UNIVERSITY OF SOUTHAMPTON

FACULTY OF DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

School of Medicine

Prebiotics and Human Immune Function

by

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ABSTRACT

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Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth, and/or activity, of one or a limited number of beneficial bacteria in the colon and thus improve host health". Prebiotics include the ß2-1 fructans, molecules which contain fructosyl-fructose linkages. A review of studies conducted in laboratory animals and in humans indicated that ß2-1 fructans may modulate some aspects of immune function, improve the host's ability to respond to certain intestinal infections, and to modify some inflammatory outcomes. Studies looking at these same outcomes in humans supplemented with probiotics were also reviewed, and overall, the picture that emerges from these studies is mixed and there appear to be large species and strain differences in effects seen. This thesis describes a study that investigated the effect of Orafti® Synergy1 on the functioning of the immune system in healthy human middle-aged adults, an age group that is underrepresented in the literature. A pilot study was conducted initially in order to develop a vaccination protocol, as response to vaccination has been identified as the most meaningful measure of the immune response. This protocol was then used in an intervention study to investigate the effect of ß2-1 fructans on human immune responses. Healthy adults aged 45 - 65 years (n = 22 in test group, n = 21 in placebo group) were supplemented with Orafti® Synergy1 (8 g/d for eight weeks) or maltodextrin, and vaccinated with the 2008/2009 seasonal influenza vaccination after four weeks. Fasting blood samples were taken at baseline (week zero) and weeks four, six and eight for analysis of various systemic immune parameters; anti-vaccine antibodies (the primary outcome), total antibody concentrations, immune cell phenotypes, neutrophil and monocyte phagocytosis and oxidative burst, ex vivo lymphocyte responsiveness (activation, proliferation and cytokine production) to the mitogen concanavalin A (ConA) and the vaccine. Faecal bifidobacteria were measured at weeks zero and four. Supplementation with Orafti® Synergy1 (8 g/d) for four weeks had a bifidogengic effect. However, no effect of the supplement was seen on immune function measured in the absence of vaccination. Furthermore, few differences were seen between groups in most immune outcomes measured post-vaccination. However, two important and novel observations were made. First, the prebiotic supplement enhanced the antibody response to the HAH3_UR strain of the vaccine ($p = 0.0019$), and enhanced the IgG1-specific antibody response to the vaccine ($p = 0.0019$). Therefore, it can be concluded that Orafti® Synergy1 is able to alter some aspects of immune function in healthy middle-aged adults.

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DECLARATION OF AUTHORSHIP

I, **Amy Lomax**,

declare that the thesis entitled

Prebiotics and Human Immune Function

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this university;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, the thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as:

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Signed: [redacted].....

Date: 21.04.2011.....

Acknowledgements

Recently, someone mentioned that my journey through my PhD was exactly how I described my experience of running the London Marathon. I had never considered this before, but when I thought about it, I realised it is true. The start was of the race was slow, and it seemed that however hard I tried and wanted to, I could not move past so many obstacles and get going. Once I did, things moved quickly, and it seemed that there was so much to take in, there was not enough time to appreciate it all. And there were times, especially toward the end, when it would have been easier to slow down, or to stop. But all the way, especially during the hard times, there were people there to support me; family, friends, colleagues, and even strangers. The last few miles of a marathon should be the toughest, when you are tiring, but the thought of the finish line and the encouragement of the crowd spur you on. Crossing the finish line was one of the best feelings in the world, one never to forget, and worth every minute of discomfort, which was soon to be forgotten afterwards and more races entered.

The similarity between running a marathon and doing my PhD that has struck me most is that of the huge support that I have received along both. I have found both experiences difficult at times, and especially for my PhD I have often doubted whether I was capable of completing it. Looking back, I can understand why I was faced with each challenge, and the lessons that each one has taught me. Every time I was faced with a difficult situation there was always someone there to help me, a colleague, a friend, family, even strangers, and I realise that even on a journey that is largely undertaken alone, you never truly are. I am incredibly grateful to the many people who have helped me along the way. Without each one of you, I know that I could not have stuck with it and made it to the end.

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This is for all of you.

"The steeper the mountain
The harder the climb
The better the view from the finishing line."

Definitions and abbreviations

ANOVA: analysis of variance
AOS: acidic oligosaccharides
APC: antigen presenting cell
BMI: body mass index
CBA: cytometric bead array
CD: cluster of differentiation
CDR: complimentarity determining region
CFSE: carboxyfluorescein diacetate succinimidyl ester
ConA: concanavalin A
CRP: C-reactive protein
CSF: colony-stimulating factor
CTLA: cytotoxic T lymphocyte antigen
DAI: disease activity index
DAPI: 4',6-diamidino-2-phenylindole
DH:- docoshexaenoic acid
DHR-123: dihdrorhodamine 123
DMSO: dimethyl sulphoxide
DP: degree of polymerization
DTH: delayed type hypersensitivity
ELISA: enzyme linked immunosorbent assay
EPA: eicosapentanoic acid
FAB: fragment antigen binding part
Fc: fragment crystalline part
FCS: foetal calf serum
FISH: fluorescence in situ hybridization
FITC: fluorescein isothiocyanate
fMLP: N-formyl-Met-Leu-Phe
FOS: fructooligosaccharides
FSC: forward scatter
GALT: gut associated lymphoid tissue
GI: gastrointestinal
GOS: galactooligosaccharides
GSH: glutathione peroxidise
HI: haemagglutination inhibition
HLA: human leukocyte antigen
HP-inulin: high performance inulin
H1N1: hemagglutinin type 1, neuraminidase type 1

H3N2: hemagglutinin type 3, neuraminidase type 2
IBS: irritable bowel syndrome
ICAM: intracellular adhesion molecule
ICU: intensive care unit
IEL: intraepithelial lymphocytes
IFN: interferon
Ig: immunoglobulin
IL: interleukin
IN: inulin
iNOS: inducible nitric oxide synthase
lc-FOS: long chain FOS
lc-PUFA: long chain polyunsaturated fatty acid
LP: lamina propria
LFA: leukocyte functional antigen
LPS: lipopolysaccharide
LTB4: leukotriene B4
MCP1: monocyte chemoattractant protein 1
MFI: mean fluorescence intensity
MHC: major histocompatibility complx
MLN: mesenteric lymph node
MOS: mannanoligosaccharides
MPO: myeloperoxidase
mRNA: messenger RNA
NK cell: natural killer cell
OF: oligofructose
PAMP: pathogen-associated molecular pattern
PBMC: peripheral blood mononuclear cell
PBS: phosphate buffered saline
PE: phycoerythrin
PGE2: prostaglandin E2
PE-Cy5: phycoerythrin-cyanine 5
PHA: phytohaemaglutinin
PI: propidium iodide
PMA: phorbyl myristic acetate
PP: Peyer's Patches
PRR: pattern recognition receptor
SCFA: short chain fatty acid
SDS: sodium dodecyl sulphate
sIgA: secretory IgA

sc-FOS: short chain FOS

sc-GOS: short chain GOS

SPS: soy polysaccharides

SSC: side scatter

Tc cell: cytotoxic T cell

TCR: T cell receptor

TGF: transforming growth factor

Th cell: helper T cell

TLR: toll-like receptor

TNBS: trinitrobenzene sulphonic acid

TNF: tumour necrosis factor

URTI: upper respiratory tract infection

VCAM: vascular adhesion molecule

VLA: very late antigen

WHO: World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 GENERAL STATEMENT

The objective of this research project is to identify the effect of ß2-1 fructan-type prebiotics on human immune function with the primary outcome being response to influenza vaccination. The project has the following aims:

1. To identify, assemble, and critically review the existing literature on ß2-1 fructans with regard to immune function, infections, inflammation and inflammatory conditions;

2. To identify, assemble, and critically review the existing literature on probiotics with regard to immune function, infections, inflammation and inflammatory conditions in humans;

3. To conduct a pilot study in order to establish an influenza vaccination protocol as a useable experimental tool;

4. To conduct an intervention study with ß2-1 fructans in human volunteers to evaluate the effect on the immune response with the primary outcome being response to influenza vaccination and secondary outcomes being faecal bifidobacteria counts and a range of immune cell responses tested ex vivo.

1.2 THE IMMUNE SYSTEM

1.2.1 INTRODUCTION

The function of the immune system is to protect the host from infectious agents which exist in the environment (such as bacteria, viruses, fungi, parasites) and from other noxious insults. The immune system has two functional divisions: the innate (or natural) immune system and the acquired (also termed specific or adaptive) immune system (Table 1).

	Natural (innate) immune system	**Acquired (specific) immune system**
Physicochemical Barriers	Skin Acid pH of stomach Commensal gut bacteria Mucous membranes	Cutaneous and mucosal immune systems Antibodies in mucosal secretions
Circulating Molecules	Complement	Antibodies
Cells	Phagocytes Natural killer cells	Lymphocytes
Soluble mediators	Macrophage-derived cytokines	Lymphocyte-derived cytokines

Table 1 : Components of the innate and acquired immune systems (from Calder 2007)(1)

When present in the bloodstream, cells of the immune system are termed white blood cells, or leukocytes, and there are two main categories of these: phagocytes (neutrophils, other granulocytes, and monocytes) and lymphocytes (T lymphocytes, B lymphocytes and natural killer (NK) cells). The relative contribution of the different cells of the immune system to total blood leukocytes in humans is shown in Table 2.

Leukocyte type	**Contribution (%)**
Neutrophils (% leukocytes)	50 - 70
Eosinophils (% leukocytes)	0 - 3
Basophils (% leukocytes)	0 - 1
Monocytes (% leukocytes)	1 - 10
Lymphocytes (% leukocytes) - T cells (% lymphocytes) - B cells (% lymphocytes)	20 - 40 70 10 - 20

Table 2 : Distribution of blood leukocytes in adult humans (from Abbas 1994)(2)

1.2.2 THE INNATE IMMUNE SYSTEM

1.2.2.1. INTRODUCTION

Innate immunity is the body's first line of defence against an infection. Innate immunity is present before the body is exposed to a pathogen, and has no memory. Thus, the activity of the innate immune system is not influenced by prior exposure to a pathogen. Innate immunity consists of physical barriers, soluble factors and phagocytic cells (Table 1). The physical barriers of the body are shown in Figure 1.

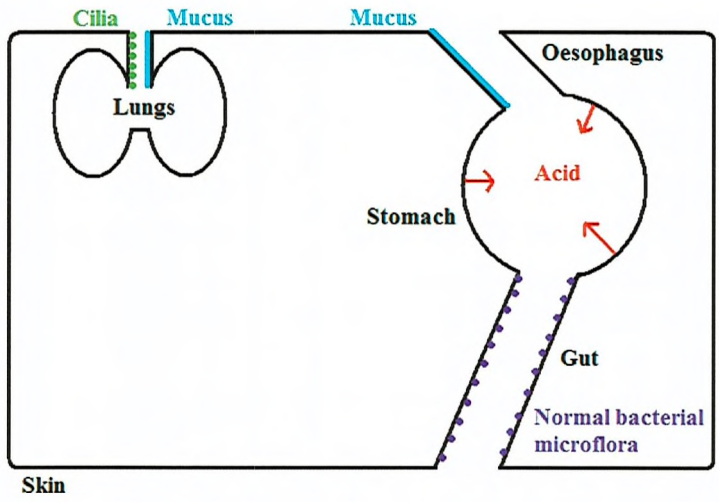


Figure 1 : The physical barriers of the immune system (adapted from Roitt 2006)(3)

1.2.2.2 PHAGOCYTIC CELLS

Phagocytic cells are classified as granulocytes (neutrophils, eosinophils, basophils and mast cells), and mononuclear phagocytes (monocytes and their differentiated derivatives macrophages).

