES

Institute of Food, Nutrition and Human Health
Private Bag 102 904,
North Shore Mail Centre,
Auckland,
New Zealand
Telephone: 64 9 443 9700
Facsimile: 64 9 443 9640

Does bovine colostrum supplementation enhance immune function?

CONSENT FORM

I have read and I understand the information sheet dated 9/6/3 for volunteers taking part in the study designed to find out the effects of bovine colostrum on enhancing immune function in athletes and non-exercising controls. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.

I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study up until the end of data collection and this will in no way affect my future health care.

I understand that my participation in this study is confidential and that no material, which could identify me, will be used in any reports on this study. I understand that the project will be submitted as part fulfilment of the completion of a PhD in Science degree. I understand the compensation provisions for this study.

I have had time to consider whether to take part. I know who to contact if I have any side effects to the study. I know who to contact if I have any questions about the study.

5/8/3
Version 2

I would like the researcher to discuss the outcomes of the study with me. YES/NO

I consent to data being stored for use in a future related study for which ethics approval will be gained by an accredited NZ ethics committee. YES/NO

I_____(full name), hereby consent to take part in this study.

Date:

Signature participant:

Full name of parent (Swim club policy):

Signature parent:

Full name of researcher:	Christine Virginia Crooks
Contact phone number for researcher:	(09) 845 5517, 027 2333 590
Project explained by:	Christine Crooks

Signature:

Name of Witness:

Signature of Witness:

(Note a copy of the consent form to be retained by the participant)

5/8/3
Version 2

Appendix 10.4
Ethics approval letter: Study 1 (Swimmers and Students)

Please include the reference no. and study title in all correspondence/telephone calls.

Auckland Ethics Committees

Private Bag 92522
Wellesley Street
Auckland
Delivery Address:
C/O Ministry of Health
3rd Floor, Unisys Building
650 Great South Road, Penrose
Phone (09) 580 9105
Fax (09) 580 9001
Committee X Email: pat_chainey@moh.govt.nz
Committee Y Email: yvonne_erixon@moh.govt.nz

29 August 2003.

Ms Christine Crooks
Institute of Food Nutrition & Human Health
Albany Campus
PB 102 904
North Shore Mail Centre
Auckland

Dear Christine,

AKX/03/07/182 The effects of bovine colostrum supplementation on levels of s-IgA in elite athletes and non-exercising controls: PIS/Cons V#2 6/8/03.

Thank you for your response to the points raised by the Committee – received 27 August 2003. The above study has been given ethical approval by the Auckland Ethics Committee under the process as set out in the National Application Form for Ethical Approval of a Research Project, Guidelines for Completion, May 2002.

Approved Document:
- Information Sheet/Consent Form V#2,

Certification:
The Committee certifies that it is satisfied this trial is conducted principally for the benefit of the manufacturer or distributor of the medicine or item in respect of which this trial is carried out. Ethical approval is given on the understanding participants in the proposed research project will receive an acceptable level of compensation from Fonterra Co-operative Group Ltd in the event of injury to participants resulting from their involvement in the proposed research study.

Accreditation:
The Auckland Ethics Committee is accredited by the Health Research Council and is constituted and operates in accordance with the Operational Standard for Ethics Committees 2002.

Progress Reports:
The study is approved until June 2004. The Committee will review the approved application in ? of next year and notify the researcher if it withdraws approval. A form to assist with this should come off our data base two months prior to the due date. However, it is your responsibility to ensure that a yearly progress report is submitted to the Ethics Committee.

Please advise when the study is completed and a final report is also required at the conclusion of the study.

..../2

Requirements for SAE Reporting:
Please inform the Committee as soon as possible of the following:
- Any study in another country or NZ that has stopped due to serious or unexpected adverse events.
- Withdrawal of investigational product for continued development.
- Withdrawal from the market for any reason.
- All serious adverse events which result in the investigator or sponsor breaking the blinding code at the time of the SAE or which may result in hospitalisation or death.

General:
All correspondence, protocol amendments, SAE reports and progress reports should be forwarded to the Committee.

It should be noted that Ethics Committee approval does not imply any resource commitment or administrative facilitation by any healthcare provider, within whose facility the research is to be carried out. Where applicable, authority for this must be obtained separately from the appropriate manager within the organisation.

Yours sincerely,

Pat Chainey
Administrator, Committee X

Appendix 10.5
Training and wellness diary: Study 1 (Swimmers)

Massey University
Albany Campus

TRAINING & WELLNESS DIARY

'Immunity Study'

For further information, contact:
Christine Crooks
c.v.crooks@massey.ac.nz
Phone: 09-414 0800 xtn.9803

Institute of
Food,
Nutrition &
Human Health

Training and Wellness Diary
'Immunity Study'

ID Number:

This diary is designed to record your daily training and wellness over the period of the study including the two weeks before the intervention begins, the 12 weeks of intervention and the two weeks after.

Instructions

Training:

Please record your swim training in distance (KM) and time (minutes) for each session. Rate the intensity of the sessions:

Intensity:

Endurance = high volume/low intensity
Quality = mod volume/high intensity
Taper = decreasing volume/high intensity
Rest = low volume/low intensity

Record your dry land training in minutes.

Wellness:

Please record your daily health status. If you have no health problems, tick the nil box. If you have upper respiratory symptoms (URS) please tick the type of symptom and rate the severity.

URS:

Nasal symptoms (includes runny nose, congestion, sneezing)
Fever, Sore throat, Coughing

Severity:

1 = Very mild, no change to daily training
2 = Moderate, did not train today
3 = Severe, confined to bed

All information provided in this diary will be treated with strictest confidence. No outside study will have access to it.

Thank you for participating in this study. I really appreciate the time you are giving!

Christine Crooks
Phone: 09-414 0800 xtn.9803 *Mobile*: 027-2333 590
christinecrooks@massey.ac.nz

DATE BEGINNING OF WEEK: ....

TRAINING INFORMATION

WELLNESS RECORD

	KM	Time	Endurance	Quality	Taper	Rest
am						
pm						

Day:..................................	Tick if present	Severity 1	2	3
Nil				
Nasal symptoms				
Fever				
Sore throat				
Coughing				
Other				

	KM	Time	Endurance	Quality	Taper	Rest
am						
pm						

Day:..................................	Tick if present	Severity 1	2	3
Nil				
Nasal symptoms				
Fever				
Sore throat				
Coughing				
Other				

	KM	Time	Endurance	Quality	Taper	Rest
am						
pm						

Day:..................................	Tick if present	Severity 1	2	3
Nil				
Nasal symptoms				
Fever				
Sore throat				
Coughing				
Other				

Endurance:
Endurance = high volume/low intensity
Quality = mod volume/high intensity
Taper = decreasing volume/high intensity
Rest = low volume/low intensity

Symptoms: nasal (includes runny nose, congestion, sneezing), fever, sore throat, coughing
Severity
1 = Very mild, no change to daily training
2 = Moderate, did not train today
3 = Severe, confined to bed

	KM	Time	Endurance	Quality	Taper	Rest
am						
pm						

Day:..................................	Tick if present	Severity 1	2	3
Nil				
Nasal symptoms				
Fever				
Sore throat				
Coughing				
Other				

	KM	Time	Endurance	Quality	Taper	Rest
am						
pm						

Day:..................................	Tick if present	Severity 1	2	3
Nil				
Nasal symptoms				
Fever				
Sore throat				
Coughing				
Other				

	KM	Time	Endurance	Quality	Taper	Rest
am						
pm						

Day:..................................	Tick if present	Severity 1	2	3
Nil				
Nasal symptoms				
Fever				
Sore throat				
Coughing				
Other				

	KM	Time	Endurance	Quality	Taper	Rest
am						
pm						

Day:..................................	Tick if present	Severity 1	2	3
Nil				
Nasal symptoms				
Fever				
Sore throat				
Coughing				
Other				

Appendix 10.6
Food diary: Study 1 (Swimmers and Students)

Massey University
Albany Campus

FOOD DIARY

'Immunity Study'

For further information, contact:
Christine Crooks
c.v.crooks@massey.ac.nz
Phone: 09-414 0800 xtn.9803

Institute of Food, Nutrition & Human Health

Food Diary
'Immunity Study'

ID Number:

This diary is designed for recording food intake over the period of allocated days.

Two out of four days are to be weekdays, the other two should be weekend days.

Instructions

- *Carry the Food Diary booklet with you when you are recording your food intake.*
- *Please record all food and drink as shown on the tables in the following pages* ***just before you eat or drink*** *NOT from memory at the end of the day.*
- *Use a new line for each food and drink. You can use more than one line for food and drink.*
- *Remember to include all snacks and drinks, even tap water. Include all supplements.*
- *Use as many pages of the booklet that you need.*
- *Check the sample food record to see how it is done.*

All information provided in this diary will be treated with strictest confidence. No outside study will have access to it.

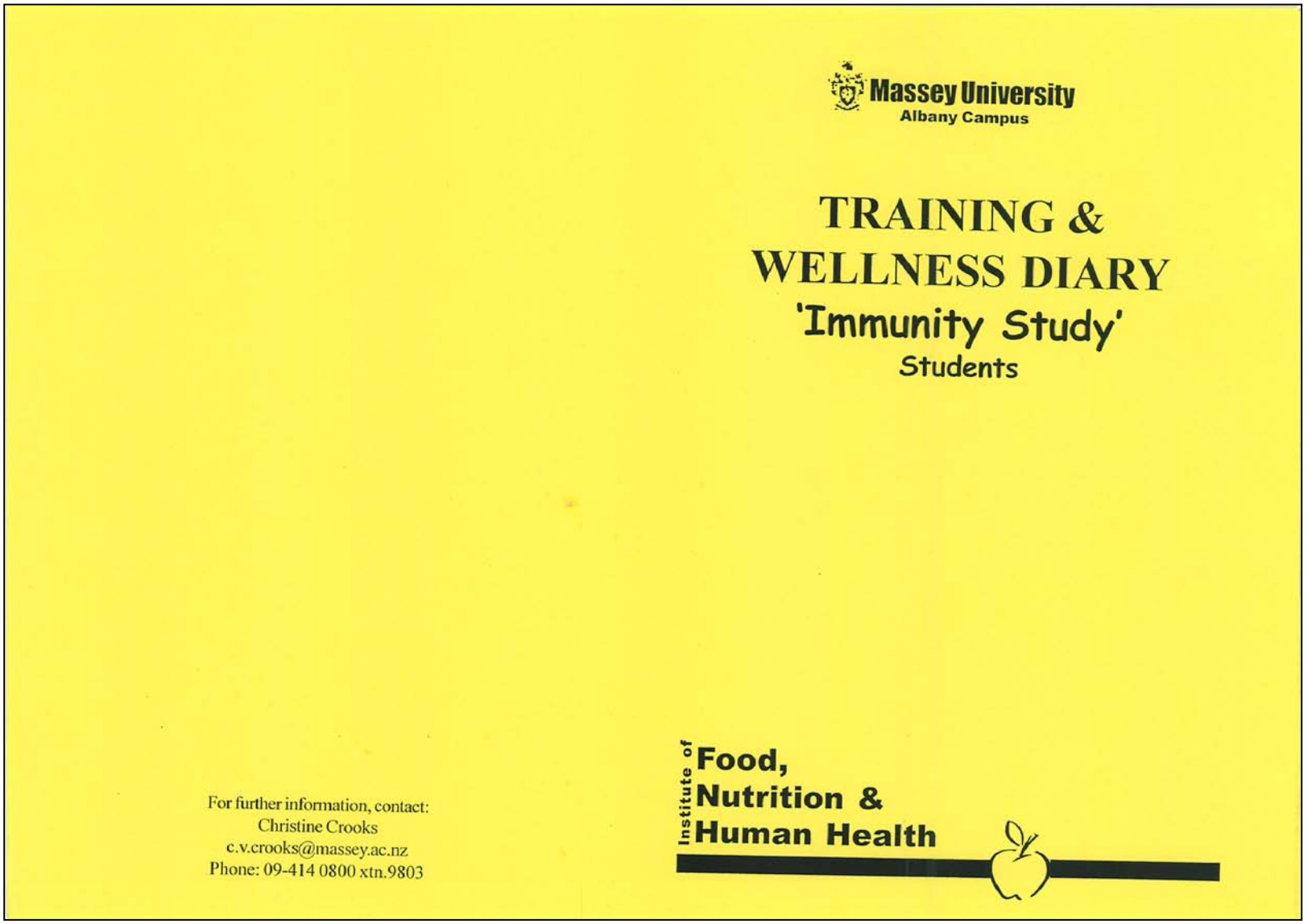
Thank you for participating in this study. I really appreciate the time you are giving!

Christine Crooks
Phone: 09-414 0800 xtn.9803 *Mobile*: 027-2333 590
c.v.crooks@massey.ac.nz

Day: Date:

Eating Time	Food or Drink (Name)	How much did you eat or drink?	Brand name of food or drink (if any)	Preparation of food eg *boiling, frying...*

Appendix 10.7
Training and Wellness Diary: Study 1 (Students)


Massey University
Albany Campus
TRAINING &
WELLNESS DIARY
'Immunity Study'
Students
For further information, contact:
Christine Crooks
c.v.crooks@massey.ac.nz
Phone: 09-414 0800 xtn.9803
Institute of
Food,
Nutrition &
Human Health

Training and Wellness Diary
'Immunity Study'

ID Number:
This diary is designed to record your daily training and wellness over the period of the study including the two weeks before the intervention begins the 12 weeks of the intervention and the two weeks after.

Instructions

Activity:
Please record the type of physical activity performed and the time (minutes) spent.
Rate the intensity the activity was performed at
Intensity:
1 = Mostly endurance e.g. walking, jogging
2 = A mixture of speed and endurance e.g. a game of netball or soccer
3 = Mostly speed, high intensity e.g. sprint session

Wellness:
Please record your daily health status. If you have no health problems, tick the nil box. If you have upper respiratory symptoms (URS) please tick the type of symptom and rate the severity.
URS:
Nasal symptoms (includes runny nose, congestion, sneezing)
Fever, Sore throat, Coughing
Severity:
1 = Very mild, no change to daily activity
2 = Moderate, did not participate in physical activity today
3 = Severe, confined to bed.

All information provided in this diary will be treated with the strictest confidence. No outside study will have access to it.

Thank you for participating in this study. I really appreciate the time you are giving!

Christine Crooks
Phone: 09 414 0800 xtn 9803 Mobile: 027 233 3590
christinecrooks@massey.ac.nz

DATE BEGINNING OF WEEK: ....

TRAINING INFORMATION — WELLNESS RECORD

	Type	Time (mins)	Intensity 1	Intensity 2	Intensity 3		Tick if present	Severity 1	Severity 2	Severity 3
am						Nil				
						Nasal symptoms				
						Fever				
pm						Sore throat				
						Coughing				
						Other				

	Type	Time (mins)	Intensity 1	Intensity 2	Intensity 3		Tick if present	Severity 1	Severity 2	Severity 3
am						Nil				
						Nasal symptoms				
						Fever				
pm						Sore throat				
						Coughing				
						Other				

	Type	Time (mins)	Intensity 1	Intensity 2	Intensity 3		Tick if present	Severity 1	Severity 2	Severity 3
am						Nil				
						Nasal symptoms				
						Fever				
pm						Sore throat				
						Coughing				
						Other				

Training Intensity:
1 = Mostly endurance
2 = A mixture of speed and endurance
3 = Mostly speed, high intensity

Symptoms: nasal (includes runny nose, congestion, sneezing), fever, sore throat, coughing

Severity
1 = Very mild, no change to daily training
2 = Moderate, did not train today
3 = Severe, confined to bed

	Type	Time (mins)	Intensity 1	Intensity 2	Intensity 3		Tick if present	Severity 1	Severity 2	Severity 3
am						Nil				
						Nasal symptoms				
						Fever				
pm						Sore throat				
						Coughing				
						Other				

	Type	Time (mins)	Intensity 1	Intensity 2	Intensity 3		Tick if present	Severity 1	Severity 2	Severity 3
am						Nil				
						Nasal symptoms				
						Fever				
pm						Sore throat				
						Coughing				
						Other				

	Type	Time (mins)	Intensity 1	Intensity 2	Intensity 3		Tick if present	Severity 1	Severity 2	Severity 3
am						Nil				
						Nasal symptoms				
						Fever				
pm						Sore throat				
						Coughing				
						Other				

	Type	Time (mins)	Intensity 1	Intensity 2	Intensity 3		Tick if present	Severity 1	Severity 2	Severity 3
am						Nil				
						Nasal symptoms				
						Fever				
pm						Sore throat				
						Coughing				
						Other				

Appendix 10.8
Information pamphlet: Study 2 (Older Adults)

This study has received ethical approval from the Massey University Ethics Committee and the Auckland Ethics Committee.

If you have any questions?

..... about the study, either now or in the future, please feel free to call. If you need an interpreter, one can be provided.