1.2.2.2.1 GRANULOCYTES

Neutrophils are the most abundant of the white blood cells; they are short lived and quickly migrate to the tissues. They are polymorphonuclear cells, and contain granules of two types: primary azurophil granules (contain myeloperoxidase and non-oxidative antimicrobial effectors) and secondary specific granules (contain lactoferrin, lysozyme, alkaline phosphatase and membrane bound cytochrome b_{558}). Neutrophils have receptors for antibodies and complement, which help with uptake of opsonized particles.

Eosinophils are also polymorphonuclear cells. They contain granules that contain several enzymes that are damaging to pathogens, express surface receptors for C3b (a component of the complement system, see section 1.2.2.5), produce a respiratory burst

upon activation, and secrete granule proteins capable of producing a transmembrane plug in the membrane of the target cell.

Mast cells and basophils are functionally similar to one another. Mast cells contain abundant granules containing inflammatory mediators (histamine), prostaglandins and leukotrienes. These are released when either C3a or C5a binds (components of the complement system, see section 1.2.2.5), or when immunoglobulin (Ig) E and antigen bind to IgE receptors (FcεRI) on the mast cell surface. There are two types of mast cell: connective tissue mast cells and mucosal mast cells. Basophils also contain granules that contain inflammatory mediators.

1.2.2.2.2 MONONUCLEAR PHAGOCYTES

Monocytes begin life as bone marrow monoblasts, which develop into promonocytes and then monocytes, at which point they move into the peripheral circulation, where they can circulate for between 36 and 104 hours (4). Monocytes are incompletely differentiated, and can develop into macrophages. These are large, long-lived (they can live for months or years) phagocytic cells. They are found in most tissues, lining serous cavities, lungs, liver, blood vessels of the spleen and lymph nodes. They have different names according to their location in the body; for example in the spleen they are called sinusoidal lining cells, in the liver Kupffer cells, and in the bone osteoclasts.

Macrophages function in both the innate and adaptive immune responses. In the adaptive immune system they act as both accessory and effector cells; in their accessory cell role they act as antigen presenting cells, processing and presenting antigenic peptides to T cells, and in their effector cell role they act as phagocytes.

Macrophages contain lysosomes, which contain many substances, such as enzymes, neutral proteases and acid hydrolases, which enable the macrophage to carry out its functions. Macrophages also produce cytokines (interleukin (IL)-1, IL-6, tumour necrosis factor (TNF)-α and interferon (IFN)-γ), prostaglandins, leukotrienes and thromboxanes, which allow communication between the macrophage and other cells within or outside the immune system.

1.2.2.3 PHAGOCYTOSIS

In a process known as phagocytosis, phagocytes can engulf pathogens, cell debris and antigens and break them down. Phagocytic cells, the main effectors of innate immunity, express surface receptors specific for bacterial surface antigens. These receptors are known as pattern recognition receptors (PRRs) and they recognise and bind to pathogen-

associated molecular patterns (PAMPs) on the pathogen. PAMPs are shared by many infectious agents, are unlikely to mutate and are distinguishable from self patterns. PRRs may be Toll-like receptors (TLRs), C-type (calcium-dependent) lectins, or scavenger receptors. PRRs do not bind to galactose or sialic groups which are commonly found at the end of the sugars on mammalian surface polysaccharides, and thus phagocytes are able to distinguish effectively self from non-self. The process of phagocytosis is shown in Figure 2.

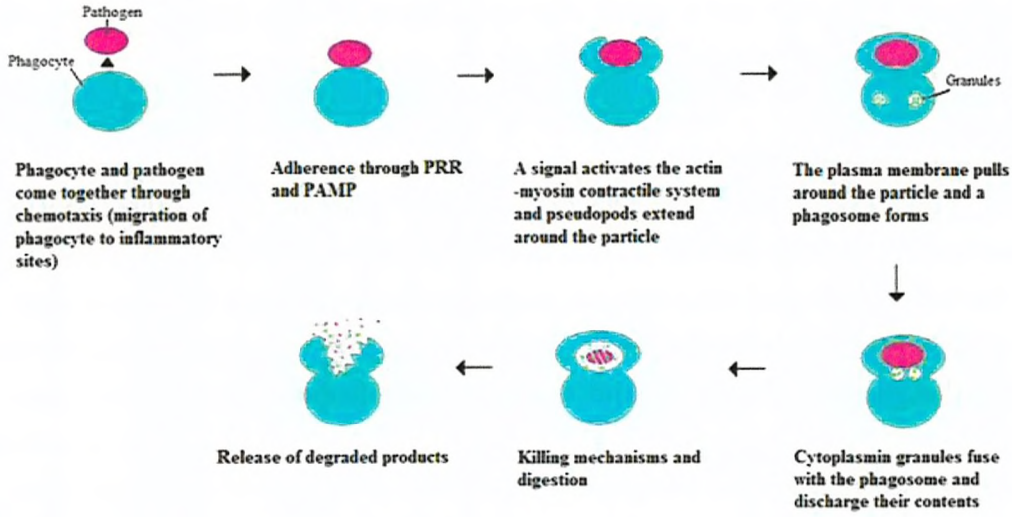


Figure 2 : The process of phagocytosis (adapted from Roitt 2006)(3)

Binding of antigen to the receptors triggers phagocytosis and subsequent destruction of the pathogenic microorganism by the following mechanisms:

- Production of reactive oxygen species (H_2O_2, $\cdot O_2^-$, ·OH and HOCl) as a result of the process termed respiratory or oxidative burst, although this can also occur without phagocytosis, for example upon stimulation by cytokines. Reactive oxygen species can be released into either vacuolar or extracellular environments.
- Production of reactive nitrogen intermediates (NO· and ·ONOO)
- Release of preformed antimicrobials (e.g. lactoferrin, lysozyme, proteolytic enzymes, hydrolytic enzymes, cathepsin G)
- Production of complement factors (see section 1.2.2.5)

1.2.2.4 NATURAL KILLER CELLS

NK cells are non-B, non-T lymphocytes that are classified as large granular lymphocytes. They are unusual lymphocytes, as they do not require antigen to be presented in association with major histocompatability complex. They are responsible for killing virally infected or tumour cells and removal of immunoglobulin-bound antigens. This is achieved by the activation of apoptosis of the target cell.

1.2.2.5 COMPLEMENT SYSTEM

Complement is a group of serum proteins that are involved in processes such as opsonization of pathogens, damage to the membranes of pathogens, removal of immune complexes and control of inflammation. The complement system is a cascade system, so that the product of one reaction catalyses the next, and so on; therefore a small stimulus is quickly amplified into a much larger response. There are different pathways by which the system can be activated: the Classical pathway, the Alternative pathway and the Lectin pathway. The most important protein in the complement system, both in quantity and in function, is C3.

C3 is spontaneously activated under normal circumstances, but in the presence of microbial polysaccharides (on the surface of microbes), the enzyme C3 is stabilised, and this catalyses the activation of C3; thus the reaction is speeded up, and the cascade is amplified. The functions of the complement system can be summarised as follows:

- C3b can coat microbes. Phagocytes have receptors for C3b (called CR1 and CR3), and so this results in the attraction of phagocytes to the microbe for destruction by phagocytosis.
- C3a and C5a stimulate the respiratory burst of phagocytes, increase expression of CR1 and CR3, and trigger release of mediators from mast cells and basophils. C3a attracts eosinophils and C5a attracts neutrophils.
- A membrane attack complex is formed from other proteins in the complement system, which can insert into the membrane of the target cell and cause cell lysis.

1.2.2.6 CYTOKINES

1.2.2.6.1 COMMUNICATION WITHIN THE IMMUNE SYSTEM

Direct cell-to-cell contact (e.g. via adhesion molecule pairs) between immune cells is one way in which cells can communicate both within the acquired immune system and between the innate and acquired systems. However, it would not be practical for this to be the only way that different cells could communicate with each other, and thus there is another mechanism by which this can occur; the cytokine system. Cytokines are proteins produced mainly by leukocytes and which act as chemical messengers between cells of the immune system (and also cells from outside of the immune system), inducing changes in growth, development, or activity of the target cell. Each cytokine can have multiple activities on different cell types (i.e. they are pleiotropic), and many cytokines have the same, or similar, effects, although most cytokines have some functions which are unique. Cytokines are able to influence the cells that produced them (i.e. they act in an autocrine manner) or other cells of the immune system, thereby acting in a paracrine manner (if the affected cell is nearby) or sometimes even in an endocrine manner (if the affected cell is far away). There are different families of cytokines, such as the interleukin (IL) family

(involved in communication between leukocytes), the colony stimulating factors (CSF; involved in proliferation of haematopoietic precursors), the tumour necrosis factors (TNF; cytotoxic to transformed cells) and the interferons (IFN; interfere with viral replication). Cytokines act by binding to specific receptors on the cell surface. Upon binding of a cytokine to its receptor, intracellular signalling pathways are activated (involving JAK and STAT proteins) which result in the transcription of genes associated with the response of the cytokine. Different JAKs and STATs are involved in the pathways induced by different cytokines and receptors. Cytokines are not stored inside a cell, they are synthesized in response to a stimulus. Given the actions of cytokines, it is important that the production is tightly regulated, and the cytokines derived from lymphocytes do not often remain in the circulation for prolonged periods of time.

1.2.2.6.2 CYTOKINES AND THE INNATE AND ADAPTIVE IMMUNE SYSTEMS

Cytokines mediate innate and acquired immunity, and are important in the communication between these two systems. Of particular note are TNF-α, IL-1 and IL-6. These cytokines are among the most important to be produced by monocytes and macrophages, and their actions include activation of neutrophils, monocytes and macrophages in order to initiate bacterial and tumour cell killing, increased adhesion molecule expression on the surface of neutrophils and endothelial cells, stimulation of T and B lymphocyte proliferation and initiation of the production of other pro-inflammatory cytokines (TNF induces production of IL-1 and IL-6, and IL-1 induces production of IL-6).

1.2.3 THE ACQUIRED IMMUNE SYSTEM

1.2.3.1 INTRODUCTION

Acquired immunity is characterized by specificity and memory. It involves the specific recognition of molecules (antigens) on an invading pathogen which distinguish it as being foreign to the host. Lymphocytes are the main cells involved in the acquired immune response, and each lymphocyte has surface receptors for a single antigen, resulting in high specificity. Yet acquired immunity also accomplishes extreme diversity; the lymphocyte repertoire in humans has been estimated to be able to recognise approximately 10^{11} antigens. Thus, this results in only a relatively small number of lymphocytes being able to recognise any given antigen, which potentially would be a problem when the body encountered a pathogen which it needed to respond to. However, the acquired immune system has developed the ability for clonal expansion; when a lymphocyte interacts with its specific antigen the lymphocyte proliferates, so that a single lymphocyte gives rise to a clone of lymphocytes. Each of these lymphocytes has the ability to recognise and destroy the source of the antigen that caused the initial response.

Because this process takes some time, the acquired immune response becomes effective over several days after the initial activation, but it also persists for some time after the removal of the initiating antigen. This gives rise to immunological memory, the second characteristic feature of acquired immunity. If the host has encountered an antigen and mounted an immune response to it, it would be beneficial if the host immune system could remember this response, so the next time the same pathogen is encountered, a stronger, more effective, and faster immune response is generated. This is the basis for vaccination, where the host is exposed to a non-toxic form of the infectious agent (such as killed, live attenuated, or subunits of the organism) to elicit a primary response to this agent. If the host is subsequently exposed to a natural form of the infection, they will generate a secondary immune response and thus will be protected from developing this disease. Eventually, the immune system will re-establish homeostasis using self-regulatory mechanisms which involves communication between cells.

Lymphocytes, which are subdivided into T and B lymphocytes, effect this form of immunity. B lymphocytes undergo development and maturation in the bone marrow before being released into the circulation, whilst T lymphocytes, although derived from the bone marrow, mature in the thymus. From the bloodstream, lymphocytes can enter peripheral lymphoid organs, which include lymph nodes, the spleen, mucosal lymphoid tissue, tonsils and gut-associated lymphoid tissue. Immune responses occur largely in these lymphoid organs, which are highly organized to promote the interaction of cells and invading pathogens.

1.2.3.2 B CELLS AND ANTIBODY PRODUCTION

B lymphocytes are characterized by their ability to produce antibodies, or antigen-specific immunoglobulins (Ig), which confer antigen specificity to the acquired immune system (i.e. the antibodies produced by B lymphocytes are specific for individual antigens). This form of protection against infections is termed humoral immunity and is conducted exclusively by B lymphocytes. B lymphocytes carry immunoglobulins, which are capable of binding an antigen, on their cell surfaces. Binding of immunoglobulin with antigen causes proliferation of the B lymphocyte and subsequent transformation into plasma cells *(Figure 3),* which secrete large amounts of antibody (or immunoglobulin) with the same structure as the membrane bound form, except that the secreted form lacks the transmembrane and intra-cytoplasmic segments.

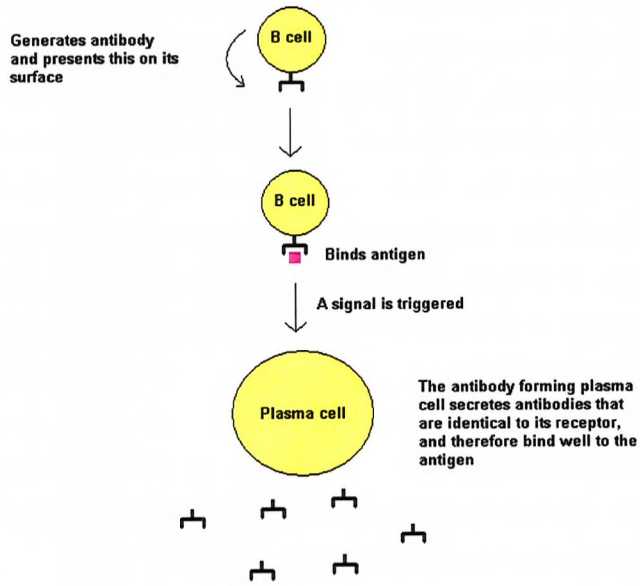


Figure 3 : Production of antibodies by B lymphocytes

Immunoglobulins recognise and bind to intact antigens or large fragments of antigens. Immunoglobulins are proteins with a basic structure of four polypeptide chains, consisting of two identical heavy chains and two identical light chains. There are five different types of heavy chain (γ, μ, α, δ, and ε) which give rise to five major classes of immunoglobulin (IgG, IgM, IgA, IgD and IgE respectively). There are two types of light chains (λ or κ). The general structure of an antibody is shown in Figure 4.

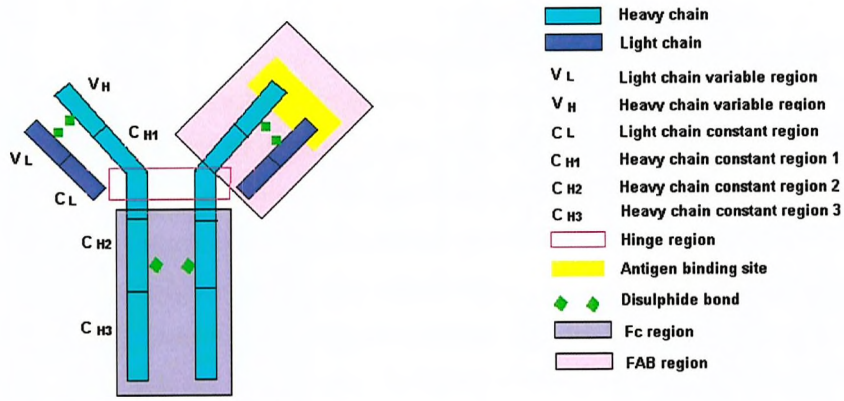


Figure 4 : General structure of antibodies

The antibody molecule can be divided into two parts; the fragment antigen binding part (FAB) or the fragment crystalline part (Fc); the FAB section is where the antigen binds, while the Fc section is where the immunoglobulin interacts with other cells of the immune system. Heavy (H) chains span the FAB and Fc fragments and light (L) chains span the FAB fragments only. As shown in Figure 4, the variable (V) regions of the light and heavy

chains make up the antigen binding site, and as the name suggests, this part of the molecule differs from one antibody to another, thus allowing recognition of different antigens. The regions where the amino acids making up the antibody differ are called complementarity determining regions (CDRs). The constant (C) regions of the heavy and light chains are largely conserved between antibodies, and are involved in the biological functions of the antibody molecule.

Antibodies work in several ways to combat invading pathogens:

1) They can 'neutralize' toxins or micro-organisms by binding to them and preventing their attachment to host cells;

2) Initiation of phagocytosis; immunoglobulins have binding sites for an antigen and receptors on phagocytic cells, thus they can promote the interaction of these two components by the process of opsonization. The type of phagocytic cell bound by the antibody will be determined by the antibody class; macrophages and neutrophils are specific for IgM and IgG, whilst eosinophils, mast cells and basophils are specific for IgE;

3) Activation of complement proteins in plasma, which in turn promote the destruction of bacteria by phagocytes.

Thus antibodies communicate between the acquired and the innate immune response: they are elicited through highly specific mechanisms, but can be translated to a form which can be interpreted by the innate immune system, enabling it to destroy the pathogen. The features of the different classes of antibodies are summarised in Table 3.

Ig class	Form	Primary location	Functions
IgG	Momomer	Serum (~12 mg/ml); Non-mucosal tissues	• Interaction with complement (Classical pathway) • Binds to Fc receptors on macrophages + neutrophils • Main antibody in the secondary immune response to most pathogens
IgM	Pentamer	Serum (~1.5 mg/ml)	• Activates complement • Main antibody component of response to T-cell independent antigens • First class to be produced during the development of the immune system and in the primary immune response
IgA	Monomer + dimer (serum), dimer (sIgA)	Serum (~3 mg/ml); sIgA is found in seromucus secretions, milk, colostrum + tears	• Links pathogens to effector cells via Fc receptors • Complement activation • sIgA protects mucosal surfaces of the body against attack by microorganisms
IgE	Monomer	Serum (0.05 µg/ml)	• Binds to Fc receptors on mast cells + basophils, causing IgE Fc receptors to cross link, and causing them to release inflammatory mediators (e.g. histamine) • No complement activation • Modulates hypersensitivity reactions, such as asthma + hay fever
IgD	Monomer	Serum (30 µg/ml); Found on surface of B cells as an antigen receptor together with IgM	• Control of lymphocyte activation + suppression • No complement activation • Present on differentiating B cells following activation, but is absent from mature antibody-forming cells

Table 3 : Antibody classes and their functions

1.2.3.3 T CELLS

Humoral immunity deals with extracellular pathogens. However not all pathogens are extracellular. For example viruses and also some bacteria infect individuals by entering cells. Therefore, humoral immunity will not eliminate these pathogens, and so there must be another mechanism by which the body deals with these pathogens. This mechanism is termed cell-mediated immunity, and is conferred by T lymphocytes. T cells can further be divided into T-helper (Th) cells and cytotoxic T (Tc) cells, which can be differentiated according to the cluster of differentiation (CD) molecules that they express. Th cells express CD3 and CD4, while Tc cells express CD3 and CD8, and these molecules are responsible for mediating the different kinds of immune response required to deal with different types of pathogen.

1.2.3.3.1 THE T CELL RECEPTOR (TCR)

Resting T cells must be activated before they can take part in an immune response. The T cell must move through the different stages of the cell cycle to become activated, and

many molecules expressed on the T cell and on accessory cells are involved in this process.

T lymphocytes express antigen-specific T-cell receptors (TCR) on their surface, which have an enormous antigen repertoire. TCR are structurally quite similar to antibodies, but they do not recognise intact antigens; they can only recognise antigens that have been processed and fragments of them "presented" on a cell surface. This is the distinguishing feature between humoral and cell-mediated immunity. T cells are also not capable of secreting the TCR, unlike B cells which can secrete antibody. The structure of the TCR is shown in Figure 5.

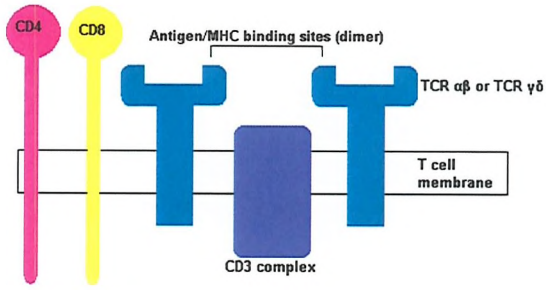


Figure 5 : Structure of the TCR

T cells cannot recognise the antigen fragments alone; the antigen fragments are transported to the surface of the infected cell and expressed there in conjunction with proteins called major histocompatibility complex (MHC); in humans MHC is termed human leukocyte antigen (HLA). There are two classes of MHC, MHC I and MHC II:

- Peptides which originate from pathogen proteins synthesised within the host cell cytosol (e.g. from viruses or certain bacteria) are presented with MHCI;
- Peptides from pathogens that have been phagocytosed by macrophages or endocytosed by antigen-presenting cells (macrophages, dendritic cells, B lymphocytes) are presented with MHCII.

Thus, the TCR on the T cell recognises the MHC-peptide complex on the infected cell. As shown in Figure 6, the TCR is associated with either CD4 or CD8 molecules. Whether a T cell expresses CD4 or CD8 determines which MHC molecule the T cell can recognise. T cells expressing CD8 (cytotoxic T cells) recognise MHC I (intracellular pathogens), while T lymphocytes expressing CD4 (helper T cells) recognise MHC II (extracellular pathogens).

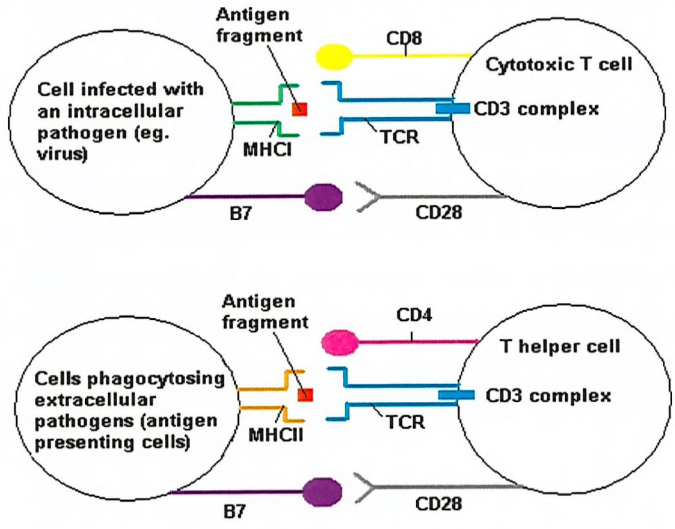


Figure 6 : Interaction of T cells with antigen presenting cells showing the roles of MHC and TCR

As shown in Figure 5, there are two types of TCR; the TCRαβ and the TCRγδ. In humans the majority of T cells in the periphery are of the TCRαβ type, with only a small percentage being of the TCRγδ type. However, in epithelial rich tissues, such as the intestines, γδ T cells make up a much larger percentage. At least some γδ T cells do not require an antigen to be presented with the MHC complex, and they may also bind to MHC related molecules without requiring the antigen. Thus, by interacting with these molecules (some of which are up-regulated on activation of αβ T cells) γδ T cells may play some kind of an immuno-regulatory role.