Christine Crooks:
Phone (09) 414 0800 xtn.9803
Phone (027) 233 3590
c.v.crooks@massey.ac.nz

Or my research supervisor,
Dr Ajmol Ali:
Phone (09) 414 0800 xtn.9638

If you have any queries or concerns about your rights as a participant in this study, you may wish to contact:

Health Advocates Trust
(Northland to Franklin) 0800 555 050

Thank you for taking the time to read this information sheet.

Version 2: 1 November 2004

Massey University
Institute of Food, Nutrition & Human Health

Compensation

The Auckland Ethics Committee has certified that this clinical trial is being conducted principally for the benefit of the manufacturer in respect of which this trial is being carried out. This means that if you suffer injury as a result of your participation, you will not be eligible for cover under accident compensation legislation. Compensation, however, will be provided by Fonterra Co-operative Group Ltd in accordance with the "New Zealand Researched Medicines Industry Guidelines on Clinical Trials - Compensation for injury resulting from participation in Industry Sponsored Clinical Trials."

These RMI Guidelines are only guidelines and until your claim is assessed by the insurers of Fonterra Co-operative Group Ltd it cannot be said with any certainty exactly what type or amount of compensation you will receive if you suffer injury as a result of your participation, or what sort of injury will be covered. The guidelines require that compensation must be provided by Fonterra Co-operative Group Ltd where the injury you suffer is serious and not just temporary and is one caused by the for dietary supplements or where you would not have suffered injury but for your inclusion in this trial.

The guidelines also require that the compensation you receive must be appropriate to the nature, severity and persistence of your injury. This means that you will be likely to receive some compensation from Fonterra Co-operative Group Ltd unless your injury is minor or temporary, however, you might not receive compensation from Fonterra Co-operative Group Ltd if your injury was caused by the investigators, if there is a deviation from the proposed plan of research, or if your injury was caused solely by you.

Effects of Bovine Colostrum Supplementation on the Immune System in Older Adults

(65 - 75 years)

Does bovine colostrum enhance the immune system?

You are invited to participate in the study *'Effects of bovine colostrum supplementation on levels of a salivary antibody (immunoglobulin A or s-IgA)'*. The study is part fulfillment of the completion of a PhD in Immunology and Nutrition at Massey University.

Screening Questionnaire is available in Te Reo if required.

Why am I doing this study?

A health concern for older people is that they may experience a higher frequency of 'colds' and other respiratory problems because of a declining immune system. Inadequate nutrition has been found to be a major cause of immune problems with ageing. It is now being recognised that an important approach to improving immune health in older people is through the use of specific dietary supplements.

One way of monitoring the immune response in the respiratory tract is to measure an antibody in the saliva. This antibody (called secretory immunoglobulin A or s-IgA) is important in the first line of defence against viral infections such as colds.

A pilot study to establish the procedures for measuring s-IgA found levels of s-IgA were increased in those taking bovine colostrum compared to those in a placebo group (aged 30-58 years). Bovine colostrum is the first milk produced by cows after the birth of their calf. The study was limited by the wide age group and the small number of participants.

The main aim of this study is to determine the effect of bovine colostrum on levels of s-IgA on a larger group of similarly aged older people.

The study also aims to investigate the normal food intake of older adults in the Auckland area and how this might affect their immune system.

By participating in this study you will help to determine whether bovine colostrum supplementation can enhance the immune system in older adults.

Who is in this study?

- Healthy older adults aged 65 to 75 years in urban Auckland
- Must not be lactose or dairy intolerant
- Must not be taking any medication for coronary heart disease, diabetes, kidney or liver problems and asthma or other respiratory diseases
- Must be physically active
- Must live independently
- Must not be taking anti-inflammatory drugs
- Must meet requirements in screening questionnaire.

What is involved?

Nutritional and immunological information will be collected from all participants in the study. Saliva samples will be collected from you at home. You will need to visit a local Diagnostic Medlab to provide a blood sample. There will be no cost to you. Travel to Diagnostic Medlab for blood samples will be covered by taxi chits.

Participants will be asked to participate in the following procedures:

- Consume daily either a bovine colostrum supplement or skim milk placebo in the form of a flavoured milkshake.
- Provide an interviewer with information on dietary intake over the previous 24 hours. This will be done once at the beginning of the trial and once at the end.
- Provide 5 blood samples. 2 blood samples taken in the 2 weeks before supplementation begins, then one at 5 weeks, 10 weeks of taking supplementation. The final one 2 weeks after supplementation has finished.
- Provide 9 saliva samples. 6 saliva samples taken weekly for 6 weeks then 3 more samples fortnightly.
- Self check of hydration status by using a urine analysis stick when saliva sample due.
- Complete an easy to use daily wellness and activity checklist.

Family/whanau may be present on visits;
Unused red blood cells may be returned if requested.

Do you have to take part in this study?

Your participation is entirely voluntary (your choice). If you agree to take part in the study, you are free to withdraw up until the end of data collection, and have the right to refuse to answer any question at any time. Your health will not be affected.

What will happen to the results?

Data collected from participants is completely confidential. No material, which could personally identify you, will be used in any reports in this study. The results will be stored by code number in a computer in a locked room throughout the study. Information will be stored for 10 years and then destroyed.

Appendix 10.9
Consent form: Study 2 (Older adults)

Massey University

Does bovine colostrum supplementation enhance immune function?

CONSENT FORM

I have read and I understand the information sheet dated 23/6/4, version 2 for volunteers taking part in the study designed to find out the effects of bovine colostrum on enhancing immune function in older adults. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.

I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study up until the end of data collection and that this will in no way affect my health

I understand that my participation in this study is confidential and that no material, which could identify me, will be used in any reports on this study. I understand that the project will be submitted as part fulfilment of the completion of a PhD in Science degree. I understand the compensation provisions for this study.

I have had time to consider whether to take part. I know who to contact if I have any side effects to the study. I know who to contact if I have any questions about the study.

Disposal of unused red blood cells

I request unused red blood cells to be returned to me YES/NO

I understand that if the red blood cells are not returned they are disposed of following the safety procedures of the laboratory for disposing of biohazardous material

YES/NO

Version 2,
1/11/4

Research results

I would like the researcher to discuss the outcomes of the study with me. YES/NO

Maori	E hiahia ana ahau ki tetahi kaiwhakamaori/kaiwhaka pakeha korereo.	Ae	Kao
Samoan	Oute manaó ia iai se faámatala upu.	Ioe	Leai
Tongan	Oku ou fiemaú ha fakatonulea.	Io	Ikai
Cook Island	Ka inangaro au I tetai tangata uri reo.	Ae	Kare
Niuean	Fia manako au ke fakaaoga e taha tagata fakahokohoko kupu.	E	Nakai
	Other languages to be added following consultation with relevant communities.		

I consent to data being stored for use in a future related study for which ethics approval will be gained by an accredited NZ ethics committee. YES/NO

I_____(full name), hereby consent to take part in this study.

Date: .....

Signature participant: .....

Full name of researcher: Christine Virginia Crooks
Contact phone number for researcher: (09) 414 0800 ex 9803, (027) 2333 590

Project explained by: Christine Crooks

Signature: .....

Name of Witness: .....

Signature of Witness: .....

(Note a copy of the consent form to be retained by the participant)

Version 2, 1/11/4

Appendix 10.10
Ethics approval letter: Study 2 (Older Adults)

Auckland
Ethics Committees

Private Bag 92522
Wellesley Street
Auckland
Delivery Address:
C/O Ministry of Health
3rd Floor, Unisys Building
650 Great South Road, Penrose
Phone (09) 580 9105
Fax (09) 580 9001
Email: pat_chainey@moh.govt.nz

Please include the reference no. and study title in all correspondence/telephone calls.

11 November 2004

Christine Crooks
Institute of Food Nutrition & Human Health
Albany Campus
Private Bag 102 904
Auckland.

Dear Christine

AKY/04/10/268 **The effect of bovine colostrum supplementation on levels of secretory immunoglobulin A (s-IgA) in older adults. PIS/ConsV#2, 1 November 2004**

Thank you for your amendments, received 3 November 2004.

The above study has been given ethical approval by Auckland Ethics Committee Y.

Certification
It is certified as not being conducted principally for the benefit of the manufacturer or distributor and may be considered for coverage under ACC.

Accreditation
This Committee is accredited by the Health Research Council and is constituted and operates in accordance with the Operational Standard for Ethics Committees, March 2002.

Documents Approved:
- Information Sheet/Consent Form, version 2, dated 1 November 2004.

Progress Reports
The study is approved until 31 October 2005, subject to the Committee's review of the approved application annually. A progress report is required for this study on 31 October 2005. A form will come off our database requesting this information prior to the review date. Please note that failure to complete and return this form may result in the withdrawal of ethical approval.

Please advise the Committee when the study is completed and a final report is also required at the conclusion of the study.

Requirements for SAE Reporting
Please advise the Committee as soon as possible if there are any serious adverse events which relate to this study.

.../2

General:
All amendments to the study must be advised to the Committee prior to their implementation, except in the case where immediate implementation is required for reasons of safety. In such cases the Committee must be notified as soon as possible of the change.

It should be noted that Ethics Committee approval does not imply any resource commitment or administrative facilitation by any healthcare provider, within whose facility the research is to be carried out. Where applicable, authority for this must be obtained separately from the appropriate manager within the organisation.

Yours sincerely

Sandra Sparks
Administrator Committee Y, email: sandra_sparks@moh.govt.nz

cc: Auckland DHB

Appendix 10.11
Study 2: Screening questionnaire for Older Adults

Massey University

SCREENING QUESTIONNAIRE FOR PARTICIPANTS IN IMMUNITY STUDY- Does bovine colostrum enhance immune function?

This questionnaire is to be completed by interested participants before they can be accepted onto the study. It is not a medical requirement and medical examination is not necessary to participate in the study.

Name :……Age……. Date…….
Ethnicity:……

1	**Known medical conditions-are you taking medication for any of the following?**				
a	Heart problems	**Yes**		**No**	
b	Blood pressure	**Yes**		**No**	
c	Asthma or any other respiratory condition	**Yes**		**No**	
d	Kidney or liver problems	**Yes**		**No**	
e	Diabetes	**Yes**		**No**	
f	Digestive	**Yes**		**No**	
g	Stroke	**Yes**		**No**	
h	Blood disorder	**Yes**		**No**	
i	Inflammatory diseases e.g. arthritis, coeliac disease	**Yes**		**No**	
j	A major allergic reaction	**Yes**		**No**	
h	Irregular bowel movements?	**Yes**		**No**	
2	**In the past five years, have you had any illnesses that have required you to:**				
a	Consult your GP	**Yes**		**No**	
b	Attend a hospital outpatient department	**Yes**		**No**	
c	Be admitted to hospital	**Yes**		**No**	
d	Have you ever been told that you have high blood pressure?	**Yes**		**No**	
e	Have you ever been told that you have had a heart attack?	**Yes**		**No**	
f	Have you ever been told that you have heart problems?	**Yes**		**No**	
g	Have you ever been told that you have a stroke?	**Yes**		**No**	
h	Have you ever been told you have epilepsy?	**Yes**		**No**	
i	Have you ever been told you have Parkinsons disease?	**Yes**		**No**	
j	Have you ever been told that you have diabetes mellitus?	**Yes**		**No**	
k	Have you ever been told you have any other chronic condition?	**Yes**		**No**	
3	**In the past five years have you had surgical treatment for:**				
a	Coronary heart disease	**Yes**		**No**	
b	Stroke	**Yes**		**No**	
c	Cancer	**Yes**		**No**	

4 Are you sensitive to:

a	Lactose in cow's milk	**Yes**		**No**	
b	Other dairy products e.g. yoghurt, cheese	**Yes**		**No**	

5 Are you taking any:

a	Whey protein dietary supplements	**Yes**		**No**	
b	Pre biotic or probiotic dietary supplements	**Yes**		**No**	

6 Other health related questions

a	Do you smoke?	**Yes**		**No**	
b	Do you drink more than 20 standard alcohol drinks per week?	**Yes**		**No**	
c	Do you live alone?	**Yes**		**No**	
d	Do you live with a partner/ husband /wife /other	**Yes**		**No**	

If you do smoke,

How long have you smoked……..

How much do you smoke…….

If you answered yes in part 1 or 2 briefly describe the medical condition and list any medication here

Thank you for taking the time to complete this questionnaire

Appendix 10.12
Activity and wellness diary: Study 2 (Older Adults)

ACTIVITY & WELLNESS DIARY

'Effects of Bovine Colostrum Supplementation on the Immune System in Older Adults'

(65 - 75 years)

Massey University

Institute of Food, Nutrition & Human Health
Massey University
Albany

Activity and Wellness Diary
'Immunity Study'
65 - 75 years

ID Number:

This diary is designed to record your daily activity and wellness over the period of the study including two weeks before the intervention begins, the 10 weeks of intervention and the two weeks after.

Instructions

Activity:
Please record activity and time (minutes) for each day. Rate the intensity of the sessions:

1 = Gentle eg. stretching, yoga
2 = Moderate eg. golf, walking
3 = More effort eg jogging, dancing

Wellness:
Please record your daily health status. If you have no health problems, tick the nil box. If you have upper respiratory symptoms (URS) please tick the type of symptom and rate the severity.

URS:
Nasal symptoms (includes runny nose, congestion, sneezing)
Fever, Sore throat, Coughing

Severity:

1 = Very mild, no change to daily activities
2 = Moderate, was not active
3 = Severe, confined to bed

All information provided in this diary will be treated with strictest confidence. No outside study will have access to it.

Thank you for participating in this study.
I really appreciate the time you are giving!

Christine Crooks
Phone: 09-414 0800 xtn.9803 *Mobile:* 027-2333 590
c.v.crooks@massey.ac.nz

ACTIVITY	TYPE	TIME (minutes)	WELLNESS	If Yes, rate the severity of symptom	Severity		
					1	2	3
Monday			'Cold or Flu' symptoms	Nasal symptoms			
Date:			☐ Yes	Fever			
				Sore throat			
			☐ No	Coughing			
				Other:			

ACTIVITY	TYPE	TIME (minutes)	WELLNESS	If Yes, rate the severity of symptom	Severity		
					1	2	3
Tuesday			'Cold or Flu' symptoms	Nasal symptoms			
Date:			☐ Yes	Fever			
				Sore throat			
			☐ No	Coughing			
				Other:			

ACTIVITY	TYPE	TIME (minutes)	WELLNESS	If Yes, rate the severity of symptom	Severity		
					1	2	3
Wednesday			'Cold or Flu' symptoms	Nasal symptoms			
Date:			☐ Yes	Fever			
				Sore throat			
			☐ No	Coughing			
				Other:			

ACTIVITY	TYPE	TIME (minutes)	WELLNESS	If Yes, rate the severity of symptom	Severity		
					1	2	3
Thursday			'Cold or Flu' symptoms	Nasal symptoms			
Date:			☐ Yes	Fever			
				Sore throat			
			☐ No	Coughing			
				Other:			

Appendix 10.13
Diet recall interview questionnaire: Study 2 (Older Adults)

Massey University

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Diet Recall Immunity Study

Name/Code: **Date recorded:**

Time	Food (and brand where possible)	Amount	Preparation

Check these items

Fruit
Bread
Cereal
Sugar
Jam, peanut butter and other spreads
Butter/marg
Milk (green, blue etc)
Water/tea/coffee (with milk? sugar added?)
Takeaways
Red meat/chicken/ fish
Eggs
Nuts (other protein alternatives ?)
Supplements ???