1.2.3.3.2 T CELL ACTIVATION

Initial adherence of the antigen presenting cell and the T cell involves the attachment of intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and leukocyte functional antigen (LFA)-3 molecules on the antigen presenting cell to their receptors (LFA-1, very late antigen (VLA)-4 and CD2, respectively) on the T cell. The subsequent interaction of the MHC-antigen peptide complex and the TCR is not sufficient to fully activate resting T cells; three signals are required:

1. Interaction of MHC-antigen peptide complex and the TCR. This association is strengthened by CD4 or CD8 molecules. If only this first signal is present, this will lead to anergy (i.e. lack of a response);
2. Interaction of B7 on the antigen presenting cell with CD28 on the T cell. CTLA-4 is an alternative ligand for B7 that is not expressed on resting T cells. Upon activated

T cells, CTLA-4 becomes expressed, and can down regulate T cell activation as it has a higher affinity for B7 than CD28 does;

3. Cytokines produced by the antigen presenting cell (see section 1.2.2.62.).

Signals originating from the TCR are transmitted and amplified throughout the cell, leading to changes in the transcription of specific genes, eventually resulting in activation of the T cell. This cell then undergoes clonal proliferation and differentiates into an effector cell. CD69 expression is induced by activation of T cells. This is the earliest cell surface glycoprotein expressed during lymphocyte activation. CD69 plays a role in signal transduction in activated T cells, and also in monocytes and platelets. It contributes to calcium influx and induction of many genes including those for some cytokines and cytokine receptors.

1.2.3.3.3 CYTOTOXIC (Tc) CELLS

These are a subset of T cells which possess cytotoxic activity, and which are involved in the protection against viruses and possibly tumour cells. Cytotoxic T (Tc) cells recognise antigen presented with MHCI molecules, and often require help from the Th cells. The Th cell and the cytotoxic precursor cell may bind to the same antigen presenting cell, with the Th cell binding to antigen associated with MHCII and the cytotoxic precursor cell binding to antigen associated with MHCI. The Th cell releases cytokines, which cause the cytotoxic precursor cell to differentiate into a Tc cell (Figure 7, signal 1). This interaction does not have to occur at the same time, so the Th cell can bind first, activate the antigen presenting cell (by CD40; thus up-regulating co-stimulatory molecules and cytokine production), so that when the cytotoxic T cell precursor binds later on, it is able to differentiate into a Tc cell (Figure 7, signal 2).

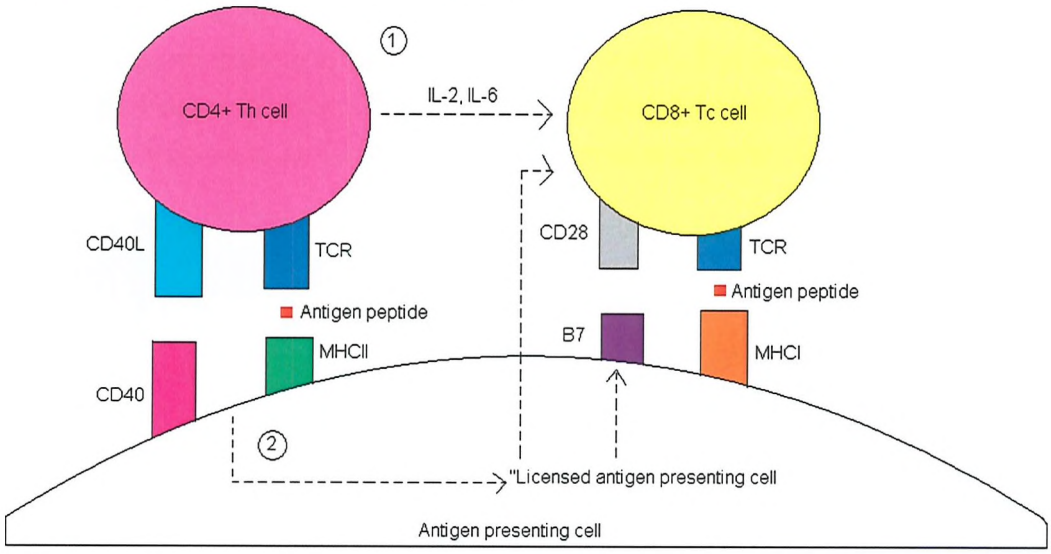


Figure 7 : Activation of Tc cells

Tc cells contain cytotoxic granules, which themselves contain perforin and granzymes. After the Tc cell becomes activated, these granules move along the microtubule system inside the Tc cell, to the place where the Tc cell and its target cell meet. Exocytosis of the cytotoxic granules results in the cytotoxic proteins arriving in the target cells cytosol, to induce apoptosis of the target cell, within about 60 minutes. The Tc cell can then detach itself from the target cell and move away to perform the same function on another target cell. The apoptotic target cell is then engulfed by phagocytic cells, thus ensuring that its contents (maybe including viruses) do not leak out and damage surrounding cells. This mechanism needs to be switched off at the appropriate point, as to have this inflammatory process inappropriately activated would clearly be damaging to the host. IL-10 is largely responsible for this, through its inhibitory effects on IL-1 and TNF release from macrophages and Th1 cells, and IL-4 is also involved.

Tc cells can be divided into Tc1 and Tc2, although all Tc cells have the same cytolytic function. Clones of Tc1 cells have been shown to secrete IFN-γ but not IL-4 and induce Th1 cells when co-cultured with $CD4^+$ T cells, and clones of Tc2 cells have been shown to secrete IL-4 but not IFN-γ and induce Th2 cells when co-cultured with $CD4^+$ T cells.

1.2.3.3.4 T HELPER (Th) CELLS

Helper T lymphocytes can be sub-divided into two broad categories according to the pattern of cytokines they produce, as described in Figure 8. Naïve T cells produce mainly IL-2 upon initial encounter with antigen, and under the regulation of cytokines these cells differentiate into Th0 cells, and then further into either Th1 or Th2 cells. The type of pathogen that the antigen presenting cell has encountered determine the type of cytokines it produces, and hence whether the Th0 cell differentiates into a Th1 or Th2 cell. Th1 and Th2 cells themselves have relatively restricted profiles of cytokine production, as shown in Figure 8. Th1 cells are responsible for protection against intracellular infections via cell-mediated immunity, and Th2 cells are responsible for protection against extracellular pathogens via humoral immunity, eosinophils and mast cells.

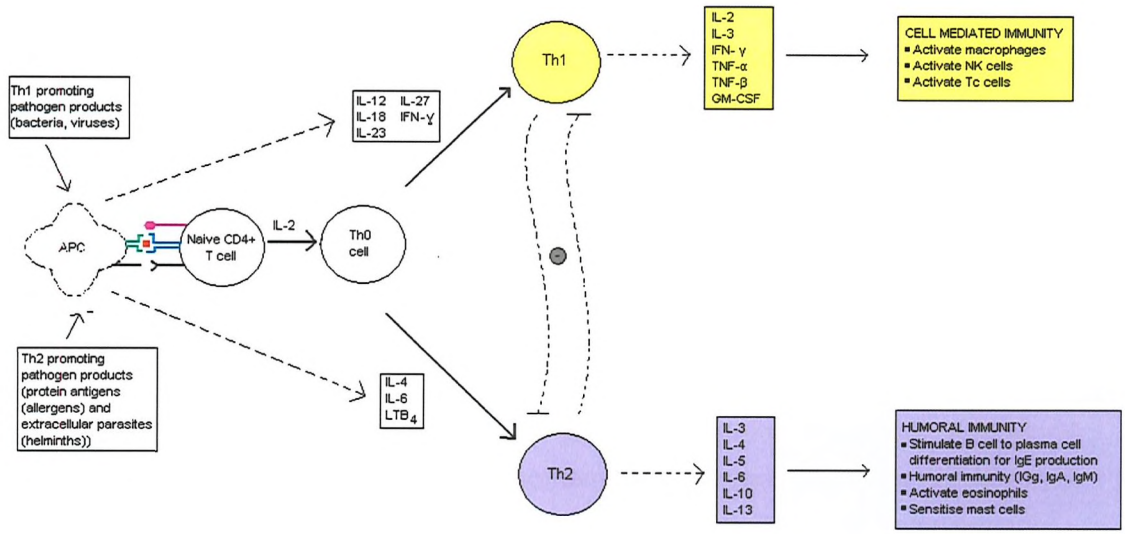


Figure 8 : Differentiation of a naïve helper T cell to a Th1 or Th2 cell

The patterns of cytokine secretion are probably not as clearly defined as this, and activated T cells are likely to produce a wide spectrum of cytokines, with a skewing of their cytokine profile to either a Th1 or Th2 dominated pattern. A summary of the functions of Th cells is given in Figure 9.

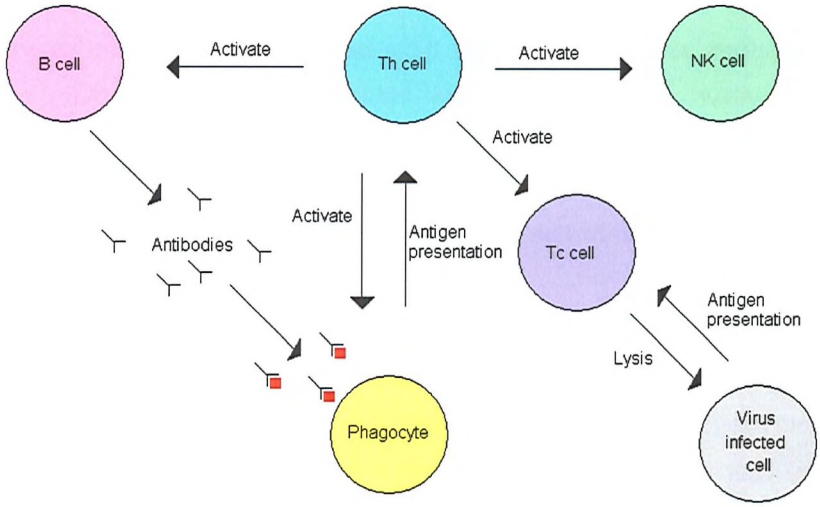


Figure 9 : Schematic overview of the immune response showing the central role of Th cells

1.2.3.3.5 REGULATORY T CELLS

In recent years regulatory T cells have been described. These cells have been shown to suppress cell-mediated immunity and some inflammatory processes (5). There are different subsets of regulatory T cells, such as the natural $CD4^+CD25^+$ T_{regs}, IL-10 secreting T_R1 cells, and TGF-ß secreting Th3 cells. In the intestines, the body is constantly exposed to a huge number of bacteria, many of which are commensal, and so it is important that

the body does not mount an immune response against them, thus preventing chronic intestinal inflammation from occurring. Unique subsets of antigen presenting cells in the gut-associated lymphoid tissue (GALT) present antigen to $CD4^+CD25^+TGF\text{-}\beta^+$ T_{regs} and to T_R1 cells, which provide the necessary immuno-suppression (5).

1.2.4 VACCINATION

Vaccination is the active induction of protective immunity against a pathogen, and relies on immunological memory. The objectives of vaccination are to:

1. Establish adequate levels of antibody against the vaccine antigen;
2. Establish a primed population of memory cells which will expand the next time the body comes into contact with the antigen.

Vaccination, or the idea that it is based on, has been practised throughout history, by many different cultures. The modern history of vaccination began with Jenner (1749 - 1823) who showed that cowpox vaccination (non-virulent to humans) protected humans from contracting smallpox (the virulent form of the disease for humans). This type of vaccine is known as a living heterologous vaccine; these vaccines produce a milder but cross-protecting form of the disease. Later on Pasteur (1822 - 1895) discovered that attenuated viruses could offer protection against virulent forms of the disease. Live attenuated vaccines use organisms that have been modified in some way, but that still mimic the behaviour of the live organism. Attenuation methods include using high temperatures, anaerobic conditions and recombinant DNA technology. However, these kinds of vaccines also have disadvantages, in that a virus could revert to its virulent form, and there are difficulties with the storage of these vaccines. Other kinds of vaccination include:

- Killed organisms - the microbes are no longer able to cause disease, but the antigenic structure remains. These vaccines are generally safe, but not as effective as a vaccination with a live organism.
- Subunit vaccines - many antigens make up a bacterium/parasite/virus, but not all of them are involved in the protective response of the host, but they can still cause problems (for example, suppress the response to the protective antigens). So, by isolating the protective antigens and vaccinating with only these will avoid these complications. Influvac® is a vaccine against seasonal influenza. It was introduced onto the market in the early 1950s, and since 1983 has been a sub-unit vaccine. Influvac® contains purified surface antigens, haemagglutinin and neuraminidase prepared from certain selected strains of the influenza virus and distributed by the World Health Organisation (WHO), on the basis of their latest recommendations. Because the influenza virus has a high mutation rate, the composition of the vaccine

has to be changed each year. The WHO has a Global Influenza Surveillance Network, which has centres in 83 countries, whose role is to isolate the local influenza viruses. The viruses are analysed and from this analysis, the WHO recommends which strains of the virus are most likely to cause influenza outbreaks during the coming winter, in both the Northern and Southern hemispheres. Current vaccines are trivalent, and contain two subtypes of influenza A and one subtype of influenza B, which are selected for the annual vaccine. For the 2007/2008 influenza season, the strains used were: an A/Solomon Islands/3/2006(H1N1)-like virus, an A/Wisconsin/67/2005 (H3N2)-like virus and a B/Malaysia/2506/2004-like virus. For the 2008/2009 influenza season, the strains used were: an A/Brisbane/59/2007 (H1N1)-like virus, an A/Brisbane/10/2007 (H3N2)-like virus and a B/Florida/4/2006-like virus.

1.2.5 THE GUT-ASSOCIATED LYMPHIOD TISSUE (GALT)

The mucosa-associated lymphoid tissues of the gut make up the gut-associated lymphoid tissue (GALT). GALT includes specialised areas where immune cells are concentrated: the lamina propria (where there are high numbers of lymphocytes) and the Peyer's Patches (where there are high numbers of IgA producing B cells and plasma cells; Figure 10). In the columnar epithelium covering the GALT there are microfold "M" cells, which are specialised antigen transporting cells. These cells take up antigen from the intestinal lumen and transport it (by transcellular transport) to the Peyer's Patches in the GALT underneath. Antigen presenting cells then recognise antigen, process it and present it to the appropriate lymphocytes, which then become activated.

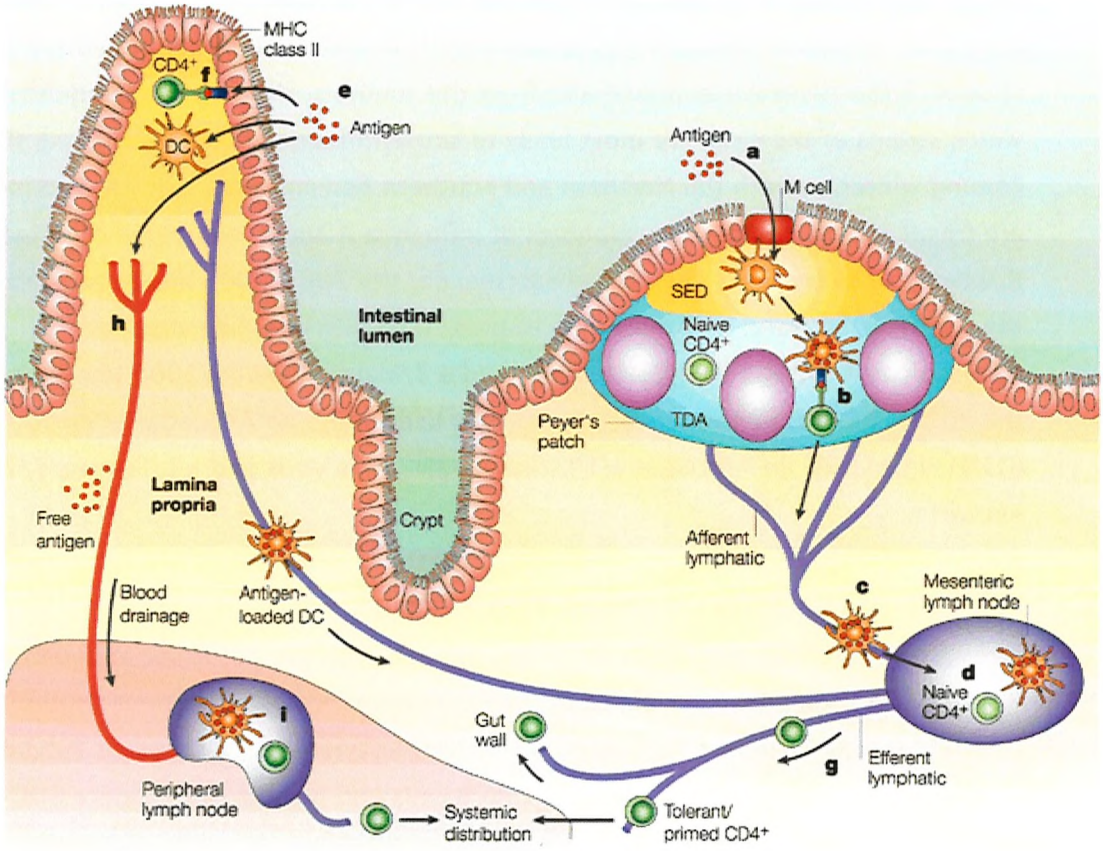


Figure 10 : Location of function of the GALT (taken from Mowat 2003)*(6)*

After activation of lymphocytes has occurred, activated lymphocytes travel via the lymph to mesenteric lymph nodes, where they may become activated further and proliferate. Then, they travel through the thoracic duct and into the blood, where they are now able to travel to several mucosal sites around the body (such as the lungs, salivary glands, genito-urinary tract, the lactating mammary gland), or back to the gut to the lamina propria. In the lamina propria the activated T cells assist IgA forming B cells which are now able to protect the whole gut by secretion of the antibody specific to the pathogen they were first stimulated within the Peyer's Patches. This is shown in Figure 11.

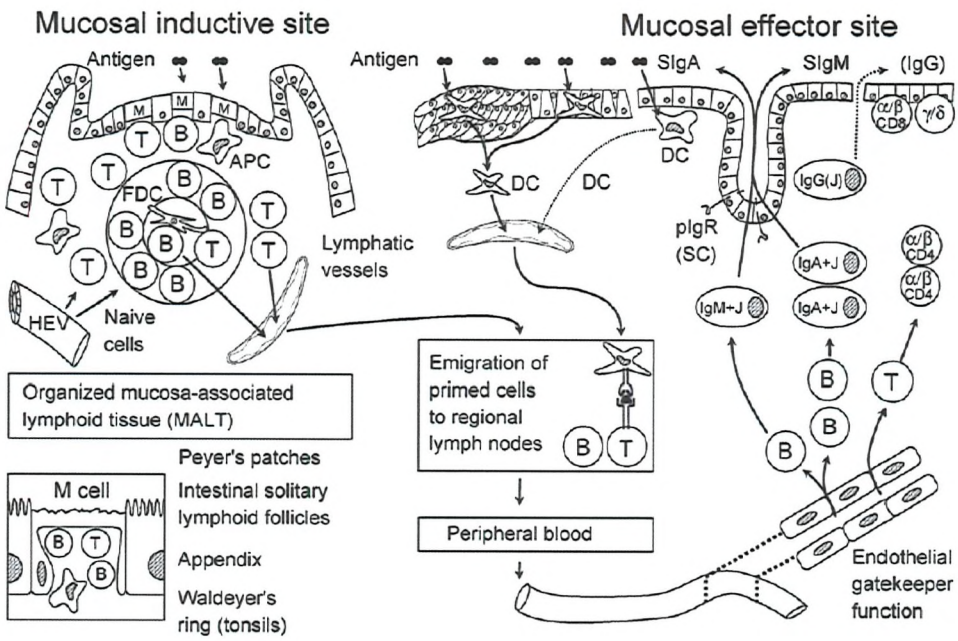


Figure 11 : Movement of activated lymphocytes around the GALT and systemic immune systems (taken from Cummings et al 2004)(7)

In the typical adult human gut there reside approximately 10^{14} of a variety of indigenous aerobic and anaerobic bacteria, all of which have been derived from the environment beginning from the moment of birth. Many of these bacteria are commensal; they pose no threat and may even be beneficial. However, pathogenic bacteria are also present. Thus the GALT needs to be able to distinguish between the harmless and the harmful bacteria. It is important that the immune system does not respond actively to the commensal bacteria, as this will result in an inappropriate immune response, which is thought to be responsible for some intestinal inflammatory conditions, such as Crohn's disease (8). The immune system is thought to recognise bacteria through TLRs that act as PRRs on the surface of antigen presenting cells. TLRs can recognise conserved molecular products on micro-organisms, for example TLR-4 recognises lipopolysaccharide (LPS) and TLR-2 recognises lipoteichoic acid (9).

Intraepithelial lymphocytes (IELs) are mostly T cells, about 10% of which are γδ T cells (in humans). Of the αβ T cells, most are $CD8^+$, and are either CD8αβ (conventional T cells) or CD8αα. The CD8αα is almost exclusive to IELs, and is not restricted by the classical MHCI molecules, unlike conventional $CD8^+$ T cells. Most of the γδ T cells in the intestine also are also of the CD8αα type, and are thought to act as a first line of defence at the outer surfaces of the body.

1.3 MARKERS TO MEASURE THE ACTIVITY OF THE HUMAN IMMUNE SYSTEM

As described previously, the immune response involves a complex interaction between many different cell types. Many factors influence the functioning of the immune system. These include age (10), sex hormones, stage of the menstrual cycle, and pregnancy (11), obesity (12), psychological stress (13), physical stress (14), composition of diet (15), and previous history of exposure to pathogens. Therefore, it is important when measuring immune function, that a variety of parameters are measured, and that the potentially confounding factors are taken into account (10). The following paragraphs summarise some of the available immune markers, with focus on those which will be used in this study.

1.3.1 CONSIDERATIONS WHEN DESIGNING STUDIES AIMING TO MEASURE IMMUNE FUNCTION

1.3.1.1 SUBJECT-SPECIFIC FACTORS AND THEIR RELEVANCE

Age - a specific age range should be specified, as many aspects of immune function have been shown to vary with age (10).

Sex - this should be considered when recruiting subjects to ensure either recruitment of one sex only or of an equal distribution of men and women across groups, to limit any sex specific differences that may occur in immune function.

Obesity - unless the effect of obesity is being studied, recruitment should be restricted to those within the normal BMI range, as those defined as obese may show changes in immune function (12) and inflammatory markers (16).

Background diet - although it is not normally possible to assess and control for a subjects' background diet, it is likely that this could affect their immune function. Where possible important aspects of the background diet should be specified or controlled. For example, if the effect of prebiotics is to be studied, then prebiotic (other than those under study) and probiotic containing foods should be eliminated from the diet during the trial period and preferably for a pre-trial run-in period

Presence of infections or other diseases - this can be controlled for at recruitment, with the exclusion of subjects presenting with these conditions.