Appendix 10.14
Formulation of bovine colostrum and placebo blends used in supplementation trials

Appendix 10.14: Formulation of bovine colostrum and placebo blends used in supplementation trials

Ingredients	BOVINE COLOSTRUM BLEND			PLACEBO BLEND		
	Chocolate	Berry	Malt	Chocolate	Berry	Malt
Colostrum Powder (AN15)	40.0%	40.0%	40.0%			
Skim Milk Powder	30.1%	31.7%	31.0%	59.0%	40.0%	38.0%
Milk Protein Concentrate Powder					31.1%	32.0%
Calcium Caseinate				11.0%		
Whole Milk Powder	0.0%	2.0%	2.0%	1.6%	3.0%	2.9%
Sugar	20.0%	20.0%	14.5%	20.0%	20.0%	15.0%
Flavours	3.8%	1.1%	2.5%	2.3%	0.7%	2.1%
Colour		0.2%	1.5%		0.2%	1.5%
Cocoa Powder	5.0%			5.0%		
Stabilisers	1.1%	1.0%	1.0%	1.1%	1.0%	1.0%
Malt Powder			7.5%			7.5%
Maltodextrin		4.0%			4.0%	
	100.0%	*100.0%*	*100.0%*	*100.0%*	*100.0%*	*100.0%*

Predicted Compositions	BOVINE COLOSTRUM BLEND			PLACEBO BLEND		
	Chocolate	Berry	Malt	Chocolate	Berry	Malt
Protein	31.9%	31.8%	31.6%	31.8%	31.8%	31.6%
Fat	1.5%	1.5%	1.5%	1.6%	1.5%	1.5%
Total Carbohydrates	56.0%	57.8%	57.9%	56.2%	57.6%	57.7%
Ash	6.1%	5.7%	5.7%	5.8%	5.8%	5.7%
Moisture	4.5%	3.2%	3.3%	4.6%	3.3%	3.5%
TOTAL	*100.0%*	*100.0%*	*100.0%*	*100.0%*	*100.0%*	*100.0%*

Colours: Berry=Beet Powder, Malt=Caramel Powder

Appendix 10.15
Levels of endogenous bioactive measured in the bovine colostrum (BC) and placebo blends (supplied by Fonterra)

Appendix 10.15: Levels of endogenous bioactive measured in the bovine colostrum (BC) and placebo blends (supplied by Fonterra)

Bioactive	AN15	BC (calculated)	BC (malt)	BC (chocolate)	BC (berry)	Placebo (malt)	Placebo (chocolate)	Placebo (berry)
IgG (HPLC-MC) (% w/w)[1]	4.6	1.9	1.7	1.6	1.7	<0.01	<0.01	0.1
IgG (RID) (% w/w)[2]	8.2	3.3	2.9	2.9	3.2	<0.03	<0.03	<0.03
IgA (mg/g)[3]	9.1	3.7	3.0	3.3	1.7	0.07	<0.01	0.04
IgM (mg/g)[4]	0.8	0.32	0.22	0.23	0.09	ND	ND	ND
Lf (mg/g)[5]	0.65	0.28	0.2	0.2	0.1	<0.01	<0.01	<0.01
IGF-1 (ng/g)[6]	144.5	57.8	53.1	53.7	64.0	2.8	ND	4.5
IGF-2 (ng/g)[7]	89.3	35.7	35.3	29.3	31.6	ND	ND	ND
TGF-B1 (ng/g)[8]	69.6	27.8	45.3	19.7	27.7	44.1	21.7	31.0
TGF-B2 (ng/g)[9]	283.3	113.3	162.0	102.7	135.5	101.7	56.8	93.6
Oligosaccharides (g/100g)[10]	0.18	0.07	5.92	2.64	3.89	5.64	1.52	3.38

[1]Analysed according to NZTM3, Method 11.23, IgG by HPLC for colostrum containing powders, skim milk powders and whey protein concentrates with casein precipitation.
[2]Analysed according to NZTM3, Method 24.8, Bovine IgG by Radial Immunodiffusion for whey protein concentrate and skim milk powder.
[3]Analysed using Bethyl Laboratories Ltd. Bovine IgA Elisa Quantitation Kit (Cat. No. E10-121), using attached kit method.
[4]Analysed using Bethyl Laboratories Ltd. Bovine IgM Elisa Quantitation Kit (Cat. No. E10-101), using attached kit method.
[5]Analysed using Bethyl Laboratories Ltd. Bovine Lactoferrin Elisa Quantitation Kit (Cat. No. E10-126), using attached kit method
[6]Analysed using R&D Systems DuoSet Elisa Kit (Human IGF-1, Cat. No. DY291), using attached kit method and adapted preparation and activation steps.
[7]Analysed using R&D Systems DuoSet Elisa Kit (Mouse IGF-2, Cat. No. DY782), using attached kit method and adapted preparation and activation steps.
[8]Analysed using R&D Systems DuoSet Elisa Kit (Human TGF-B1, Cat. No. DY240), using attached kit method and adapted preparation and activation steps.
[9]Analysed using R&D Systems DuoSet Elisa Kit (Human TGF-B2, Cat. No. DY302), using attached kit method and adapted preparation and activation steps.
[10]Analysed according to NZTM3, Method 5.10, Oligosaccharide analysis for milk powders using HPLC.

AN15= Bovine colostrum powder AN15
ND=Not detected

Appendix 10.16
Enzyme Linked Immunosorbent Assay (ELISA) method for human IL-6

Appendix 10.16: ELISA method for human IL-6

1. Dilute 33.3µL reconstituted capture antibody (360µg/mL) in 6000µL Phosphate buffer solution (PBS) (1x) to give working concentration of 2.0µg/mL.
   Aliquot 50µL of the diluted capture antibody into each well of a 96 well ELISA plate (plate). Cover plate with foil and place at 4^{o}C overnight.

2. Wash plate 3 times with wash buffer.

3. Block unbound sites by adding 200µl blocking buffer to each well and incubate at room temperature for 60minutes (min).

4. Wash plate 2-3 times with wash buffer.

5. Add 50µl blank, prediluted standard or undiluted plasma sample into the appropriate well, cover and incubate at room temperature for 2 hours.
   Blank - Reagent diluent (RD)
   Standard - Dilute human recombinant standard (70 ng/mL) serially in RD.

Volumes for each standard:

2 stage dilution: Dilution factor=512/70,000pg/mL=0.00731=1/136.72
1st 1/10 (20/180) =***(x)***
2nd 1/13.672=0.0731 x 400µL=29.26µL

Standard (pg/mL)	**512** (stock)	**256**	**128**	**64**	**32**	**16**	**8**	**4**	**2**
Quantity RD (µL)	385.4	200	300	200	200	200	200	200	200
Amount of previous standard (µL)	29.26 ***(x)***	200	100	200	200	200	200	200	200

6. Wash plate 5 times with wash buffer. The 1st wash is quick and completed within 10 seconds. The 2nd to 5th washes remain on the plate for 60 seconds before removing.

7. Dilute 33.3µL reconstituted detection antibody (36µg/mL) to 100ng/mL in 6000µL reagent diluent.
   Aliquot 50µL of the diluted detection antibody (200ng/mL) in reagent diluent into each well of the ELISA plate, cover with fresh foil and incubate for 60min at room temperature.

8. Wash plate 5 times with wash buffer as in (6).

9. Dilute Streptavidin-Horse radish peroxidase (Streptavidin-HRP) in RD, 1:200 for working dilution (30µL in 6mL). Cover with foil until ready to use. Add 50µL of the working dilution of Streptavidin-HRP per well, cover with fresh foil and incubate at room temperature in drawer for 30min.

10. Wash plate 5 times with wash buffer as in (6).

11. Aliquot enough Tetramethylbenzidine substrate solution (TMB) to centrifuge tube and cover in foil until ready to use.
    Add 50µL TMB to each well, cover plate with fresh foil and incubate in drawer for 20min at room temperature.

12. Stop reaction by adding 25µL 1.0N hydrochloric (HCL) acid. Pop any bubbles with a fresh pipette tip.

13. Read at 450nm

Reagents and Consumables

Phosphate buffered solution (PBS) - single strength concentration (1x)
pH 7.2–7.4 (store in fridge away from light)

NaCl (Sodium Chloride; Merk, 10404)	8.0g
KCl (Potassium Chloride; Merk, 104936)	0.2g
KH_2PO_4 (Potassium dihydrogen orthophosphate, BDH 102034B)	0.2g
Na_2HPO_4 (Disodium hydrogen orthophosphate, Merk 106586)	1.13g

Adjust to pH 7.2 with either 1.0M HCl acid or 1.0M NaOH.
Make up to 1L with deionised water.

(Make 10 x concentration by weighing out 10 times the weight of each reagent and making up to 1L. Adjust pH to 7.2 with either 1.0M HCl acid or 1.0M NaOH. The 10 x concentration will keep longer and can be diluted to single strength concentration (1x) as needed; store at room temperature).

Wash buffer
0.05% Tween 20 (Polyoxyethylenesorbitan monolaurate tween 20 [Sigma P9416]) in PBS (1x)

Blocking Buffer
1% BSA (BSA Fraction V, IgG free (Bovine Serum Albumin [Gibco 300063-572]) in PBS (1x)

Reagent Diluent (RD)
0.1% BSA in PBS (1x).

TMB+ Substrate-Chromogen
3,3',5,5'-tetramethylbenzidine(TMB)+ substrate-chromogen, Dako (S1599).

96 well plate: Microlon 600 high affinity flat bottomed plates (Greiner 655061).

Human IL-6 (DuoSet DY206, RnD systems)
Capture antibody: Part 840113, mouse anti-human IL-6 antibody. One vial is reconstituted to 360 µg/mL with 1.0mL of PBS (1x).
Detection antibody: Part 840114, biotinylated goat anti-human IL-6 antibody. One vial is reconstituted to 36µg/mL in 1.0mL of RD.
Human IL-6 Standards: Part 840115, recombinant human IL-6, lypholised. One vial is reconstituted to 70ng/mL in 0.5mL deionised water.
Streptavidin–HRP: Part 890803, streptavidin peroxidase conjugated to horse radish peroxidase. Stored at 2 8°C. DO NOT FREEZE.

Appendix 10.17
ELISA method for human IL-1ra

Appendix 10.17: ELISA method for human IL-1ra

1 Dilute 33.3µL reconstituted capture antibody (1800µg/mL) in 6000µL PBS (1x) to give working concentration of 10µg/mL.
Aliquot 50µL of the diluted capture antibody into each well of a 96 well ELISA plate (plate). Cover plate with foil and place at 4^{o}C overnight.

2 Wash plate 3 times with wash buffer.

3 Block unbound sites by adding 200µL blocking buffer to each well. Cover with fresh foil and incubate at room temperature for 60minutes (min).

4 Wash plate 2-3 times with wash buffer.

5 Add 50µL blank, prediluted standard or undiluted plasma sample into the appropriate well, cover and incubate at room temperature for 2 hours
Blank - Reagent diluent (RD).
Standard - Dilute human recombinant standard (70ng/mL) serially in RD.

Volumes for each standard:

2 stage dilution 1920/70,000pg/mL=0.0274=1/36.5
1st 1/10 (20/180) =***(x)***
2nd 1/3.65 (volume of 1920pg/mL standard = 400µL, need 109.6 µL of stock)

Standard (pg/mL)	**1920**	**960**	**480**	**240**	**120**	**60**	**30**	**15**	**7.5**
Quantity RD (µL)	290.4	200	200	200	200	200	200	200	200
Amount of previous standard (µL)	109.6 ***(x)***	200	200	200	200	200	200	200	200

6 Wash plate 5 times with wash buffer. The 1st wash is quick and completed within 10 seconds. The 2nd to 5th washes remain on the plate for 60 seconds before removing.

7 Dilute 33.6µL reconstituted detection antibody (18µg/mL) to 100ng/mL.

Aliquot 50µL of the diluted detection antibody (100ng/mL) into each well of the plate, cover with fresh foil and incubate for 60min at room temperature.

8 Wash plate 5 times with wash buffer as in (6).

9 Dilute Streptavidin–HRP in RD, 1:200, for working dilution (30µL in 6mL). Cover with foil until ready to use.
Add 50µL of the working dilution of Streptavidin-HRP per well, cover with fresh foil and incubate at room temperature in drawer for 30min.

10 Wash plate 5 times with wash buffer as in (6).

11 Aliquot enough TMB substrate solution (TMB) to centrifuge tube and cover in foil until ready to use.
Add 50µL TMB to each well, cover with fresh foil and incubate in drawer for 20min at room temperature.

12 Stop reaction by adding 25µL 1.0N HCL acid. Pop any bubbles with a fresh pipette tip.

13 Read at 450nm

Reagents and Consumables

Phosphate buffered solution (PBS)- single strength concentration (1x)
pH 7.2–7.4 (store in fridge away from light)

NaCl (Sodium Chloride; Merk, 10404)	8.0g
KCl (Potassium Chloride; Merk, 104936)	0.2g
KH_2PO_4 (Potassium dihydrogen orthophosphate, BDH 102034B)	0.2g
Na_2HPO_4 (Disodium hydrogen orthophosphate, Merk 106586)	1.13g

Adjust to pH 7.2 with 1.0M HCl acid or 1.0M NaOH.
Make up to 1L with deionised water.
(Make 10 x concentration by weighing out 10 times the weight of each reagent and making up to 1L. Adjust pH to 7.2 with 1.0M HCl acid acid or 1.0M NaOH. The 10 x concentration will keep longer and can be diluted to single strength concentration (1x) as needed; store at room temperature).

Wash buffer
0.05% Tween 20 (Polyoxyethylenesorbitan monolaurate tween 20 [Sigma P9416]) in PBS (1x).

Blocking Buffer
1% BSA (BSA Fraction V, IgG free (Bovine Serum Albumin [Gibco 300063-572]) in PBS (1x).

Reagent Diluent (RD)
0.1% BSA in PBS (1x).

TMB+ Substrate-Chromogen
3,3',5,5'-tetramethylbenzidine(TMB)+ substrate-chromogen, Dako (S1599).

96 well ELISA plate: Microlon 600 high affinity flat bottomed plates (Greiner 655061).

Human IL -1ra (DuoSet: DY280, RnD systems)
Capture antibody: Part 840270, mouse anti-human IL-1ra. One vial is reconstituted to 1800μg/mL in 1mL of PBS. 50μL aliquots are stored at -80°C.
Detection antibody: Part 840721, biotinylated goat anti-human IL-1ra antibody. One vial is reconstituted to 18μg/mL with 1.0mL RD. 50μL aliquots are stored at -80°C.
Streptavidin–HRP: Part 890803, streptavidin peroxidase conjugated to horse radish peroxidase. Stored at 2–8°C. DO NOT FREEZE.
Human IL-1ra Standard: Part 840272, recombinant human IL-1ra. One vial is reconstituted to 70ng/mL with 0.5mL RD. 50μL aliquots are stored at -80°C.

Appendix 10.18
ELISA method for IFN-α

Appendix 10.18: ELISA method for human interferon-alpha (IFN-a)

1 Prepare final wash solution: Add 50mL of bottle A to 1000mL deionised water, store in the fridge. Use ice cold for washing.

2 Add 50µL blank, prediluted standard or undiluted plasma sample into each appropriate well of the pre-coated plate, cover and incubate at room temperature for 1 hour.

Standard (pg/mL)	**1000** (vial B)	**500**	**200**	**100**	**50**	**25**	**12.5**	**0**
Quantity RD Bottle C (µL)	-	950	600	500	500	500	500	1000
Amount of previous standard (µL)	-	50	400	500	500	500	500	-

RD=reagent diluent

3 Wash plate 3 times in wash buffer.

4 Dilute 60µL antibody concentrate (bottle D) to 6000µL antibody diluent (bottle I). Add 50 µL per well, cover and incubate at room temperature for 60 minutes (min).

5 Wash plate 3 times in wash buffer.

6 To prepare working solution of Streptavidin-HRP:
Dilute 7µL Streptavidin-HRP concentrate in 49µL conjugate diluent (bottle F).
Further dilute 32µL of this 'diluted' Streptavidin-HRP concentrate in 288µL conjugate diluent (bottle F) (1/10 dilution) = **X**.
Dilute 150µL of **X** in 6000µL conjugate diluent (bottle F) = Streptavidin-HRP working solution.

Aliquot 50µL Streptavidin-HRP working solution into each well of the ELISA plate, cover and incubate for 60min at room temperature.

7 Wash plate 5 times in wash buffer.

8 Add 50µL TMB substrate solution (bottle G) and incubate 15 min in the dark at room temperature.

9 Stop reaction by adding 50µL stop solution (bottle H) per well. Mix by tapping plate gently.

10 Read at 450nm.

Consumables
Human interferon alpha ELISA kit for human serum samples, Five Plate (480 tests)
Product# 41110-2

Appendix 10.19
List of metabolic equivalents (METs) for physical activities performed by the participants

Appendix 10.19: List of metabolic equivalents (METs) for activities for the students, older adults and training for the swimmers. METS are from *Ainsworth et al.,* 1993

Students and Older adults					Swimmers			
Activity	S Intens	OA Intens	AC	METs	Training	Sw Intens	AC	METs
Walking	N/A	1	17150	2.0	Swimming	1	18240	8.0
	1	2	17170	3.0		2	18230	10.0
	2	3	17200	4.0		3	18280	11.0
	3	N/A	17080	6.0	Gymn WO	2	02050	6.0
Running	1	N/A	12010	5.0		3	02040	8.0
	N/A	3	12020	7.0	Running	1	12060	11.0
	2	N/A	12030	8.0		2	12080	12.5
	3	N/A	12060	11.0		3	12100	14.0
Cycling	1	2	01010	4.0	WPolo		18360	10.0
	2	3	02013	7.0	IM Surf		18190	12.5
	3	N/A	01040	10.0				
Swimming	1	2	18350	4.0				
	2	3	18240	8.0				
	3	N/A	18340	10.0				
Body Pump	1	N/A	02130	3.0				
	2	N/A	03021	7.0				
Netball	2	N/A	15060	7.0				
	3	N/A	15490	10.0				
Soccer	2	N/A	15060	7.0				
	3	N/A	15490	10.0				
Touch Rugby	2	N/A	15610	7.0				
	3	N/A	15560	10.0				
Bowls	N/A	1	15160	2.5				
Golf	1	2	15270	3.0				
Keep fit class	N/A	2	02100	4.0				
	N/A	3	02072	7.0				
Dancing	N/A	2	03025	4.0				
Tennis	N/A	2	15675	7.0				
Shopping	N/A	1	05065	2.0				
	N/A	2	05066	2.5				
Housework	N/A	2	05040	2.5				
	N/A	3	05140	4.0				
Fishing	N/A	1	04030	2.5				
	N/A	2	04040	3.5				
Manual labour	N/A	1	11170	2.5				
	N/A	2	11630	4.0				
	N/A	3	11570	7.0				
Choir singing	N/A	1	13035	2.0				

AC = Activity code
S = Students
OA = Older adults
Sw = Swimmers
Intens = Intensity
Gymn WO = Workout in gymnasium
WPolo = Water Polo
IM Surf = Ironman surf lifesaving training

Appendix 10.20
Mean weekly activity factor (mean wAF) converted to Physical Activity Level (PAL) by cohort

Appendix 10.20: Mean weekly activity factor (Mean wAF) converted to PAL by cohort.

Students				Swimmers				Older adults			
ID	Mean wAF	M/F	PAL	ID	Mean wAF	M/F	PAL	ID	Mean wAF	M/F	PAL
31	0.46	F	1.6	219	3.26	F	2.0	94	0.78	F	1.7
163	2.84	F	2.0	249	4.04	F	2.3	133	0.24	F	1.5
213	0.40	F	1.6	266	2.39	M	2.0	161	1.11	F	1.8
294	0.34	F	1.6	267	3.20	F	2.0	200	0.94	F	1.8
303	0.35	M	1.7	272	3.85	M	2.0	204	0.58	M	1.7
328	0.54	M	1.7	335	4.12	M	2.3	211	2.14*	M	1.9
351	0.78	F	1.8	428	1.17	M	1.9	278	2.27*	M	1.9
366	1.01	F	1.8	463	2.66	F	2.0	299	0.41	F	1.6
369	0.36	M	1.7	466	4.35	M	2.3	336	6.58*	M	2.2
372	0.04	M	1.4	504	4.57	M	2.3	342	0.23	F	1.5
402	0.16	M	1.5	521	1.86	F	1.8	343	1.33	F	1.8
414	0.18	F	1.5	539	3.48	F	2.0	353	1.30	F	1.8
431	0.05	F	1.4	571	4.22	F	2.1	365	0.86	F	1.7
448	0.83	F	1.7	583	2.77	M	2.1	378	1.36	M	1.8
484	0.66	F	1.7	607	3.38	F	2.0	385	0.82	F	1.7
486	0.67	F	1.7	633	4.09	M	2.3	427	0.75	F	1.7
550	0.68	F	1.7	634	4.49	M	2.3	442	0.47	F	1.6
560	1.11	F	1.8	635	3.26	F	2.0	473	0.24	F	1.5
627	0.42	F	1.6	651	4.07	F	2.3	495	1.13	M	1.8
649	0.24	F	1.5	668	0.80	M	1.8	501	0.92	F	1.8
744	0.60	F	1.6	682	4.09	M	2.3	515	0.53	M	1.6
770	0.49	F	1.6	708	3.51	F	2.0	558	0.22	M	1.5
833	0.30	M	1.5	743	2.69	F	2.0	580	0.46	F	1.6
856	0.15	M	1.4	762	2.97	M	2.0	601	5.64*	M	2.0
974	0.33	F	1.6	786	1.60	F	1.8	616	2.90*	M	2.0
1073	0.38	M	1.7	1175	0.93	M	2.1	636	1.07	M	1.8
1177	0.75	M	1.8					691	0.68	F	1.7
1301	0.52	F	1.6					709	2.01*	M	1.9
								711	0.67	F	1.7
								714	0.85	M	1.8
								727	1.26	M	1.8
								760	2.47*	F	1.8
								790	1.59	F	1.7
								846	1.06	M	1.9
								884	0.38	F	1.7
								1028	0.23	M	1.5
								1044	1.09	M	1.9
								1049	0.36	M	1.7
								1089	0.71	F	1.8
								1090	1.17	M	1.9
								1305	0.38	M	1.7
								1348	0.98	F	1.7
								1351	0.71	F	1.8
								1406	0.76	F	1.8
								1442	0.83	F	1.8

*Older adults: For mean wAFs greater than 2.0, a PAL value from the lower end of the 'Heavy range' was assigned to account for their better record keeping e.g. Older Male 336 with mean wAF=6.58 was assigned 2.2

Appendix 10.21
Haematological parameters and liver function enzymes

Appendix 10.21: Haematological parameters (swimmers and students). Mean and standard error of the mean (SEM) of baseline (Base) and post-supplementation (Post) parameters. Results for bovine colostrum (BC) and placebo (Pl) groups within cohorts. Median (Med) and interquartile range (IQ range) used for non-normal distributions. *P*=significance levels for differences between BC and Pl within cohorts

	Students				Swimmers			
Haematological parameter	BC (n=16)		Pl (n=11)		BC (n=12)		Pl (n=12)	
Ferritin (µg/L)	Med	IQ range	Med	IQ range	Med	IQ range	Med	IQ range
F	39.0	32.5-53.5	39.75	17.75-48.8	52.3	40.8-80.9	74.8	30.0-128.0
M	74.5	59.8-103.8	99.0	55.0-153.0	73.0	44.5-85.4	130.5	48.5-197.5
P (within cohort)	*0.869*				*0.827*			
Haemoglobin (g/L)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	134.0	1.87	136.4	3.38	137.2	2.07	142.1	1.99
M	153.4	2.88	157.1	1.08	160.3	2.77	156.2	3.41
P (within cohort)	*0.227*				*0.312*			
Red Cell Count (x 10^9 /L)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	4.58	0.077	4.56	0.102	4.55	0.099	4.83	1.99
M	5.24	0.116	5.31	0.071	5.38	0.092	5.34	3.41
P (within cohort)	*0.227*`				*0.248*			
MCV (fL)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	87.41	0.77	89.33	0.70	90.50	1.09	88.00	1.27
M	86.10	1.29	86.60	0.37	87.25	0.66	86.29	1.10
P (within cohort)	*0.303*				*0.735*			

MCV=Mean cell volume
M=Male
F=Female

Appendix 10.21 continued: Haematological parameters and liver function enzymes (older adults). Mean and SEM of baseline and post-supplementation parameters. Results for bovine colostrum (BC) and placebo (Pl) groups. Median (Med) and interquartile range (IQ range) used for non-normal distributions. *P*=significance levels for differences between BC and Pl within the older adult cohort

	Older adults			
	BC (n=22)		Pl (n=23)	
Ferritin (µg/L)	Med	IQ range	Med	IQ range
F	70.5	59.8-288.8	127.5	82.8-288.8
M	150.0	79.8-326.6	182.3	88.8-326.6
P (within cohort)	*0.140*			
Haemoglobin (g/L)	Mean	SEM	Mean	SEM
F	132.7	1.44	136.0	2.96
M	146.2	4.23	141.3	4.32
P (within cohort)	*0.027*			
Red Cell Count (x 10^9 /L)	Mean	SEM	Mean	SEM
F	4.61	0.061	4.83	0.12
M	4.84	0.10	5.34	0.12
P (within cohort)	*0.026*			
MCV (fL)	Mean	SEM	Mean	SEM
F	90.50	1.09	88.00	1.27
M	87.25	0.66	86.29	1.10
P (within cohort)	*0.995*			
Gamma GT (U/L)	Mean	SEM	Mean	SEM
F	32.94	8.36	24.21	4.35
M	29.05	4.21	24.58	3.21
P (within cohort)	*0.407*			
ALP (U/L)	Mean	SEM	Mean	SEM
F	79.19	4.29	79.71	5.34
M	75.75	5.43	81.53	3.99
P (within cohort)	*0.322*			
AST (U/L)	Mean	SEM	Mean	SEM
F	27.10	4.64	23.63	0.92
M	26.90	2.27	22.48	1.70
P (within cohort)	*0.288*			
ALT (U/L)	Mean	SEM	Mean	SEM
F	32.30	11.40	21.67	1.87
M	29.73	4.32	20.47	1.56
P (within cohort)	*0.215*			

F=Female
M=Male
MCV=Mean cell volume
Gamma GT=Gamma glutamyl transpeptidase
ALP=Alkaline phosphatase
AST=Aspartate transaminase
ALT=Alanine transaminase

Appendix 10.22
Salivary immunoglobulins, albumin and osmolality

Appendix 10.22: Salivary immunoglobulins, albumin (Alb) and osmolality (Osm). Mean and SEM for salivary s-IgA (s-IgA), immunoglobulin M (IgM), immunoglobulin G (IgG), Alb, Osm. *P*=significance levels for the main effects of BC/Pl, gender (Gen) and time.

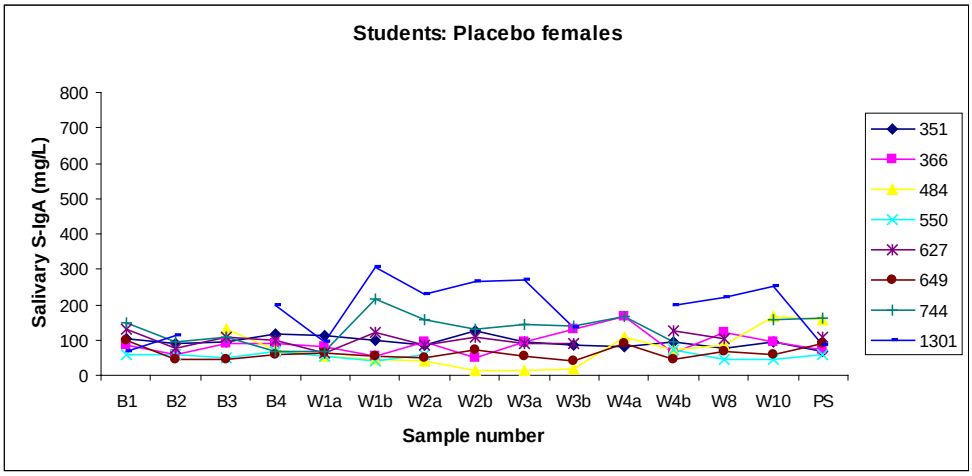
		Students				Swimmers				Older adults			
Salivary parameter		BC (n=16)		Pl (n=12)		BC (n=12)		Pl (n=13)		BC (n=22)		Pl (n=23)	
s-IgA (mg/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	82.22	5.19	89.82	5.37	97.76	10.51	93.65	7.17	152.46	14.37	123.35	15.95
	4wks	85.30	5.75	106.84	10.55	67.89	8.41	104.88	9.37	142.33	21.60	117.43	18.43
	8wks	74.25	5.37	110.90	15.92	119.81	21.97	114.78	15.22	113.47	10.46	94.09	10.14
	10wks	95.53	8.59	108.12	19.44	108.16	19.94	115.77	18.67	145.92	22.57	110.95	13.71
	Post	95.92	10.18	108.82	15.60	102.62	27.15	109.71	16.04	142.94	19.12	100.71	15.11
P (within cohort)	*BC/Pl*	*0.154*				*0.914*				*0.249*			
	Gen	*0.601*				*0.259*				*0.576*			
	Time	*0.050*				*0.609*				*0.004*			
IgM (mg/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	9.97	0.51	9.12	0.39	12.74	1.78	9.89	0.74	7.62	1.33	8.94	2.84
	4wks	9.20	0.55	9.40	0.58	9.02	1.27	9.17	0.85	6.85	1.47	7.14	1.32
	8wks	9.83	1.01	8.71	0.64	15.33	3.87	11.38	1.68	6.63	1.48	6.32	1.37
	10wks	9.99	0.89	10.58	1.39	12.31	3.59	8.97	1.20	8.94	2.50	6.59	1.10
	Post	10.85	1.15	10.747	2.99	14.52	4.67	9.80	0.99	7.66	1.66	6.30	1.01
P (within cohort)	*BC/Pl*	*0.890*				*0.557*				*0.846*			
	Gen	*0.971*				*0.797*				*0.524*			
	Time	*0.211*				*0.121*				*0.064*			
IgG (mg/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	8.70	0.85	16.22	4.02	18.92	2.17	21.38	4.43	28.66	4.33	22.51	4.23
	4wks	8.59	1.22	18.84	5.36	13.54	2.22	28.62	7.44	28.14	6.64	27.35	8.03
	8wks	7.49	0.98	13.55	3.60	40.40	18.57	29.23	9.77	24.33	5.59	27.93	4.96
	10wks	10.21	2.21	11.05	2.72	22.44	8.34	29.02	9.43	37.62	11.08	24.70	5.18
	Post	11.53	2.89	18.55	9.64	19.80	4.43	25.84	6.74	24.60	5.68	18.75	3.07
P (within cohort)	*BC/Pl*	*0.262*				*0.966*				*0.642*			
	Gen	*0.439*				*0.191*				*0.520*			
	Time	*0.611*				*0.189*				*0.168*			

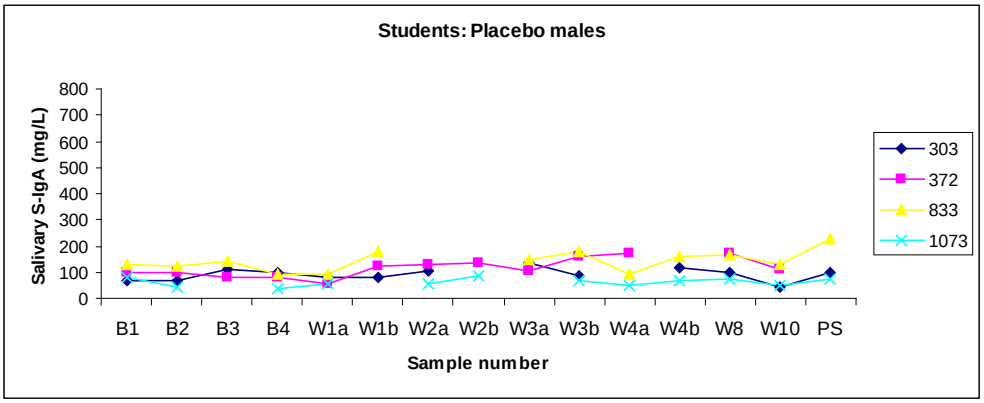
Appendix 10.22 continued: Salivary immunoglobulins, albumin and osmolality. Mean and SEM for salivary IgA (s-IgA), immunoglobulin M (IgM), immunoglobulin G (IgG), albumin (Alb), osmolality (Osm). *P*=significance levels for the main effect of bovine colostrum/placebo (BC/Pl), gender (Gen) and time.