Vaccination and infection history - for trials using a vaccination, this is crucial. Thus, for example, in a trial involving seasonal influenza vaccination, individuals who have received

an influenza vaccine previously should be evaluated for the year they received this, and subsequently the strains used in the vaccine and in the study vaccine compared. If any strains are the same, it is likely subjects will respond to the vaccine differently than someone who has not received it before, and therefore they should not be included in the trial.

Medications - anti-inflammatory drugs may affect the normal immune response. Medications for other underlying conditions often indicate that the individual may not be in optimal health, and thus should be considered on an individual basis to assess suitability for inclusion.

Physical exercise, smoking, psychological stress, alcohol - these have all been shown to affect the immune response (13, 14, 17, 18). However, it is more difficult to control for these factors, as realistically it is not possible to ask people to change their whole lifestyle for the duration of a study.

1.3.1.2 TECHNICAL FACTORS AND THEIR RELEVANCE

Subject population and controls - due to the subject-specific variations in immune function described previously, ideally subjects should act as their own control. However, in studies where a crossover design is not possible (e.g. when a subject is vaccinated), a control group that is properly matched to the test group should be used instead.

Timing of sample collection - immune cells have a circadian rhythm (19), and thus to avoid this confounding results, samples should be collected at a consistent time. This will usually be in the morning, as blood samples generally need to be taken in the fasted state. This also ensures that the fasting time before each sample is approximately the same (approximately 10 - 12 hours since the evening meal).

Season - the season also affects immune function (19) so if possible the study should account for this, although this is likely to be difficult in shorter studies. Each year, the new seasonal influenza vaccines become available in the Northern Hemisphere from September, with a shelf life lasting until the end of June. Therefore, it is not possible to include the summer months, and most of the autumn, as no vaccine is available during this time.

Length of intervention period - should be sufficient to see changes in immune function.

Bioavailability of the nutrient or food ingredient being studied - should be assessed. For prebiotics this is done by assessing the faecal microflora, as prebiotics have been shown to increase levels of beneficial bacteria in the gut, which is reflected in levels in the stool.

1.3.2 ASSESSMENT OF MARKERS

The purpose of the following section is to summarise the markers which are to be used to assess the immune system in this study. An Expert Group of the Nutrition and Immunity in Man Task Force of the International Life Sciences Institute Europe, analysed all available markers to measure the immune system in humans, and categorised them according to their biological relevance, biological sensitivity and feasibility (10). Biological relevance refers to how well the immune marker correlates with relevant clinical endpoints, biological sensitivity refers to the ability to detect changes in immune function, taking into account variation between and within subjects, and feasibility refers to how practical the method is to carry out. Upon evaluation of these points, each marker was assigned a suitability score of high, medium or low, and these are stated below.

1.3.2.1 IN VIVO MEASURES

1.3.2.1.1 RESPONSE TO VACCINATION

The body's response to an infection is the most relevant way in which to assess the functioning of the immune system, and as this response occurs *in vivo*, it is an accurate representation of how the immune system functions in a physiologically natural environment. As natural exposure to pathogens is uncommon and deliberate infection is unethical, a vaccination is a convenient way in which to challenge the immune system. This is considered to be the gold standard method for measuring the functioning of the immune system (10). Response to vaccination can be assessed in several ways:

i) By looking at vaccine-specific antibody concentrations in serum, plasma, or saliva. These can be measured by haemagluttination assays or ELISA. It is a good idea to measure baseline responses to the vaccine, as factors such as natural infections can result in high variability in the population. Repeated measurements can be used to look at the kinetics of this response over several weeks or months, and the study period should encompass at least the time taken to reach the plateau phase of the response. Between-subject variability is normally quite high for this marker. This method of assessing response to vaccination reflects an integrated, *in vivo* immune function, and is categorised as having high suitability.

ii) Response to vaccination can also be assessed by vaccine-specific T cell responses, *ex vivo*. T cells isolated from the blood can be stimulated with the vaccine antigen

and various measurements, such as T cell proliferation, cytokine production and T cell activation can be assessed.

iii) The delayed type hypersensitivity response is another *in vivo* measure that is used to assess the cell-mediated immune response to the vaccine. This is an intracutaneous test, where the skin is pricked, and a tiny amount of the antigen is placed on the break in the skin. The inflammatory response to the antigen is then assessed by measuring the size of the wheal 24 - 48 hours later. This could be used in vaccination studies, several weeks or months after the vaccination was given and the nutritional intervention completed, in order to see if the intervention had any long-lasting effects on the immune response to vaccination. This marker is considered to have high suitability.

1.3.2.2 EX VIVO MEASURES

This involves *in vitro* assays. The cells are studied outside of their environment, and thus the physiological relevance of responses observed is not as clear as in vivo responses. However, ex vivo measures are a useful and standardised way to assess immune function. In cell culture work using immune cells from the blood, peripheral blood mononuclear cells can be isolated, and then cryopreserved. Although desirable to use fresh cells, this is not always possible due to technical and logistical reasons. Also, by cryopreserving cells and using batch culture at the end of an intervention, day-to-day variability in the culture conditions and in human technical error is limited. It has been shown previously that cryopreservation and thawing does not distort mononuclear cell immune responses (20). The cells are then cultured in some form of medium, frequently containing foetal calf serum.

Only around 2% of lymphocytes are circulating in the blood at one time (21), thus evaluation of the immune system in the blood may not be an accurate representation of what is going on in other parts of the body. However, in humans, this is usually the only possible way to look at immune function, due to ethical constraints.

1.3.2.2.1 PHAGOCYTIC FUNCTION

Phagocytosis by polymorphonuclear neutrophils and monocytes is an important aspect of the host defence against bacterial or fungal infections. Modern methods to measure phagocytosis use flow cytometry to analyse these functions in whole blood. A method known as "Phagotest" measures leukocyte phagocytosis (ingestion of fluorescently labelled *E. coli*), and both the percentage of phagocytes that ingest *E. coli* and the activity of the phagocytes (the number of *E. coli* they ingest) can be measured. Internalised bacteria can be distinguished from membrane bound bacteria (22). Another method

termed "Phagoburst" can measure leukocyte oxidative burst. Granulocytes and monocytes produce reactive oxygen metabolites when stimulated, which destroy the fluorescently labelled *E. coli* that have been internalised inside the phagosome. Dihydrorhodamine (DHR) 123 is oxidised by these reactive oxidants, and so the addition of DHR123 and its oxidation to rhodamine 123 (which is fluorescent), mean that percentage of phagocytes producing reactive oxidants (conversion of DHR123 to rhodamine 123), and their enzymatic activity (amount of rhodamine 123 per cell) can be monitored. Both Phagotest and Phagoburst require that the conditions of the assay are strictly controlled, such as the incubation time of phagocytes with the *E. coli,* the temperature of the incubation, and stimuli used. Phagotest is considered to have low suitability, while Phagoburst is considered to have medium suitability.

1.3.2.2.2 LYMPHOCYTE PROLIFERATION

T cells can be stimulated by a variety of stimuli including the general stimulants phytohaemaglutinin (PHA) and concanavalin A (ConA), antibody combinations such as anti-CD3 and anti-CD28 that mimic physiological activation, and specific stimulants, such as vaccine antigens. Traditionally, ^{3}H-thymidine incorporation into the DNA of proliferating cells has been used to measure this parameter, but this has its drawbacks, such as high within-subject variability and the handling of radioisotopes. An alternative method has been developed, where the T cells are stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). CFSE consists of a fluorescein molecule containing two acetate moities and a succinimidyl ester functional group, and passively diffuses into cells. It is colourless and non-fluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, to form fluorescent conjugates. Excess CFSE and by products passively diffuse to the extracellular medium, where they are then washed away.

The fluorescent conjugates that have been formed in the labelled cells are retained by the cells throughout development and meiosis, and so the label is inherited by daughter cells. As cells divide, the CFSE is split equally between the daughter cells, which results in a diminished CFSE signal detection, which occurs with each cell division. Assessment of lymphocyte proliferation is considered to have medium suitability.

1.3.2.2.3 LYMPHOCYTE ACTIVATION

This can be assessed by cell culture using a variety of stimulants (again, vaccine-specific stimulation can be used), and subsequent analysis of the cell surface expression of activation markers. Activation markers that can be used include CD69 (an early activation marker), HLA-DR and CD95 (late activation markers); the length of culture depends upon

which marker is being used. The analysis is performed via flow cytometry. This assay is considered to have low suitability.

1.3.2.2.4 T CELL CYTOKINE PRODUCTION

The function of Th cells can be assessed by the production of cytokines; Th1 cells produce predominantly IL-2, IL-3, IFN-γ, and TNF-α, while Th2 cells produce predominantly IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13, and the level of production is related to the level of activity of these cells. This is a good *ex vivo* measure of cell mediated immunity. Again, the cells will be cultured, as above, and a variety of stimulants (including vaccine antigen) can be used. The concentrations of cytokines in the supernatant can be measured. This can be done using ELISA kits, or by flow cytometry using cytometric bead arrays (CBA). CBA kits can be used to measure levels of multiple cytokines simultaneously in small sample volumes. For each cytokine, a set of beads is coated with cytokine specific antibodies, and each cytokine specific set of beads has a fluorescence intensity that is different from the beads for other cytokines. The beads are incubated with the cell culture supernatants, washed, and then incubated with another, phycoerythrin (PE) labelled, cytokine antibody to distinguish between beads positive for cytokines in the kit or not in the kit. This is then analysed by flow cytometry. This marker is considered to have medium suitability.

1.3.2.2.5 ANTIBODY PRODUCTION

The *in vivo* production of antibodies by B cells can be measured by ELISA, and the detection of vaccine-specific antibodies is considered the easiest way to assess the adaptive immune response to the vaccine antigen. Total antibody class (IgG, IgA, IgM etc), and antibodies with a specific antigen specificity, such as anti-influenza specific antibodies, can be measured. Antibody production has medium suitability.

1.3.2.2.6 BLOOD LYMPHOCYTE POPULATIONS

This is measured by flow cytometric analysis. Different populations of cells can be enumerated on small amounts of whole blood using cell-specific fluorescent antibodies. The information obtained is useful for the interpretation of functional assays that have been done on the same samples, but has a low suitability as an immune function marker.

1.3.2.2.7 SECRETORY IgA

IgA is the main immunoglobulin secreted at cell surfaces, and IgA secreted here is termed secretory IgA (sIgA), and is an indicator of mucosal immunity. Many factors have been shown to affect sIgA production, and so samples should be collected at a consistent time, at rest, and in the fasted state (23). When flow rate increases due to chewing, salivary IgA

concentration falls. sIgA concentrations in saliva are measured by ELISA, and both vaccine-specific IgA and non-specific IgA are considered to have high suitability.

Although several of the markers described are classified as having low suitability, they can still be valuable. It is best to use a combination of markers that look at the function of one type of immune cell, and consider all these markers when analysing the function of this particular cell. Also using a combination of markers to look at a variety of functions, even if the markers are of different suitability, gives greater confidence in the findings.

1.4 PREBIOTICS

1.4.1 INTRODUCTION

Prebiotics have been defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth, and/or activity, of one or a limited number of beneficial bacteria in the colon and thus improves host health" (24). Prebiotics may include the ß2-1 fructans (inulin (IN) and oligofructose (OF)), galactooligosaccharides (GOS), glucooligosaccharides, isomaltooligosaccharides, lactulose, mannanoligosaccharides (MOS), nigerooligosaccharides, oat ß-glucans, raffinose, soybean oligosaccharides, transgalacto-oligosaccharides and xylooligosaccharides. ß2-1 fructans, GOS, lactulose and transgalacto-oligosaccharides have all been shown to fulfil the criteria for prebiotics (25). As the prebiotic used in the study described in this thesis is a combination of IN and OF, the main focus of this section will be the ß2-1 fructan family.