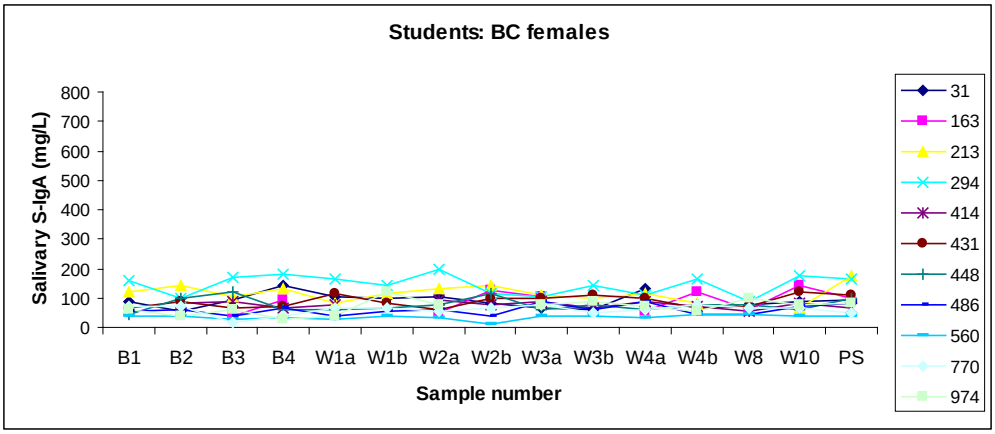
		Students				Swimmers				Older adults			
Salivary parameter		BC (n=16)		Pl (n=12)		BC (n=12)		Pl (n=13)		BC (n=22)		Pl (n=23)	
Albumin (mg/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	34.89	4.75	54.15	9.05	62.43	6.90	70.45	16.92	142.70	22.65	81.58	15.48
	4wks	33.49	6.21	60.72	14.69	44.14	7.83	117.34	34.34	99.51	16.86	106.63	29.20
	8wks	26.95	6.96	49.20	11.73	119.03	50.76	102.92	32.61	106.55	25.36	106.86	22.93
	10wks	39.16	9.79	37.64	8.98	66.19	21.33	106.94	30.94	105.96	20.75	91.63	26.67
	Post	38.16	9.54	56.25	24.49	61.96	11.70	88.96	26.87	103.73	22.66	70.27	14.12
P (within cohort)	*BC/Pl*	*0.213*				*0.698*				*0.407*			
	Gen	*0.896*				*0.285*				*0.908*			
	Time	*0.863*				*0.293*				*0.396*			
Osm (mOsm/kg)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	67.17	4.01	68.77	1.32	104.50	16.60	100.20	22.06	103.86	5.29	89.60	3.53
	4wks	61.87	3.28	72.25	4.52	81.16	8.37	83.04	5.45	100.93	6.84	97.40	5.45
	8wks	67.53	4.66	66.91	3.32	78.43	13.33	85.00	4.81	99.98	5.83	89.55	4.23
	10wks	65.63	5.21	71.00	4.20	88.82	10.05	84.69	7.79	110.41	7.94	90.78	5.10
	Post	61.94	4.22	64.91	4.32	85.00	9.29	79.64	6.69	102.91	7.23	90.13	4.86
P (within cohort)	*BC/Pl*	*0.446*				*0.646*				*0.132*			
	Gen	*0.779*				*0.932*				*0.101*			
	Time	*0.435*				*0.535*				*0.407*			

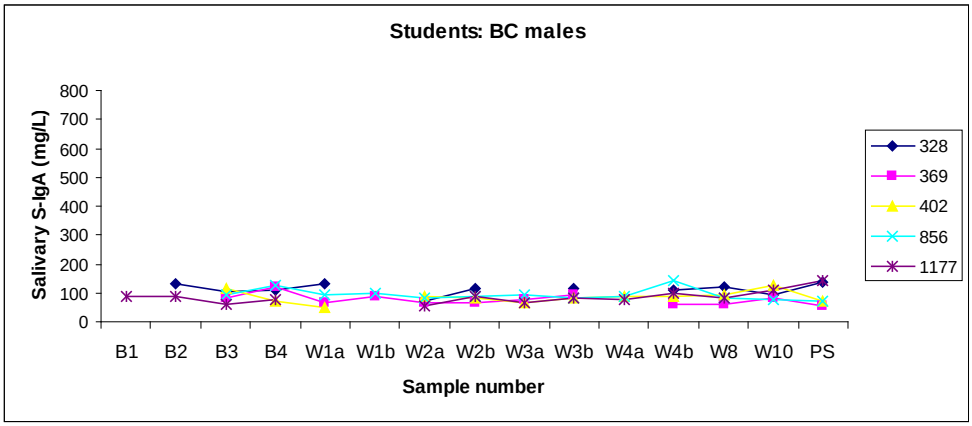
Appendix 10.23
Individual plots of salivary S-IgA by cohort and gender

Appendix 10.23: Individual plots for salivary S-IgA by supplement and gender for the students cohort

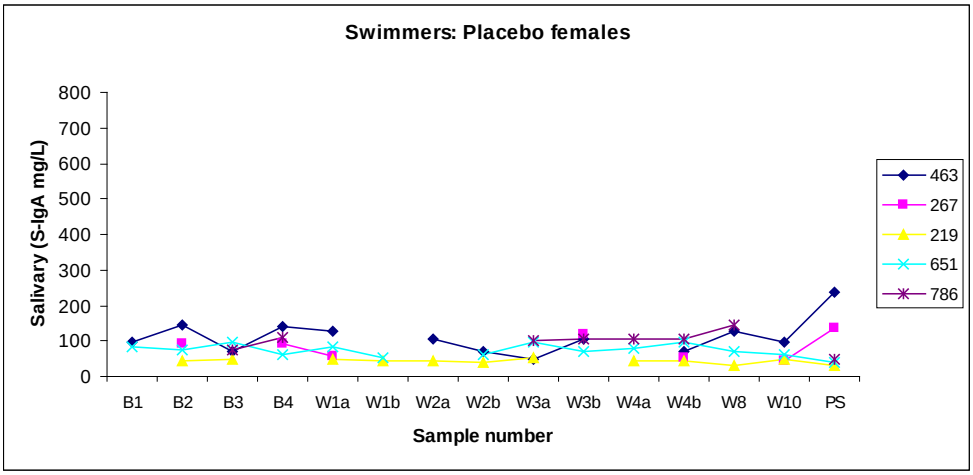


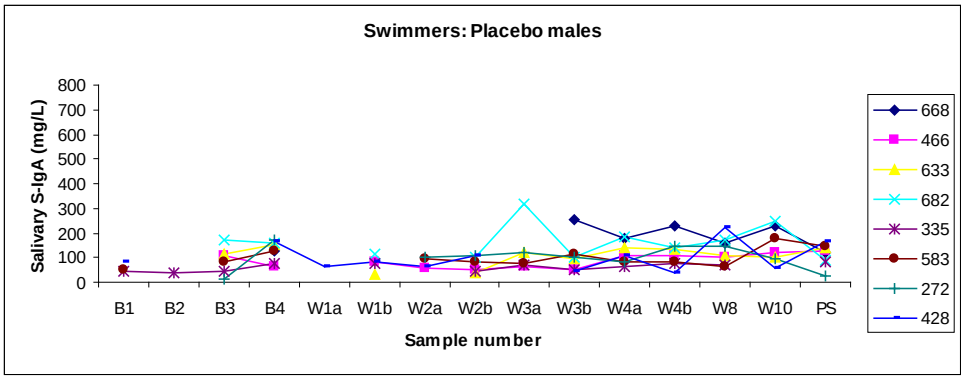


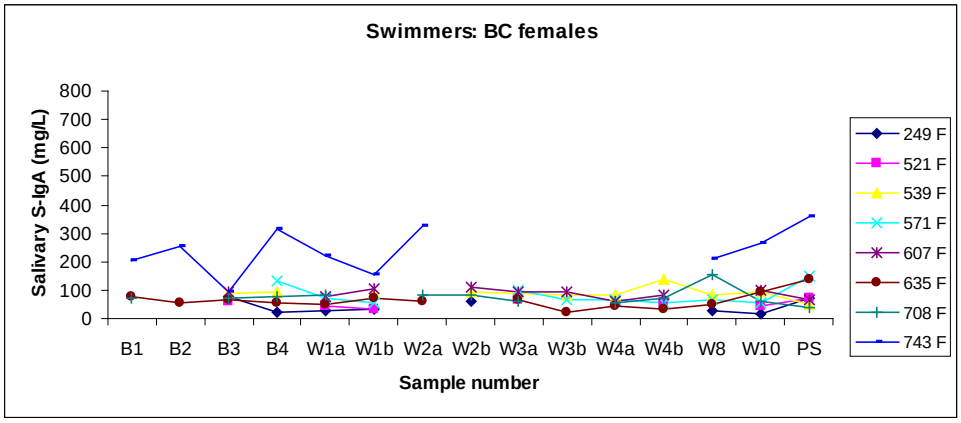


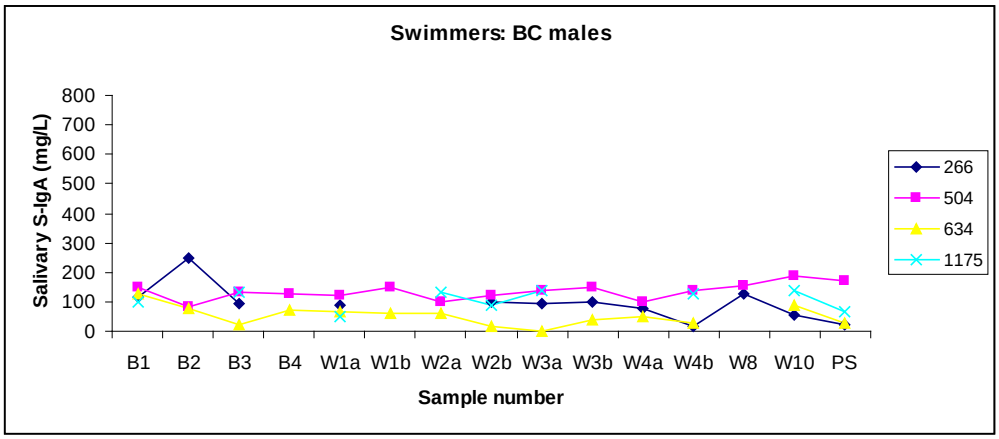


Appendix 10.23 continued: Individual plots for salivary S-IgA by supplement and gender for the swimmers cohort

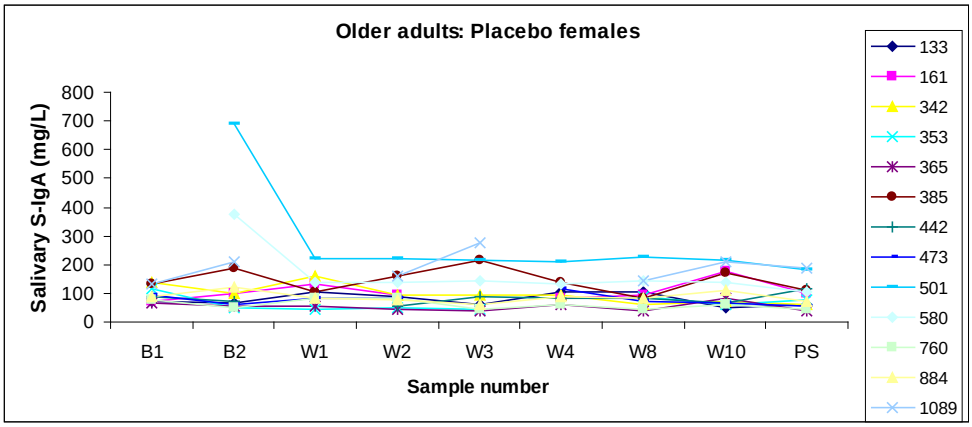


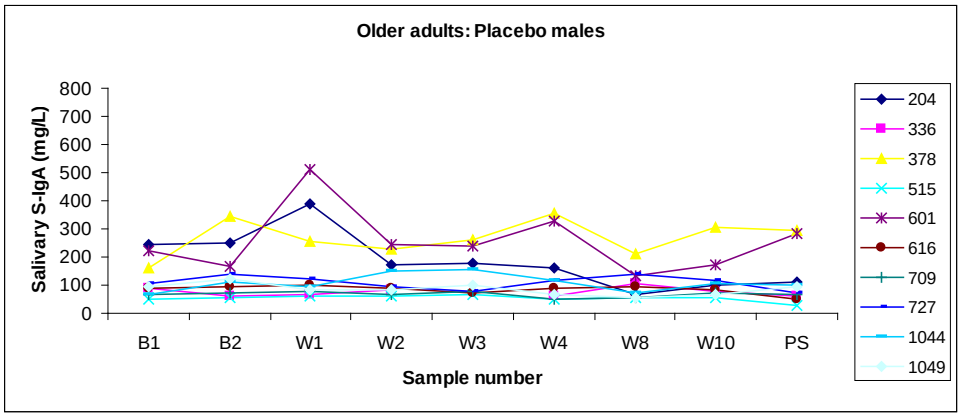


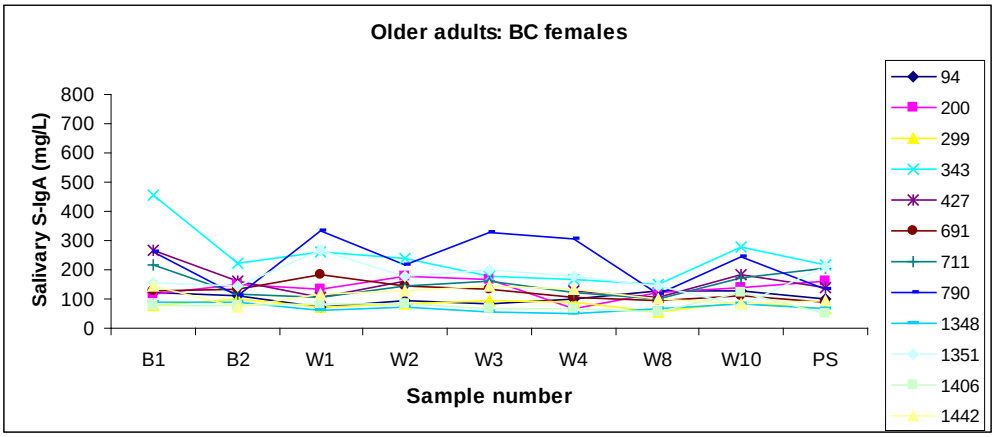


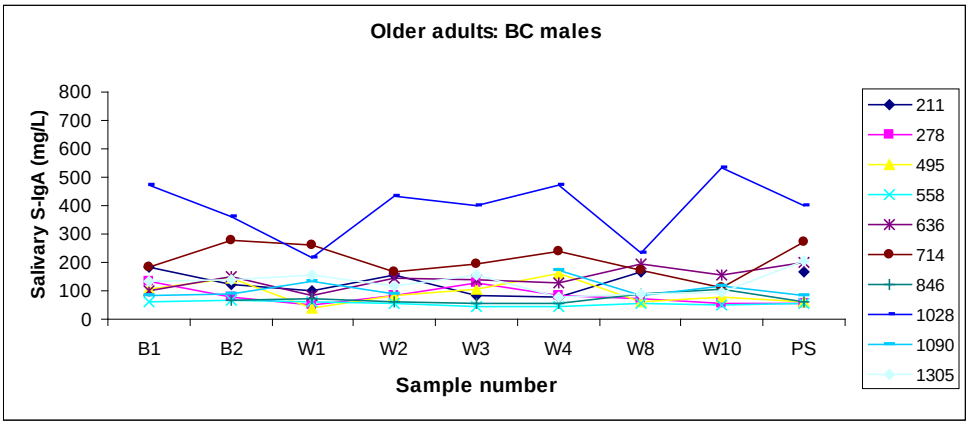


Appendix 10.23 continued: Individual plots for salivary S-IgA by supplement and gender for the older adults cohort









Appendix 10.24
Plasma immunoglobulins, albumin and C-reactive protein (CRP)

Appendix 10.24: Plasma immunoglobulins, Alb and CRP. Mean and SEM. *P*=significance levels for the main effects of BC/Pl, Gen and, time (Base, five weeks=5wks, ten weeks=10wks, Post)

Plasma parameter		Students				Swimmers				Older adults			
		BC (n=16)		Pl (n=11)		BC (n=12)		Pl (n=12)		BC (n=22)		Pl (n=23)	
IgA (g/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	1.75	0.21	2.52	0.27	1.50	0.16	1.68	0.19	2.66	0.14	2.28	0.13
	5wks	1.59	0.20	2.61	0.33	1.60	0.17	1.61	0.23	2.68	0.19	2.29	0.19
	10wks	1.39	0.12	2.53	0.30	1.52	0.20	1.62	0.23	2.74	0.20	2.27	0.20
	Post	1.36	0.11	2.48	0.34	1.54	0.46	1.61	0.23	2.73	0.21	2.39	0.23
P (within cohort)	*BC/Pl*	*0.012*				*0.430*				*0.124*			
	Gen	*0.955*				*0.076*				*0.265*			
	Time	*0.601*				*0.777*				*0.150*			
IgM (g/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	1.15	0.10	0.98	0.10	0.98	0.10	0.86	0.06	0.57	0.09	1.06	0.14
	5wks	1.16	0.11	0.92	0.14	0.99	0.12	0.82	0.07	0.63	0.13	1.08	0.16
	10wks	1.09	0.15	0.92	0.13	1.07	0.13	0.82	0.07	0.65	0.13	1.10	0.15
	Post	1.06	0.15	0.98	0.16	1.03	0.12	0.81	0.06	0.69	0.15	1.10	0.15
P (within cohort)	*BC/Pl*	*0.347*				*0.319*				*0.602*			
	Gen	*0.611*				*0.200*				*0.837*			
	Time	*0.816*				*0.183*				*0.003*			
IgG (g/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	7.68	0.29	8.47	0.96	9.37	0.62	8.49	0.47	8.04	0.28	9.57	0.44
	5wks	7.72	0.30	9.38	1.01	8.11	0.76	8.46	0.39	7.94	0.35	10.00	0.69
	10wks	7.92	0.28	9.55	1.12	8.77	0.87	8.72	0.56	8.20	0.38	10.02	0.71
	Post	7.76	0.36	9.50	1.16	9.27	0.76	8.56	0.54	8.15	0.45	10.11	0.71
P (within cohort)	*BC/Pl*	*0.045*				*0.904*				*0.024*			
	Gen	*0.319*				*0.105*				*0.670*			
	Time	*0.973*				*0.645*				*0.205*			

Appendix 10.24 continued: Plasma immunoglobulins, Alb and CRP. Mean and SEM. *P*=significance levels for the main effects of BC/Pl, Gen and, time (Base, 5wks, 10wks, Post).

Plasma parameter		Students				Swimmers				Older adults			
		BC (n=16)		Pl (n=11)		BC (n=12)		Pl (n=12)		BC (n=22)		Pl (n=23)	
Alb (g/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	42.06	0.52	42.31	0.65	43.93	0.11	43.54	0.48	40.38	0.38	40.23	0.49
	5wks	42.04	0.65	42.59	0.80	43.77	0.21	44.02	0.49	39.80	0.57	40.19	0.63
	10wks	42.04	0.48	42.51	0.80	43.55	0.23	43.58	0.45	40.29	0.51	38.31	1.69
	Post	42.13	0.42	41.55	0.89	43.07	0.21	43.84	0.60	38.44	1.85	40.38	0.45
P (within cohort)	*BC/Pl*	*0.746*				*0.606*				*0.998*			
	Gen	*0.003*				*0.037*				*0.436*			
	Time	*0.325*				*0.338*				*0.659*			
CRP (mg/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	5.41	0.62	5.47	0.96	3.84	0.32	4.08	0.25	6.19	0.69	4.98	0.03
	5wks	5.30	0.85	5.34	0.90	3.74	0.22	3.78	0.24	5.42	0.42	5.60	0.60
	10wks	5.07	0.89	4.66	0.68	3.46	0.24	3.64	0.41	5.24	0.24	5.05	0.05
	Post	6.43	2.21	6.15	1.89	3.71	0.38	4.12	0.33	6.00	0.73	5.07	0.07
P (within cohort)	*BC/Pl*	*0.832*				*0.345*				*0.306*			
	Gen	*0.294*				*0.083*				*0.233*			
	Time	*0.839*				*0.371*				*0.695*			

Appendix 10.25
Subgroup results: Plasma cortisol, interleukin-6 (IL-6), interleukin-1receptor antagonist (IL-1ra), interferon-α (IFN-α), and insulin-like growth factor-1 (IGF-1)

Appendix 10.25: Subgroup results: Plasma cortisol, IL-6, IL-1ra, IFN-α and IGF-1. Mean and SEM. *P*=significance levels for the main effect of BC/Pl, gender (Gen), and time. Base compared to levels after 5wks supplementation.

		Students				Swimmers				Older adults			
		BC (n=5)		Pl (n=5)		BC (n=3)		Pl (n=3)		BC (n=5)		Pl (n=5)	
Cortisol (nmol/L)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	772.05	69.28	651.87	86.36	749.84	65.56	872.27	27.60	373.70	52.80	326.6	32.20
	5 wks	754.48	102.25	735.04	191.17	816.73	5.43	1111.39	105.60	328.00	46.56	259.6	62.52
P (within cohort)	*BC/Pl*	*0.996*				*0.004*				*0.269*			
	Gen	*0.365*				*0.801*				*0.259*			
	Time	*0.102*				*0.026*				*0.323*			
IL-6 (pg/mL)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	32.38	12.58	12.23	4.46	7.32	4.46	24.2	22.40	10.57	3.52	7.48	3.04
	5 wks	33.53	15.89	10.34	4.72	7.49	7.50	33.27	26.94	15.92	7.45	4.88	3.20
P (within cohort)	*BC/Pl*	*0.551*				*0.576*				*0.450*			
	Gen	*0.534*				*0.419*				*0.296*			
	Time	*0.999*				*0.620*				*0.708*			
IL-1ra (pg/mL)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	195.63	113.24	15.27	8.16	162.57	83.4	137.80	87.70	92.68	79.33	9.33	93.32
	5 wks	189.66	134.64	31.29	31.24	19.98	20	130.74	130.89	120.82	53.94	26.21	26.17
P (within cohort)	*BC/Pl*	*0.559*				*0.760*				*0.251*			
	Gen	*0.643*				*0.274*				*0.471*			
	Time	*0.677*				*0.277*				*0.827*			
IFN-α (pg/mL)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	127.3	40.9	67.1	28.3	33.1	27.8	147.8	46.8	45.8	19.4	34.5	14.2
	5 wks	185	113.0	95.2	47.8	9.27	9.27	110.9	62.1	20.6	11.3	28.5	15.0
P (within cohort)	*BC/Pl*	*0.353*				*0.183*				*0.940*			
	Gen	*0.398*				*0.399*				*0.411*			
	Time	*0.804*				*0.835*				*0.277*			
IGF-1 (ng/mL)		No Ana.	No Ana.	No Ana.	No Ana.	Mean	SEM	Mean	SEM	No Ana.	No Ana.	No Ana.	No Ana.
	Base					616.70	89.20	696.20	64.20				
	5 wks					572.60	87.17	672.00	80.80				
P (within cohort)	*BC/Pl*					*0.391*							
	Gen					*0.184*							
	Time					*0.177*							

No Ana.=Analysis not performed

Appendix 10.26
Subgroup results: Leucocyte and lymphocyte subsets (absolute numbers)

Appendix 10.26: Subgroup results: Leucocytes and lymphocyte subsets. Mean and SEM. *P*=significance levels for the main effect of BC/Pl, Gen, and time on the absolute numbers of leucocytes (Base results compared to levels after 5wks supplementation)

Leucoytes and lymphocyte subsets		Students				Swimmers				Older adults			
		BC (n=5)		Pl (n=5)		BC (n=3)		Pl (n=3)		BC (n=5)		Pl (n=5)	
White blood cells x 10^9/L		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	7.49	0.51	6.41	0.39	7.77	1.27	7.51	2.31	5.01	0.23	5.67	0.50
	5 wks	6.75	0.40	6.01	0.10	6.91	1.39	10.12	7.71	5.57	0.44	6.33	0.81
P (within cohort)	*BC/Pl*	*0.270*				*0.390*				*0.384*			
	Gen	*0.937*				*0.193*				*0.797*			
	Time	*0.316*				*0.877*				*0.234*			
Segmented Neutrophil x 10^9/L		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	4.62	0.48	3.45	0.23	5.28	1.51	5.49	1.42	2.63	0.132	3.259	0.31
	5 wks	3.92	0.40	3.32	0.30	3.94	1.19	5.85	1.20	3.30	0.242	4.018	0.95
P (within cohort)	*BC/Pl*	*0.339*				*0.499*				*0.167*			
	Gen	*0.398*				*0.123*				*0.129*			
	Time	*0.333*				*0.800*				*0.275*			
Lymphocyte x 10^9/L		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	2.18	0.16	2.13	0.12	1.94	0.27	2.08	0.13	1.56	0.12	1.81	0.22
	5 wks	2.17	0.29	2.05	0.22	2.35	0.54	3.38	0.87	1.75	0.39	1.72	0.31
P (within cohort)	*BC/Pl*	*0.662*				*0.587*				*0.954*			
	Gen	*0.804*				*0.649*				*0.090*			
	Time	*0.589*				*0.254*				*0.712*			
T-cells (CD3) x 10^9/L		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	1.57	0.12	1.50	0.09	1.54	0.28	1.60	1.14	1.01	0.08	1.58	0.23
	5 wks	1.58	0.28	1.45	0.14	1.88	0.48	2.45	0.71	1.11	0.19	1.31	0.24
P (within cohort)	*BC/Pl*	*0.550*				*0.587*				*0.246*			
	Gen	*0.725*				*0.649*				*0.163*			
	Time	*0.753*				*0.254*				*0.551*			

Appendix 10.26 continued: Subgroup results: Leucocytes and lymphocyte subsets. Mean and SEM. *P*=significance levels for the main effect of BC/Pl, Gen), and time on the absolute numbers of leucocytes (Base results compared to levels after 5wks supplementation)

Leucocyte and lymphocyte subsets		Students				Swimmers				Older adults			
		BC (n=5)		Pl (n=5)		BC (n=3)		Pl (n=3)		BC (n=5)		Pl (n=5)	
Helper T-cells (CD4) x 10^9/L		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	0.97	0.07	0.87	0.05	0.78	0.09	0.924	0.11	0.70	0.07	1.18	0.19
	5 wks	0.94	0.10	0.83	0.08	0.97	0.19	1.39	0.41	0.80	0.17	0.97	0.15
P (within cohort)	*BC/Pl*	*0.429*				*0.583*				*0.140*			
	Gen	*0.963*				*0.839*				*0.103*			
	Time	*0.419*				*0.278*				*0.555*			
Cytotoxic T-cells (CD8) x 10^9/L		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	0.51	0.15	0.53	0.05	0.54	0.14	0.47	0.03	0.27	0.04	0.314	0.08
	5 wks	0.45	0.13	0.44	0.08	0.64	0.20	0.75	0.19	0.27	0.06	0.304	0.10
P (within cohort)	*BC/Pl*	*0.820*				*0.914*				*0.663*			
	Gen	*0.584*				*0.195*				*0.453*			
	Time	*0.028*				*0.206*				*0.502*			
B-cells (CD19) x 10^9/L		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	0.19	0.03	0.21	0.05	0.25	0.02	0.24	0.02	0.176	0.02	0.17	0.03
	5 wks	0.21	0.01	0.31	0.05	0.27	0.04	0.31	0.07	0.206	0.03	0.15	0.04
P (within cohort)	*BC/Pl*	*0.082*				*0.785*				*0.460*			
	Gen	*0.914*				*0.701*				*0.243*			
	Time	*0.106*				*0.363*				*0.078*			
CD4/CD8 ratio		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	1.97	0.114	1.82	0.195	1.55	0.22	1.97	0.18	3.34	0.72	3.86	0.40
	5 wks	2.13	0.151	2.11	0.298	1.60	0.17	1.87	0.23	3.56	0.90	3.77	0.48
P (within cohort)	*BC/Pl*	*0.948*				*0.097*				*0.870*			
	Gen	*0.703*				*0.394*				*0.741*			
	Time	*0.110*				*0.006*				*0.560*			

Appendix 10.27
Subgroup results: Lymphocyte subsets (percentages)

Appendix 10.27: Subgroup results: Lymphocyte subsets (percentages). Mean and SEM. *P*=significance levels for the main effect of BC/Pl, Gen, and time on percentage of lymphocyte subsets (baseline compared to percentages after 5wks supplementation).

		Students				Swimmers				Older adults			
Lymphocyte subset		BC (n=5)		Pl (n=5)		BC (n=3)		Pl (n=3)		BC (n=5)		Pl (n=5)	
%T-cells (CD3)		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	72.10	1.10	70.70	1.79	78.67	3.28	76.50	2.47	64.47	1.42	75.70	2.17
	5 wks	73.40	1.36	71.40	2.78	79.00	2.00	70.00	4.00	64.28	1.50	76.08	2.99
P (within cohort)	*BC/Pl*	*0.620*				*0.150*				*0.015*			
	Gen	*0.746*				*0.310*				*0.780*			
	Time	*0.353*				*0.096*				*0.691*			
%CD3/CD4		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	44.80	1.17	41.10	1.35	40.50	1.04	44.17	2.42	45.09	2.02	57.36	1.19
	5 wks	43.80	1.74	40.80	0.86	41.67	1.34	40.33	1.76	45.76	3.36	57.94	1.61
P (within cohort)	*BC/Pl*	*0.329*				*0.387*				*0.816*			
	Gen	*0.371*				*0.932*				*0.745*			
	Time	*0.761*				*0.056*				*0.821*			
%CD3/CD4/CD49d		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	84.53	1.211	88.204	1.121	90.05	1.06	86.57	2.20	64.03	8.13	70.03	3.03
	5 wks	83.83	2.118	85.720	1.094	86.42	1.40	89.62	1.04	71.43	5.03	69.501	3.32
P (within cohort)	*BC/Pl*	*0.061*				*0.726*				*0.903*			
	Gen	*0.217*				*0.772*				*0.664*			
	Time	*0.136*				*0.758*				*0.499*			
%CD3/CD8		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	23.10	0.88	24.30	2.02	27.00	3.50	22.83	1.64	17.39	2.50	16.35	1.79
	5 wks	20.80	0.87	21.00	3.04	26.33	2.03	22.33	3.38	16.12	3.30	16.32	2.00
P (within cohort)	*BC/Pl*	*0.966*				*0.212*				*0.973*			
	Gen	*0.661*				*0.513*				*0.980*			
	Time	*0.004*				*0.249*				*0.907*			

Appendix 10.27 continued: Subgroup results: Lymphocyte subsets (percentages). Mean and SEM. *P*=significance levels for the main effect of BC/Pl, Gen, and time on the absolute numbers of leucocytes (Base results compared to levels after 5wks supplementation)

Lymphocyte subset		Students				Swimmers				Older adults			
		BC (n=5)		Pl (n=5)		BC (n=3)		Pl (n=3)		BC (n=5)		Pl (n=5)	
%CD3/CD8/CD49d		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	97.50	1.19	98.09	2.69	100	0	98.96	1.05	94.88	0.017	95.35	0.006
	5 wks	99.17	0.83	99.17	1.86	100	0	100		95.40	0.013	95.76	0.009
P (within cohort)	*BC/Pl*	*0.769*				*0.450*				*0.707*			
	Gen	*0.695*				*0.450*				*0.721*			
	Time	*0.417*				*0.464*				*0.414*			
%CD19		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	11.88	0.62	15.57	0.87	13.00	1.73	12.33	0.955	11.26	0.70	8.85	1.56
	5 wks	10.00	0.84	14.80	1.28	12.33	2.41	10.75	1.702	12.44	1.32	8.82	2.46
P (within cohort)	*BC/Pl*	*0.042*				*0.653*				*0.363*			
	Gen	*0.841*				*0.910*				*0.871*			
	Time	*0.082*				*0.221*				*0.240*			
%CD19/CD49d		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	100	0	100	0	100	0	100	0	95.60	0.008	96.90	0.006
	5 wks	100	0	100	0	100	0	100	0	98.34	0.009	98.81	0.008
P (within cohort)	*BC/Pl*	-				-				*0.960*			
	Gen	-				-				*0.542*			
	Time	-				-				*0.197*			

Appendix 10.28
Subgroup results: Mean fluorescent intensities (MFIs) for lymphocyte subsets expressing CD49d

Appendix 10.28: Subgroup results: MFIs for lymphocyte subsets expressing CD49d. Mean and SEM. *P*=significance levels for the main effect of BC/Pl, Gen and time on MFI of CD49d (Base compared to levels after 5wks supplementation).

Lymphocyte subset		Students				Swimmers				Older adults			
		BC (n=5)		Pl (n=5)		BC (n=3)		Pl (n=3)		BC (n=5)		Pl (n=5)	
MFI CD4/49d		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	428.10	31.80	450.10	15.74	478.00	25.48	354.50	20.54	734.00	49.04	648.00	30.13
	5wks	451.60	29.63	497.60	32.46	427.00	13.24	328.80	31.17	719.20	89.05	627.00	42.02
P (within cohort)	*BC/Pl*	*0.210*				*0.041*				*0.402*			
	Gen	*0.148*				*0.220*				*0.330*			
	Time	*0.049*				*0.248*				*0.666*			
MFI CD8/49d		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	464.40	24.18	502.90	12.97	554.20	23.07	498.67	29.16	933.78	25.12	932.40	41.30
	5wks	519.60	30.75	574.40	24.40	519.33	30.20	499.25	46.31	970.80	55.47	918.00	31.67
P (within cohort)	*BC/Pl*	*0.575*				*0.505*				*0.721*			
	Gen	*0.177*				*0.821*				*0.639*			
	Time	*0.022*				*0.943*				*0.504*			
MFI CD19/49d		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	Base	469.25	19.08	420.00	14.13	520.80	26.74	481.00	24.89	617.22	23.06	669.00	35.27
	5wks	487.40	19.45	478.40	19.67	451.00	52.22	483.50	28.48	625.60	41.72	604.00	46.24
P (within cohort)	*BC/Pl*	*0.963*				*0.806*				*0.767*			
	Gen	*0.309*				*0.564*				*0.799*			
	Time	*0.022*				*0.162*				*0.226*			

Appendix 10.29
Baseline comparisons between cohorts for immunoglobulin and albumin parameters in saliva and plasma

Appendix 10.29: Baseline comparison between cohorts for immunoglobulin and albumin parameters in saliva and plasma. Mean and SEM. *P*=significance levels between the cohorts; Students saliva (n=28), Students plasma (n=27), Swimmers saliva (n=26), Swimmers plasma (n=25), Older adults (n=45).