A fructan is defined as any molecule where fructosyl-fructose linkages constitute the majority of linkages (26). Fructan-containing plants have been used as foods for as long as 5000 years, and IN was discovered in the nineteenth century. Since the end of the nineteenth century, scientific reports on the benefits of IN have been produced, and research on the potential health benefits of prebiotics has occurred over the last 15 years or so, with a recent interest in the effects on the immune system, the host's ability to fight infection, and inflammatory processes and conditions.

1.4.2 STRUCTURE OF ß2-1 FRUCTANS

Chicory IN is a polydisperse carbohydrate molecule. It contains ß-(2→1) fructosyl-fructose linkages with a terminal glucose molecule (26), and may contain between 2 and 60 fructose residues (Figure 12), with an average of 12. Although IN was thought to be a linear molecule, native chicory IN has a very small degree of branching (27). Partial enzymatic hydrolysis (by endoinulinase) of IN yields OF, which can have a terminal glucose or fructose residue (Figure 12). In OF there can be 2 to 7 (average 5) fructose residues with a terminal glucose residue or a chain of 3 to 8 (average 5) fructose residues

(28). Long-chain IN, called high performance (HP-) IN can be produced by industrial separation techniques. This product has chain lengths varying from 10 to 60, with an average degree of polymerization (DP) of 25. Orafti® Synergy1 is commercial product containing a 50:50 mixture of HP-IN and OF. Thus, different IN and OF products differ according to DP, and because of this, they have different technological properties. For example, IN and HP-IN have a bland neutral taste and are often used as fat replacers and gelling agents, while OF and Orafti® Synergy1 have a slightly sweet taste and thus are used as sugar replacers and sweeteners. Chicory IN, HP-IN and Orafti® Synergy1 are available as white colourless powders, and OF is available as powders and colourless viscous syrups.

Short-chain fructooligosaccharides (sc-FOS) may also be derived by enzymatic addition of fructose residues to sucrose, by the formation of new ß-(2-1) linkages, a reaction catalysed by ß-fructosidase (Figure 12). The products formed contain 2 to 4 fructose residues with a terminal glucose residue and a DP average of 3.6 (Figure 12). Actilight® is a commercial product containing sc-FOS. The distribution of the fructan chains in the different products is shown in Figure 13.

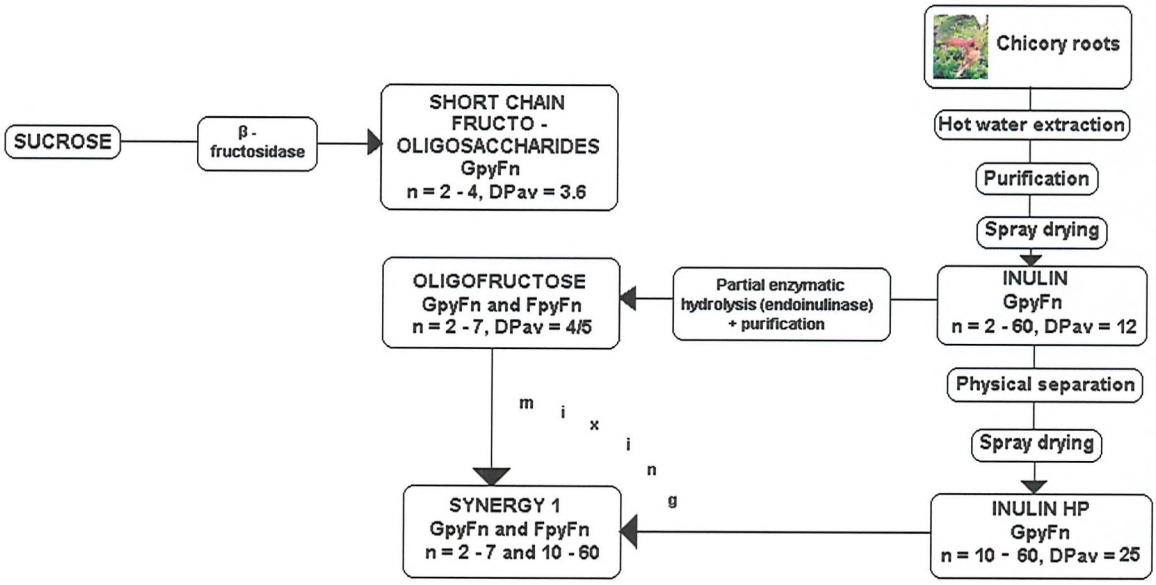


Figure 12 : The structure and extraction of ß2-1 fructans (taken from Roberfroid 2005)(29)

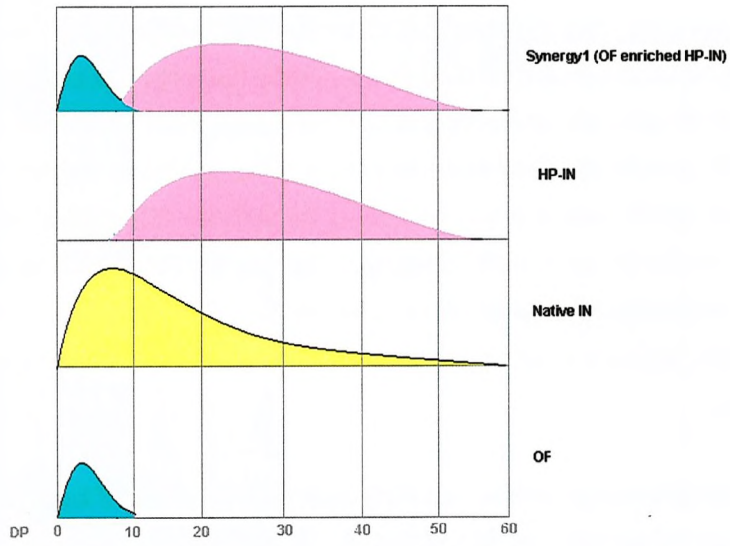


Figure 13 : Degree of polymerisation of different fructans (taken from Roberfroid 2005)(29)

A variety of trade names are used for different combinations of ß2-1 fructans in commercial products, and these are summarised in Table 4.

Product name	Description
Raftilose®, RaftiloseP95®, BeneoP95®	Commercial preparations of OF
Raftiline®	A commercial preparation of IN
Raftiline-ST®	A commercial preparation of standard IN, average DP ≥ 10
Raftiline-HP®	IN with the shorter chain molecules removed, DP ranges from 10 – 60, average DP > 23
Orafti® Synergy1	OF enriched IN, a 1:1 mixture of OF and high-performance IN
Prebio1®	A mixture of 70% Raftilose® and 30% Raftiline®
sc-FOS (Actilight®)	Made by the enzymatic addition of fructose residues to sucrose, with a DP average of 3.6, DP ranges from 2 -4

Table 4 : Commercial ß2-1 fructans products

1.4.3 SOURCES OF FRUCTOOLIGOSACCHARIDES

IN is found naturally in significant amounts in a variety of plant foods such as bananas, barley, chicory, garlic, Jerusalem artichoke, leeks, onions, and wheat (30). Chicory contains an average of 16.2 g of IN per 100 g (30), although the IN content and chain

length varies between different plants, and even in the same plants during different seasons and weather conditions and according to the age of the plant. Several physiological roles of IN have been suggested, such as a long term carbohydrate storage in underground over-wintering organisms, cryoprotection and osmotic regulation. IN has been extracted from chicory roots, Jerusalem artichoke, artichoke, dahlias and dandelions (31), although chicory is the main crop that is used for industrial IN production.

The typical daily intakes of IN for adults are estimated to be between 3 and 11 g/d in Europe, and between 1 and 4 g/d in North America (30), the most common sources in these diets being wheat, onion, banana, garlic and leeks.

Oligosaccharides, including some believed to be prebiotics, are present in human breast milk (32). They can be found in concentrations of up to 12 g/L, making them the third largest component of breast milk (33). The presence of oligosaccharides in large amounts in breast milk suggests that these compounds may play an important role in early infant development, perhaps of the gut, its microbiotia and the immune system. Breast milk contains many compounds and substances that influence gut and immune maturation and consequently has a protective role against infections (34) and possibly allergy development (35). Oligosaccharides may contribute to these protective actions. It is possible that the oligosaccharides are present in breast milk in the mix and concentrations required for optimum protection, and for the development of the immune system

1.4.4 MECHANISMS OF ACTION OF PREBIOTICS

ß2-1 fructans fulfil the three criteria which must be met in order to be classified a prebiotic, as defined by Gibson and Roberfroid (1995 (24)):

1. Resistance to hydrolysis or absorption in the upper gastrointestinal tract (as the ß-(2→1) osidic bond is not hydrolysed by mammalian digestive enzymes, which are specific for α-glycosidic bonds). This was shown in early in vitro tests, where ß2-1 fructans were incubated with rat pancreatic and small intestinal homogenates, and shown to be poorly digested (36). Fulfilment of this criterion has also been demonstrated in humans through the study of ileostomy subjects, where 87% of dietary IN was recovered in ileum (37), thus establishing the survival of IN through the upper gastrointestinal tract. The non-digestibility of ß2-1 fructans in the small intestine has also been demonstrated in healthy volunteers (38).
2. Fermented by the intestinal microbiotia. This has been demonstrated in experiments in which ß2-1 fructans were completely metabolised in microbial fermentation cultures (30, 39).

3. Selectively stimulate the growth and/or activity of beneficial intestinal bacteria, such as *Lactobacillus* species and *Bifidobacterium* species. Studies in laboratory animals and humans show that prebiotics do increase the numbers of these types of bacteria in the intestinal tract (40-45). Other experiments establish that ß2-1 fructans are selectively fermented by most *Bifidobacterium* species (46), and also by some *Lactobacillus* species (47), as these bacteria produce the intracellular fructosyl-fructofuranosidase that is needed for hydrolysis of the ß-(2→1) osidic bond in ß2-1 fructans (28).

As a result of intestinal fermentation and promotion of growth of beneficial members of the gut microbiotia, prebiotics may influence host defence (Figure 14). Firstly, by increasing the number of bifidobacteria, there will be increased competition with pathogenic bacteria for binding sites on the intestinal epithelium and for nutrients, thus inhibiting survival of the pathogenic strains. Beneficial members of the gut microbiotia may also cross the intestinal barrier into the Peyer's Patches (PPs), and activate immune cells there (48). Others suggest that it is not the beneficial bacteria themselves that cross the barrier, but microbial substances such as cell wall components and cytoplasmic antigens (49). *Bifidobacterium* species and *lactobacillus* species are able to produce antibacterial substances that can inhibit the growth and survival of pathogens (50).

Secondly, the fermentation of prebiotics by the *Bifidobacterium* species produces short chain fatty acids (SCFAs) (24), which have the following effects:

- Acidification of the colonic environment, which is detrimental to some pathogenic strains of bacteria (51) such as some pathogenic species of bacteroides, clostridia and coliforms (50).
- Acidification of the colon favouring mucin production (52). This is believed to improve mucosal morphology, so decreasing pathogenic bacterial colonization and translocation.
- Binding to SCFA receptors (G protein coupled receptors 41 and 43) on immune cells within the GALT (53-55).
- Butyrate decreases the requirement of epithelial cells for glutamine, thus sparing more for GALT (56).
- Butyrate may also alter epithelial cell gene expression, for example IL-8 and monocyte chemoattractant protein 1 (MCP-1), and this in turn would alter the signalling of the epithelial cell to the mucosal immune system (57).

Finally, prebiotics may also influence host immune function through alternative mechanisms to the modulation of beneficial bacteria in the gut. It is hypothesised that carbohydrate moieties on the prebiotic may interact with receptors on immune cells.