Salivary or plasma parameter	Mean	SEM	*P (cohort)*	*P (gender)*	Tukey	Duncan
Salivary s-IgA (mg/L)						
Students	83.71	3.52	*0.007*	*0.603*	B	C
Swimmers	95.84	5.73			B	B
Older adults	137.58	10.82			A	A
Salivary IgG (mg/L)						
Students	11.97	1.74	*0.010*	*0.971*	C	C
Swimmers	19.62	2.10			B	B
Older adults	25.17	3.01			A	A
Salivary IgM (mg/L)						
Students	9.53	0.32	*0.627*	*0.542*	-	-
Swimmers	11.70	0.89			-	-
Older adults	8.29	1.59			-	-
Salivary albumin (mg/L)						
Students	41.99	4.35	*0.002*	*0.535*	C	C
Swimmers	64.03	7.85			B	B
Older adults	110.51	13.72			A	A
Salivary osmolality (mOsmol/kg)						
Student	68.64	2.51	*0.023*	*0.010*	B	B
Swimmer	99.29	11.74			A	A
Older adults	96.38	3.21			A	A
Plasma IgA (g/L)						
Students	2.036	0.17	*0.002*	*0.741*	B	B
Swimmers	1.616	0.10			C	C
Older adults	2.464	0.10			A	A
Plasma IgG (g/L)						
Students	8.217	0.38	*0.812*	*0.892*	-	-
Swimmers	8.851	0.34			-	-
Older adults	8.821	0.28			-	-
Plasma IgM (g/L)						
Students	1.064	0.06	*0.368*	*0.290*	-	-
Swimmers	0.960	0.06			-	-
Older adults	1.037	0.07			-	-
Plasma albumin (g/L)						
Students	42.40	0.40	*0.001*	*0.028*	B	B
Swimmers	43.61	0.28			A	A
Older adults	40.30	0.31			C	C

Multiple comparison procedure:
This procedure involves testing all possible pairs of cohort results. The cohort means were ranked from highest to lowest (A to C, A was the highest rank). Cohorts that had the same letter were not significantly different to each other. If the Tukey (being more conservative) multiple ranking procedure gave a different ranking to the Duncan procedure, the result for the Tukey procedure was used due to the very small sample size.

Tukey *(Tukey's honest significant difference, HSD*): Tests the null hypothesis when all possible pairs of treatment means are equal when the samples are of the same size. More conservative than the 'Duncan' test.
Duncan (*Duncan's New Multiple Range test):* A variant of the Student Newman Keuls method that uses the studentised-range statistic to compare sets of means. Is protective against Type II error at the expense of Type I.
(Experimental Design and Analysis of Experiments for Researchers, Massey University, 2004)

Appendix 10.30
Subgroup results: Baseline comparison between cohorts for absolute numbers of leucocytes and lymphocyte subsets

Appendix 10.30: Subgroup results: Baseline comparison between cohorts for absolute numbers of leucocytes and lymphocyte subsets. Mean and SEM. *P*=significance levels between the cohorts. Students (n=10), Swimmers (n=9), Older adults (n=10)

Immune Parameter	Mean	SEM	*P (cohort*	*P (gender)*	Tukey	Duncan
Leucocytes x 10^9/L						
Students	6.85	0.33	*0.003*	*0.075*	B	B
Swimmers*	8.48	0.47			A	A
Older adults	5.31	0.28			C	C
Neutrophils x 10^9/L						
Students	3.92	0.410	*0.001*	*0.024*	B	B
Swimmers*	5.74	0.650			A	A
Older adults	2.95	0.250			C	C
Monocytes x 10^9/L						
Students	0.405	0.024	*0.603*	*0.633*	-	-
Swimmers	0.436	0.033			-	-
Older adults	0.461	0.028			-	-
Lymphocytes x 10^9/L						
Students	2.19	0.15	*0.249*	*0.673*	-	-
Swimmers	2.07	0.10			-	-
Older adults	1.69	0.18			-	-
CD3 x 10^9/L						
Students	1.55	0.49	*0.070*	*0.850*	-	-
Swimmers	1.70	0.57			-	-
Older adults	1.31	0.42			-	-
CD3/CD4 x 10^9/L					-	-
Students	0.920	0.06	*0.674*	*0.115*	-	-
Swimmers	0.894	0.06			-	-
Older adults	0.840	0.09			-	-
CD3/CD8 x 10^9/L						
Students	0.517	0.05	*0.024*	*0.816*	A	A
Swimmers	0.481	0.06			A	A
Older adults	0.303	0.06			B	B
CD19 x 10^9/L						
Students	0.202	0.04	*0.057*	*0.980*	A	AB
Swimmers	0.257	0.02			A	A
Older adults	0.174	0.02			A	B
CD4/CD8 ratio						
Students	1.90	0.16	*0.004*	*0.526*	B	B
Swimmers	2.10	0.33			B	B
Older adults	3.61	0.54			A	A

*Results known to be acutely affected by exercise. Swimmers blood sampled within 30 minutes of their 2-hour morning training session.
Multiple comparison procedure:
This procedure involves testing all possible pairs of cohort results. The cohort means were ranked from highest to lowest (A to C, A was the highest rank). Cohorts that had the same letter were not significantly different to each other. If the Tukey (being more conservative) multiple ranking procedure gave a different ranking to the Duncan procedure, the result for the Tukey procedure was used due to the very small sample size.

Tukey *(Tukey's honest significant difference, HSD*):
Duncan (*Duncan's New Multiple Range test):*
(Experimental Design and Analysis of Experiments for Researchers, Massey University, 2004)

Appendix 10.31
Subgroup results: Baseline comparison between cohorts for percentages of lymphocyte subsets, plasma cytokines, plasma cortisol

Appendix 10.31: Subgroup results: Baseline comparison between cohorts for percentages of lymphocyte subsets, plasma cytokines, plasma cortisol. Mean and SEM. *P*=significance levels between cohorts: Students (n=10), Swimmers (n=9), Older adults (n=10).

Immune Parameter	Mean	SEM	*P (cohort)*	*P (gender)*	Tukey	Duncan
%CD3						
Students	71.40	1.04	*0.152*	*0.781*	-	-
Swimmers	76.73	1.75			-	-
Older adults	70.41	1.85			-	-
%CD3/CD4						
Students	42.95	1.37	*0.011*	*0.451*	B	B
Swimmers	42.87	1.58			B	B
Older adults	51.83	2.99			A	A
% CD3/CD4/CD49d						
Students	86.37	1.28	*0.001*	*0.450*	A	B
Swimmers	89.54	1.75			A	A
Older adults	70.56	3.51			B	C
%CD3/CD8						
Students	23.70	1.53	*0.026*	*0.749*	A	A
Swimmers	23.20	2.41			A	A
Older adults	16.40	2.03			B	B
%CD3/CD8/CD49d						
Students	97.79	1.01	*0.009*	*0.62*	A	A
Swimmers	99.14	0.76			A	A
Older adults	95.13	1.18			B	B
%CD19						
Students	13.60	0.77	*0.075*	*0.580*	-	-
Swimmers	12.53	0.89			-	-
Older adults	9.99	0.22			-	-
%CD19/CD49d						
Students	100	0	*0.001*	*0.425*	A	A
Swimmers	100	0			A	A
Older adults	97.2	0.68			B	B
Plasma cortisol (nmol/L)						
Students	708.79	77.7	*0.0001*	*0.922*	B	B
Swimmers	811.14*	60.9			A	A
Older adults	314.72	43.8			C	C
Plasma IL-6 (pg/mL)						
Students	21.30	6.15	*0.574*	*0.255*	-	-
Swimmers	14.00*	6.88			-	-
Older adults	9.02	2.34			-	-
Plasma IL-1ra (pg/mL)						
Students	118.01	69.4	*0.904*	*0.473*	-	-
Swimmers	129.16*	44.5			-	-
Older adults	59.31	48.3			-	-
Plasma IFN-α (pg/mL)						
Students	97.22	25.0	*0.959*	*0.787*	-	-
Swimmers	102.73	33.2			-	-
Older adults	106.05	43.4			-	-

*Results known to be acutely affected by exercise. Swimmers blood sampled within 30 minutes of their 2-hour morning training session.

Multiple comparison procedure:

This procedure involves testing all possible pairs of cohort results. The cohort means were ranked from highest to lowest (A to C, A was the highest rank). Cohorts that had the same letter were not significantly different to each other. If the Tukey (being more conservative) multiple ranking procedure gave a different ranking to the Duncan procedure, the result for the Tukey procedure was used due to the very small sample size.

Tukey *(Tukey's honest significant difference, HSD*); Duncan (*Duncan's New Multiple Range test) (Experimental Design and Analysis of Experiments for Researchers, Massey University, 2004)*

Appendix10.32
Reported daily energy intake (RDEI) and estimated daily energy expenditure (EDEE)

Appendix 10.32: RDEI and EDEE**.** Harris-Benedict (HB) and Schofields (Sc) equations were used to estimate basal metabolic rate (BMR). *P(ave)*=significance level of differences in mean intake between BC and Pl groups within each cohort. *P(diff)* =significance levels for difference in intake at 10wks compared to 1wk of the supplementation between BC and Pl.

	Students				Swimmers				Older adults			
	BC (n=16)		Pl (n=12)		BC (n=12)		Pl (n=13)		BC (n=22)		Pl (n=23)	
RDEI(kJ)	Med.	IQ range	Med	IQ range	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	7,575	6,939-8,460	7,686	7,120-11,329	10,171	1.085	12,153	870	7,148	500	8,174	463
M	10,700	9,027-13,747	12,651	8,008-14,780	13,020	1,543	15,348	1,738	9,160	1,041	10,274	824
P (ave)	*0.705*				*0.055*				*0.181*			
P (diff)	*0.290*				*0.263*				*0.806*			
EDEE (HB)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	9,917	429	10,171	337	12,783	552	13,578	398	9,463	329	9,056	481
M	11,780	520	11,852	552	17,773	1,129	16,952	718	12,938	830	12,698	496
P (ave)	*0.781*				*0.243*				*0.890*			
P (diff)	*0962*				*0.308*				*0.645*			
EDEE (Sc)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	9,896	487	10,091	442	12,529	612	13,550	703	9,509	330	9,080	452
M	11,752	482	12,119	510	16,113	775	15,769	699	11,607	515	11,735	388
P (ave)	*0.743*				*0.225*				*0.645*			
P (diff)	*0.941*								*0.965*			
% difference (HB)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	-29.0	8.0	-19.87	9.8	-34.1	12.6	-13.99	8.37	-37.6	9.16	-11.9	8.7
M	-8.0	8.7	-7.13	14.6	-43.9	23.5	-23.50	16.2	-63.3	15.1	-29.9	10.6
P	*0.562*				*0.431*				*0.015*			
%difference (Sc)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	-28.6	8.3	-19.87	9.8	-31.5	12.7	-13.7	9.47	-39.3	9.4	-14.7	7.89
M	-7.4	7.8	-7.13	14.6	-31.0	22.1	-13.9	13.6	-29.9	13.9	-19.9	9.01
P	*0.603*				*0.225*				*0.027*			

Appendix 10.33
Reported daily macronutrient and fibre intake adjusted for energy differences

Appendix 10.33: Reported daily macronutrient and fibre intake adjusted for energy differences. Mean and SEM of dietary intake at 1wk and 10wks of the intervention. *P(ave)*=significance level of differences of average intake between BC and Pl groups within each cohort. *P(diff*) =significance levels of the difference in intake at 10wks compared to 1wk of intervention between BC and Pl.

	Students				Swimmers				Older adults			
Macronutrient	BC (n=16)		Pl (n=12)		BC (n=12)		Pl (n=13)		BC (n=22)		Pl (n=23)	
CHO g/kg BM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	4.7	0.18	4.7	0.33	6.1	0.50	6.1	0.17	4.1	0.21	4.5	0.24
M	5.2	0.31	3.6	0.20	6.9	0.52	6.3	0.52	4.0	0.27	4.3	0.29
P (ave)	*0.165*				*0.974*				*0.147*			
P (diff)	*0.647*				*0.906*				*0.266*			
Ptn g/kg BM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	1.7	0.09	1.8	0.11	2.2	0.14	1.9	0.21	1.6	0.10	1.6	0.058
M	1.8	0.16	1.4	0.09	1.8	0.07	2.0	0.13	1.8	0.09	1.7	0.108
P (ave)	*0.443*				*0.405*				*0.767*			
P (diff)	*0.726*				*0.025*				*0.396*			
Fat % E	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	36.4	3.96	33.5	2.74	39.6	4.45	32.7	4.18	39.5	3.26	28.5	2.80
M	31.8	2.64	31.9	6.63	40.3	3.68	39.6	7.67	41.8	3.73	37.1	3.74
P (ave)	*0.625*				*0.655*				*0.023*			
P (diff)	*0.007*				*0.998*				*0.410*			
Fibre (g)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F	24.0	2.12	25.7	2.03	24.3	1.76	26.7	2.21	25.0	2.04	28.8	1.58
M	24.2	3.36	18.5	2.66	44.3	4.19	35.2	3.14	30.1	2.15	33.5	3.68
P (ave)	*0.806*				*0.766*				*0.148*			
P (diff)	*0.996*				*0.445*				*0.147*			

CHO g/kg BM = grams of carbohydrate per kilogram body mass
Ptn g/kg BM = grams of protein per kilogram body mass
Fat % E = dietary fat intake as a percentage of total energy intake

Appendix 10.34
Reported daily micronutrient intake adjusted for energy differences

Appendix 10.34: Reported daily micronutrient intake adjusted for energy differences. Mean and SEM of dietary intake at 1wk and 10wks of the intervention. *P(ave)* =significance level of differences in average intake between BC and PI groups within each cohort. *P(diff)*=significance levels for differences in intake at 10wks compared to 1wk of intervention between BC and PI

Micronutrient	Students				Swimmers				Older adults			
	BC (n=16)		PI (n=12)		BC (n=12)		PI (n=13)		BC (n=22)		PI (n=23)	
Calcium (mg)	Mean	SEM	Mean	SEM	*Med	IQ range	*Med	IQ range	**Mean	**SEM	**Mean	**SEM
F	1,379	93.2	1,604	209	1,265	1,088-1,401	1,704	1,401-2,139	1,304	112	1,625	162
M	1,150	159.0	737	49.1	1,787	1,420-2,299	1,237	1,207-2,214	1,159	125	1,596	139
P (ave)	*0.865*				**1.000*				*0.007*			
P (diff)	*0.030*				**0.819*				*0.756*			
Iron (mg)	Mean	SEM	Mean	SEM	*Med	*IQ range	*Med	*IQ range	*Med	*IQ range	*Med	*IQ range
F	18.4	2.54	21.2	2.05	16.8	13.3-16.0	15.5	13.7-68.5	13.6	12.35-15.78	13.6	12.56-16.11
M	17.4	0.95	13.9	1.90	19.6	15.2-29.0	22.3	18.2-27.6	17.1	13.44-24.46	16.9	14.87-24.71
P (ave)	*0.722*				*0.116*				*0.884*			
P (diff)	*0.103*				*0.890*				*0.758*			
Vitamin A (µg)	**Mean	SEM	**Mean	SEM	Mean	SEM	Mean	SEM	*Med	*IQ range	*Med	*IQ range
F	1021	153	1310	432	1056	107	1213	165	1083	770-1623	1023	508-1298
M	685	71	614	159	1576	262	1171	166	1279	1022-1852	1319	995-1716
P (ave)	*0.886*				*0.866*				**0.884*			
P (diff)	*0.492*				*0.068*				*0.431*			
Vitamin C (mg)	**Mean	**SEM	**Mean	**SEM	**Mean	**SEM	**Mean	**SEM	*Med.	*IQ range	*Med.	*IQ range
F	160.5	26.5	123.8	26.4	751.0	423	199.6	47.1	105.1	60.9-161.3	131.7	111.3-291.6
M	117.3	54.7	96.3	34.3	292.4	97.7	295.9	80.2	146.5	79.9-192.7	154.7	81.9-256.0
P (ave)	*0.377*				*0.251*				**0.295*			
P (diff)	*0.779*				*0.859*				*0.721*			

Appendix 10.34 continued: Reported daily micronutrient intake adjusted for energy differences continued. Mean and SEM of dietary intake at week 1 and week 10 of the intervention. *P(ave)*=significance level of differences in average intake between bovine colostrum (BC) and placebo (Pl) groups within each cohort. *P(diff)* =significance levels for differences in intake at week 10 compared to week 1 of intervention between BC and Pl

Micronutrient	Students				Swimmers				Older adults			
	BC (n=16)		Pl (n=12)		BC (n=12)		Pl (n=13)		BC (n=22)		Pl (n=23)	
Vitamin E (mg)	*Med	*IQ range	*Med	*IQ range	**Mean	**SEM	**Mean	**SEM	*Med.	*IQ range	*Med.	*IQ Range
F	10.7	8.97-13.41	13.0	9.51-27.31	31.6	22.4	6.24	3.34	9.2	7.17-10.34	9.03	7.08-17.6
M	8.1	5.68-14.37	9.9	8.10-10.75	29.4	20.4	6.48	5.81	9.8	8.22-15.53	12.4	10.0-25.6
P (ave)	**0.705*		*0.705*		*0.127*				**0.295*			
P (diff)	**0.863*		*0.863*		*0.819*				*0.758*			
Zinc (mg)	Mean	SEM	Mean	SEM	**Mean	**SEM	**Mean	**SEM	Mean	SEM	Mean	SEM
F	13.9	1.22	15.5	1.98	13.11	4.03	6.43	3.38	14.44	0.885	19.20	4.54
M	17.2	1.79	14.3	1.40	12.29	3.88	9.59	4.02	21.68	2.0	20.56	3.59
P (ave)	*0.946*				*0.181*				*0.529*			
P (diff)	*0.585*				*0.480*				*0.242*			
Selenium (µg)	Mean	SEM	Mean	SEM	**Mean	**SEM	**Mean	**SEM	**Mean	**SEM	**Mean	**SEM
F	61.79	6.74	74.10	10.30	73.80	30.90	63.60	13.00	44.27	9.25	43.00	11.70
M	64.20	12.10	69.52	5.70	67.30	24.00	79.50	19.40	55.30	11.00	74.00	18.90
P (ave)	*0.285*				*0.643*				*0.795*			
P (diff)	*0.472*				*0.395*				*0.255*			

Micronutrient data that was not normally distributed was treated as follows-

* Would not normalise with transformation. Moods median test used to test differences.

** Right-skewed and normalised with logging. Significance levels are on logged data, Mean and SEM are on back transformed data.

Appendix 10.34 continued: Reported daily micronutrient intake adjusted for energy differences. Mean and SEM of dietary intake at 1wk and 10ks of the intervention. *P(ave)*=significance level of differences in average intake between BC and Pl groups within the older adult cohort. *P(diff)*=significance levels for differences in intake at week 10 compared to week 1 of intervention between BC and Pl.

Micronutrient	Older adults			
	BC (n=22)		Pl (n=23)	
Vitamin B6 (mg)	*Median	*IQ range	*Median	*IQ range
F	2.03	1.55-2.40	2.46	1.91-4.26
M	2.40	1.86-3.01	2.30	1.62-4.28
P (ave)	**0.652*			
P (diff)	**0.031*			
Vitamin B12 (µg)	Mean	SEM	Mean	SEM
F	8.58	1.34	8.11	1.94
M	10.11	1.78	17.96	8.24
P (ave)	*0.435*			
P (diff)	*0.763*			

Micronutrient data that was not normally distributed was treated as follows:
* Would not normalise with transformation. Moods median test used to test differences.

Appendix 10.35
Contribution to daily intake of micronutrients from nutrition supplementation (Students)

Appendix 10.35: Contribution to daily intake of micronutrients from nutrition supplementation (Students). Identity code=ID, Food record=FR

						Minerals				Vitamins				
ID	M/F	FR	Supplement	Intake reported	Assumption made	Ca (mg)	Fe (mg)	Zn (mg)	Se (µg)	A (µg)	B6 (mg)	B12 (µg)	C (mg)	E (µg)
163	F	1	Ferrogradumet (105mg elemental iron)	1 tablet on 1 out of 4 days.	105mg/4 days= 26.25mg/day		26.3							
649	F	1	Iron tablet (Healtheries)	1 tablet daily			20						40	
649	F	2	Iron tablet (Healtheries)	1 tablet daily			20						40	
974	F	1	Multivitamin (Healtheries), prescription	1 tablet on 3 out of 4days.	Fe=5/3=1.7, Zn =1.5/3=0.5, VitA=2100/3=700, VitC-7.5/3=2.5, VitE=10/3=3.3		1.7	0.5		700			2.5	3.3
1301	F	1	LifePak Women supplement (Pharmanex)	1 tablet on 3 out of 4 days.			1.7	2.5		2500			83.3	50

Appendix 10.36
Contribution to daily intake of micronutrients from nutrition supplementation (Swimmers)

Appendix 10.36: Contribution to daily intake of micronutrients from nutrition supplementation (swimmers)

ID	M/F	FR	Supplement	Intake reported	Assumption made	Minerals				Vitamins				
						Ca (mg)	Fe (mg)	Zn (mg)	Se (µg)	A (µg)	B6 (mg)	B12 (µg)	C (mg)	E (µg)
272	M	1	Beta carotene antioxidant (Blackmores)	1 tablet on 2 out of 4 days.	A=2000/4=500, E=230/4=115					500				115
272	M	1	Zinc organic (Thomsons)	1 tablet 2 out of 4 days.	15/2=7.5mg/d			7.5						
335	M	1	Vitamin C, 1000mg (Red Seal)	Daily	Assume same as Red Seal 'Effergise Effervescent' 1000mg								1000	
335	M	1	Multivitamin (Healtheries)	1 tablet on 1 out of 4 days.	Ca=16/4=4, Fe =5/4 =1.3, C=250/4=62.5, E=35/4=8.8, Zn=6/4=1.2	4	1.3	1.2					62.5	8.8
633	M	1	Iron. Ferrous fumarate 25mg(Healtheries)	1 tablet on 2 out of 4 days	1 tablet has 7.5 mg Fe. 7.5/2=3.75mg/d		3.75							
633	M	1	Vitamin C 500mg (Healtheries)	1 tablet on 2 out of 4 days.									250	
633	M	1	Multivitamin (Healtheries)	Daily		10	12	2		500			150	50

Appendix 10.36 continued: Contribution to daily intake of micronutrients from nutrition supplementation (swimmers)

						Minerals				Vitamins				
ID	M/F	FR	Supplement	Intake reported	Assumption made	Ca (mg)	Fe (mg)	Zn (mg)	Se (µg)	A (µg)	B6 (mg)	B12 (µg)	C (mg)	E (µg)
651	F	1	Ferrogradumet (105mg elemental Fe)	Daily			105							
651	F	1	Vitamin B complex	1 tablet on3 out of 4 days	Nutriway B complex									
651	F	2	Vitamin B (Nutriway B complex)	1 tablet on 3 out of 4 days	Fe=20x0.75=15, Zn=0.4x0.75=0.3, Se=40x0.75=30, B6=100x0.75=75, B12=100x0.75=75		15	0.3	30		15	75	75	
651	F	2	Evening Primrose Oil	1 tablet 2 out of 3 days	B6=13x0.66=8.6, B12=12x0.66=7.9, C=50x.66=33, E=41x0.66=27						8.6	7.92	33	27.1
651	F	2	Ferrogradumet (105mg elemental Fe)	Daily	Fe=105		105							
708	F	1	Multivitamin (Healtheries)	1 tablet daily	C=300, E=100, A=1200, Ca=40	40				1200			300	100

Appendix 10.37
Contribution to daily intake of micronutrients from nutrition supplementation (older adults)

Appendix 10.37: Contribution to daily intake of micronutrients from nutrition supplementation (older adults). Diet recall=DR

ID	M/F	DR	Supplement	Intake reported	Assumption made	Minerals				Vitamins				
						Ca (mg)	Fe (mg)	Zn (mg)	Se (µg)	A (µg)	B6 (mg)	B12 (µg)	C (mg)	E (µg)
133	F	2	Calcium Carbonate	4 x 250mg		1000								
161	F	1	Multivitamin generic 'Albany Village Chemist'	1 x 1000mg	750µgvitA, 15mgvitC					750			15	
161	F	1	Ca Osteo-500	2 x 500mg	Equivalent to 4 x 250mg [a]	1000								
336	M	1	Garlic Pearlies 'Healtheries'	1 tablet	Contains 0.1g garlic [b]									
343			Vitamin B6 'Golden Glow' 200mg	1 x 200mg	[c]						200			
385	F	1	Ocuvite Preservision	4 tablets	4x(A=1989µg, E=67µg, C=113mg, Zn=17.4mg)			139		7956			904	536
385	F	1	Equazen Eye Q Evening Primrose Oil	1 capsule	[d]			2.5					50	325
385	F	2	Ocuvite Preservision	4 tablets	4x(A=1989µg, E=67µg, C=113mg, Zn=17.4mg)			139		7956			904	536

Appendix 10.37 continued: Contribution to daily intake of micronutrients from nutrition supplementation (older adults)

						Minerals				Vitamins				
ID	M/F	DR	Supplement	Intake reported	Assumption made	Ca (mg)	Fe (mg)	Zn (mg)	Se (µg)	A (µg)	B6 (mg)	B12 (µg)	C (mg)	E (µg)
442	F	2	Calcium Complete 'Nutralife'	4x250mg		1000								
473	F	1	Selenium+A+C+E antioxidant 'Kordells'	1-2 per week	0.2 tablet/day 0.2x(A=250, C=261, E=71, Se=100, Zn=7.5)			1.5	20	50			52.5	14.20
515	M	1	Reliv Now supplement	17g	17g (A=15,060µg, E=21.75mg, Fe=17mg, Zn=14.8mg).		17	14.8		15,060				21.75
515	M	1	Reliv Fibfrstore	14g	14g(fibre=10g, Zn=14.77mg, E=220.5µg)			14.8						220.5
515	M	2	Reliv. Now supplement	17g	17g (A=15,060µg, E=21.75mg, Fe=17mg, Zn=14.8mg).		17	14.8		15,060				21.75
515	M	2	Reliv Fibfrstore	14g	14g (fibre=10g, Zn=14.77mg, E=220.5µg)			14.8						220.5
727	M	1	Eagle Anoxant	1g	1g (A=667µg, C=165mg, E=55µg)					667			165	55
790	F	2	Vitamin B complex Berrocca	1 tablet	1 tablet (C=1000mg, B6=10mg, B12=10µg)						10	10	1000	

Appendix 10.37 continued: Contribution to daily intake of micronutrients from nutrition supplementation (older adults)

ID	M/F	DR	Supplement	Intake reported	Assumption made	Minerals				Vitamins				
						Ca (mg)	Fe (mg)	Zn (mg)	Se (µg)	A (µg)	B6 (mg)	B12 (µg)	C (mg)	E (µg)
884	M	1	USANA multi-mineral	2 tablets	1 tablet=1g. (Zn = 6.7mg, Se= 8.6mg)			13.4	17.2					
884	M	1	USANA antioxidant	2 tablets	1 tablet=1g (A=500µg, B6=9mg, B12=20µg, C= 438mg, E=12.4µg)					1000	18	40	868	24.8
1028	M	1	Calcium complete 'Nutralife'	2 x 250mg		500								
1028	M	1	Multivitamin generic 'Albany Chemist'	1 tablet	1 tablet =(A=750µg, C=15mg)					750			15	
1049	M	2	Oral B12 spray 'Vitamist'	8spray	Equivalent to 8g (160µgVitB12)						160			
1090	M	1	USANA antioxidant	1 tablet	1 tablet =1g (A=500µg, B6=9mg, B12=20µg, C= 433.7mg, E= 12.4µg)					500	9	20	433.7	12.4
1090	M	1	USANA multivitamins	1 tablet	1 tablet=(A=2,100µg, B6=1.6mg, B12=2µg, C=75mg, E=10µg, Fe= 5mg, Zn=1.5mg)		5	1.5		2,100	1.6	2	75	10
1305	M	1	Folic Acid Plus 'Cenovis'	1 tablet	1 tablet=0.56g (C=100mg, B6=21mg, B12=5µg, Fe =5mg)		5				21	5	100	

Appendix 10.37 continued: Contribution to daily intake of micronutrients from nutrition supplementation (older adults)

ID	M/F	DR	Supplement	Intake reported	Assumption made	Minerals				Vitamins				
						Ca (mg)	Fe (mg)	Zn (mg)	Se (µg)	A (µg)	B6 (mg)	B12 (µg)	C (mg)	E (µg)
1348	M	1	Metamucil 'Proctor & Gamble'	1Tbsp	Wt 1Tbsp=11g									
1351	F	1	Calcium complete 'Nutralife	4 x 250mg		1000								
1406	F	2	Iron melts	1 tablet	1 tablet =(Fe=5mg, B12=10µg, C=50mg)		5					10	50	

[a] = Same as Calcium Complete 250mg 'Nutralife'
[b] = Equivalent to Odourless Garlic 1000mg 'Kordells'
[c] = Equivalent to 2 x 100mg Vitamin B6 'Naturally'
[d] = Equivalent to Evening Primrose Oil Plus

Appendix 10.38
Guidelines for dietary intake for athletes in New Zealand

Appendix 10.38: Guidelines for dietary intake for athletes in New Zealand *(Howe et al., 2002)*

Nutrient	Specific requirement	Guideline
Carbohydrate	60 minutes moderate to high intensity	5-6 g/kg BM
	60 to 120 minutes moderate to high intensity	7-8 g/kg BM
	2 to 5 hours of intense training	8-10 g/kg BM
	More than 5 hours	10 g/kg BM
Protein	All athletes	1.2-1.4 g/kg BM
Fat	All athletes	80-100 g day
Iron	Adolescents (12–18 years)	10-13 mg/day
	Women (19–54 years)	12-16 mg/day
	Men (19+ years)	7 mg/day
Calcium	Adolescents	1000-1200 mg/day
	Adults	800-1000 mg/day

BM = body mass

Appendix 10.39:
Summary of Nutrient Reference Values (NRVs) for Australia and New Zealand (NHMRC, 2006)

Appendix 10.39: Summary of NRVs for Australia and New Zealand (*NHMRC, 2006)*

		14–18 years			19–30 years		
Nutrient	M/F	EAR	RDI	UL	EAR	RDI	UL
Protein (g/kg BM)	M	0.76	0.99	-	0.68	0.84	-
	F	0.62	0.77	-	0.60	0.75	-
*Total fat	M	-	30-33%	-	-	30-33%	-
	F	-	30-33%	-	-	30-33%	-
*Carbohydrate	M	-	50-55%	-	-	50-55 %	-
	F	-	50-55%	-	-	50-55%	-
Fibre (g)	M	28	-	NP	30	-	NP
	F	22	-	NP	30	-	NP
Calcium (mg)	M	1,050	1,300	2,500	840	1,000	2,500
	F	1,050	1,300	2,500	840	1,000	2,500
Iron (mg)	M	8	11	45	6	8	45
	F	8	15	45	8	18	45
Zinc (mg)	M	11	13	35	12	14	40
	F	6	7	35	6.5	8	40
Selenium (µg)	M	40	70	400	60	70	400
	F	50	60	400	50	60	400
Vitamin A (µg)	M	630	900	2,800	625	900	3,000
	F	485	700	2,800	500	700	3,000
Vitamin C (mg)	M	28	40	NP	30	45	NP
	F	28	40	NP	30	45	NP
Vitamin E (mg)	M	10	-	250	10	-	300
	F	8	-	250	7	-	300
Vitamin B6 (mg)	M	1.1	1.3	40	1.1	1.3	50
	F	1.0	1.2	40	1.1	1.3	50
Vitamin B12 (µg)	M	2.0	2.4	NP	2.0	2.4	NP
	F	2.0	2.4	NP	2.0	2.4	NP

* = percentage of total energy intake
EAR = estimated average requirement
RDI = recommended dietary requirement
UL = upper level of intake
NP = not possible to calculate
* Food and nutrition guidelines for healthy adults, 2002.

Appendix 10.40:
Summary of NRVs for Australia and New Zealand - Older adults (NHMRC, 2006)

Appendix 10.40: Summary of NRVs for Australia and New Zealand - Older Adults *(NHMRC, 2006)*

		51-70 years			> 70 years		
Nutrient	M/F	EAR	RDI	UL	EAR	RDI	UL
Protein	M	0.68	0.84	-	0.86	1.07-	NP-
(g/kg BM)	F	0.60	0.75	-	-0.75	0.94	NP
*Total fat	M	-	30-33%	-	-	30-33%	-
	F	-	30-33%	-	-	30-33%	-
*Carbohydrate	M	-	50-55%	-	-	50-55%	-
	F	-	50-55%	-	-	50-55%	-
Fibre (g)	M	30	-	NP	30	-	NP
	F	25	-	NP	25	-	NP
Calcium (mg)	M	840	1,000	2,500	1,100	1,300	2,500
	F	1,100	1,300	2,500	1,100	1,300	2,500
Iron (mg)	M	6	8	45	6	8	45
	F	5	8	45	5	8	45
Zinc (mg)	M	12	14	40	12	14	40
	F	6.5	8	40	6.5	8.	40
Selenium (µg)	M	60	70	400	50	60	400
	F	50	60	400	50	60	400
Vitamin A (µg)	M	625	900	-	625	900	-
	F	500	700	-	500	700	-
Vitamin C (mg)	M	30	45	-	30	45	-
	F	30	45	-	30	45	-
Vitamin E (mg)	M	10	-	300	7	-	300
	F	10	-	300	7	-	300
Vitamin B6 (mg)	M	1.4	1.7	50	1.4	1.7	50
	F	1.3	1.5	50	1.3	1.5	50
Vitamin B12 (µg)	M	2.0	2.4	NP	2.0	2.4	NP
	F	2.0	2.4	NP	2.0	2.4	NP

* = percentage of total energy intake
EAR = estimated average requirement
RDI = recommended dietary requirement
UL = upper level of intake
NP = not possible to calculate