Although a specific fructose receptor has not yet been identified, receptors for ß-glucan (58, 59) and mannose (60) have been identified on immune cells, and in vitro, fructose has been shown to alter non-opsonic phagocytosis (61), suggesting that a receptor for fructose on immune cells may exist. In addition, some oligosaccharides, for example OF, can bind to receptors on pathogenic bacteria and prevent them from attaching to this same sugar on the epithelial membrane, thus preventing adherence (62).

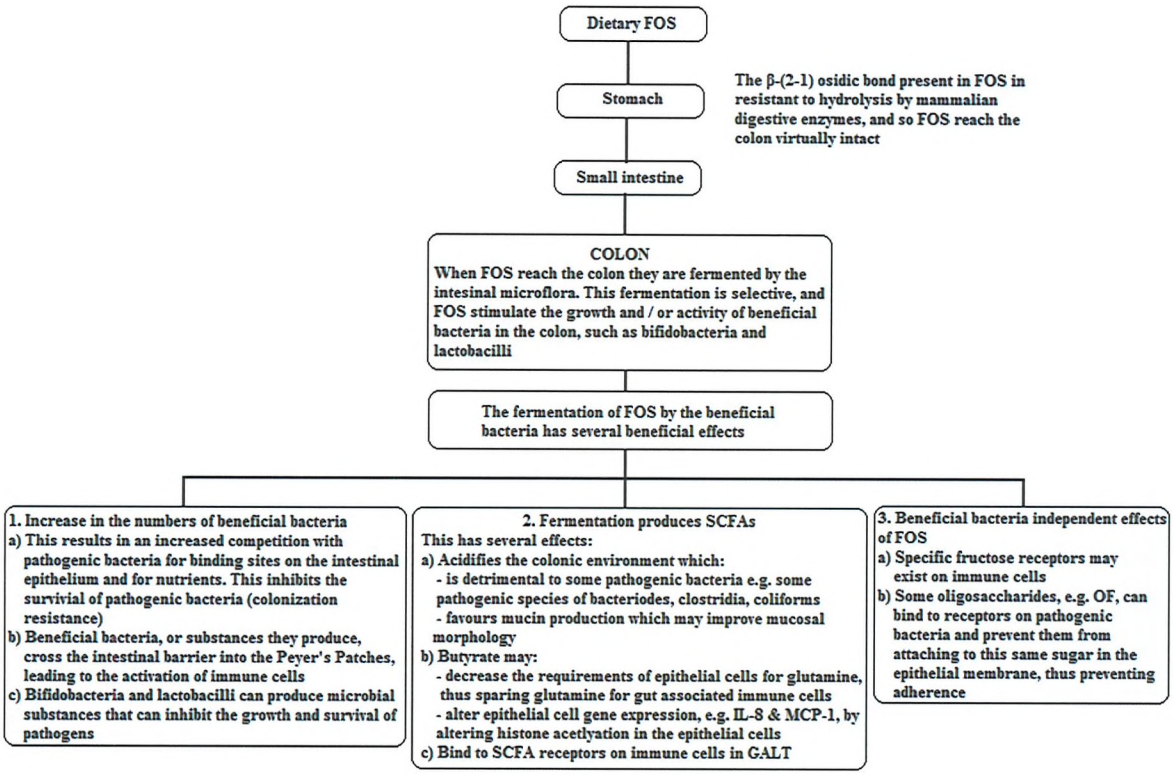


Figure 14 : Mechanisms of action of ß2-1 fructans (taken from Lomax and Calder 2009)(63)

1.4.5 EFFECTS OF ß2-1 FRUCTANS ON IMMUNUE FUNCTION, INFECTION AND INFLAMMATORY CONDITIONS

As mentioned, research on the potential health benefits of prebiotics has occurred over the last 15 years or so, with a recent interest in the effects on the immune system, the host's ability to fight infection, and inflammatory processes and conditions. This section will review all available studies investigating the effect of ß2-1 fructans on these outcomes, and is a summary and update of a comprehensive review article published elsewhere (Lomax and Calder 2009a (63)); the review article is included in this thesis as Appendix 1. A table summarising studies investigating the effect of ß2-1 fructans on the

relevant outcomes that have been published since this review article was prepared (February 2008) and up to June 2010 is included at the end of this section (Table 5).

1.4.5.1 ß2-1 FRUCTANS AND IMMUNE FUNCTION

This section reviews studies in experimental animals and in humans that investigate the effects of increased consumption of ß2-1 fructans on aspects of immune function.

1.4.5.1.1 STUDIES IN LABORATORY ANIMALS

Studies conducted in laboratory animals are useful because they can be highly controlled, thus eliminating sources of variation in diet and in immune response. 35 studies of ß2-1 fructans reporting immune outcomes were identified in mice, rats, pigs, dogs, fish and chickens and are summarised in Appendix 1 (Lomax and Calder 2009a(63)) and Table 5. Many of these studies show benefits of ß2-1 fructans to some aspects of immune function, while showing no effect on other aspects. Thus, ß2-1 fructans may have specific effects upon different components of the immune system. Here, the studies are separated into those which investigate the GALT and those which investigate the systemic immune system.

1.4.5.1.1.1 THE GUT-ASSOCIATED LYMPHIOD TISSUE: INNATE IMMUNE RESPONSE

The effect of ß2-1 fructans upon macrophage number and function has been studied, with the results suggesting that number (64) and function (65-67)) are enhanced by the addition of ß2-1 fructans to the diet. However, in chickens supplemented with OF, there was no effect on the proportion of functional phagocytes or percentage of macrophage phenotypes in caecal tonsil (68). MHC II expression was increased on antigen presenting cells (APCs) in the mesenteric lymph nodes (MLNs) of rats upon OF and IN supplementation (65). NK cell activity in the GALT does not seem to be affected by ß2-1 fructan supplementation (69, 70).

Thus, from the animal studies available, it appears that the innate immune system of the gut may be improved by ß2-1 fructan intake, which could result in a beneficial effect on the host's primary response to infection. However, studies measuring NK cell activity did not find any effect upon this component of the innate immune system, which plays a major role in the anti-tumour immunity and destruction of virus infected cells. Future studies should build upon those reported here, to create a more complete picture of how ß2-1 fructans affect the innate immune system.

1.4.5.1.1.2 THE GUT-ASSOCIATED LYMPHOID TISSUE: ADAPTIVE IMMUNE RESPONSE

In healthy and endotoxemic mice supplemented for a short time with FOS, B cell numbers were increased in the PPs (71). There was a decrease in percentage of B cell phenotypes in caecal tonsil of chickens supplemented with OF (68).

Several studies report an increase in intestinal or faecal IgA levels upon ß2-1 fructan supplementation (67, 70, 72-75). As IgA present at the mucosal surface of the gut prevents adherence of pathogens to the gut mucosa, these findings would indicate improved health of the host upon ß2-1 fructan supplementation. However, several other studies do not show an effect of ß2-1 fructan supplementation on intestinal or faecal IgA levels (66-68, 74, 76-79). Thus, there is some disagreement about the effects of ß2-1 fructans on IgA levels in the gastrointestinal tract, although four out of five mouse studies show some enhancement (67, 72, 73, 75) and a single study reports no effect (66). These studies were all conducted in young mice. None of the three studies that were conducted in adult dogs showed an effect upon faecal IgA concentrations (74, 76, 78), but studies regarding ileal IgA concentrations are inconclusive with one showing an effect (74), and another reporting no effect (77). Thus it seems that the animal used and age may be important in determining whether or not ß2-1 fructan supplementation is beneficial on this aspect of immune function. There may be a greater effect in younger animals as their gut immune system is still developing and may therefore be more susceptible to modulation. Other explanations for why there is disparity in the results reported could include a) that faecal IgA may not be an accurate marker of what is happening inside the gut, and b) that the level of IgA that is reported would depend, perhaps, on the site of the gut at which IgA is measured. If ß2-1 fructans enhance the immune system through promotion of the growth of beneficial members of the gut microbiotia, and if a prebiotic, by definition, is specific with respect to the beneficial bacteria it stimulates, then there will be parts of the gut where these beneficial bacteria are most abundant and therefore where the largest effect upon the immune response would be observed. This may partly explain why results reporting IgA at different locations vary.

ß2-1 fructan supplementation has been reported to have effects upon T cell subsets and function, but these effects vary depending upon the anatomical site of origin of the cells, and the animal model used (65, 68-71, 80).

FOS has been reported to increase the response of intraepithelial and MLN T cells to mitogens, but this response was decreased for PP and lamina propria (LP) cells in dogs supplemented with FOS plus fermentable fibres (69), and no effect of Orafti® Synergy1 was seen on MLN or PP lymphocyte proliferation in rats (70). There was a reduction in the

stimulation index of caecal tonsil lymphocytes when stimulated with ConA in chickens supplemented with OF (68).

Alteration of T cell cytokine production has been reported in the GALT with ß2-1 fructan supplementation (70, 72, 81-83). However, other studies have reported no effect (68, 70, 82, 84)). The site of measurement and the cytokines measured seem to be important in determining the effect seen.

Taken together these findings do not present a clear picture of the effects of ß2-1 fructans on T cell numbers in GALT or on T cell responses. It is possible that the effects of prebiotic supplementation upon cell-mediated immunity in the GALT are dependent upon the site of origin of the cells and the animal model used.

1.4.5.1.1.3 THE SYSTEMIC IMMUNE SYSTEM: INNATE IMMUNE RESPONSE

The systemic immune system has been more widely studied in the context of ß2-1 fructan supplementation than the GALT. As observed in the GALT, after OF or IN supplementation, MHCII expression was increased in antigen presenting cells (APCs) in the spleen and thymus of male rats (65) and mean fluorescence intensity of $MHCII^+$ cells in the spleen of mice also increased, although the percentage of $MHCII^+$ cells did not change (67). In the systemic immune system, there seems to be little effect of ß2-1 fructans upon phagocytic function (70, 79), although when given in a synbiotic, whole blood phagocyte activation was increased (79). There is also little effect on phagocyte numbers (74, 77, 85), although neutrophil numbers have been shown to decrease (76).

NK cytotoxicity in the peripheral blood was not altered by FOS supplementation in adult dogs (69), similar to the effects seen in the GALT, although one study showed this function to be non-significantly increased with Orafti® Synergy1 (70). In the spleen, NK cell function appears to be enhanced by ß2-1 fructans (66, 81). Thus, in the spleen, at least, NK cell function may be enhanced by ß2-1 fructan supplementation.

1.4.5.1.1.4 THE SYSTEMIC IMMUNE SYSTEM: ADAPTIVE IMMUNE RESPONSE

In adult dogs, the proportion of B cells in the peripheral blood was decreased when a high fermentable fibre diet including FOS was fed (69).

Several studies measuring the effect of ß2-1 fructans on serum immunoglobulins show no effect. This was observed in mice (42, 67, 72), rats (80) and dogs (74, 76-78, 85), with supplementation of FOS and sc-FOS alone, FOS in combination with MOS, IN alone or IN in combination OF or MOS or GOS. Antibodies measured included total serum immunoglobulins, IgA, IgE, IgG, IgG1, IgG2a, IgM, and vaccine-specific antibodies to

influenza vaccine (total IgG, IgG1 and IgG2a). One study reported a decrease in serum antibody concentrations upon FOS supplementation (72), and a study in ovalbumin sensitized mice showed a non-significant decrease in serum IgE with IN and short chain GOS (86). Three studies report increases in systemic immunoglobulins (67, 68, 75) with ß2-1 fructans. Thus, the results from studies reporting the effect of ß2-1 fructans on systemic immunoglobulins are mixed.

T cell subpopulations may be altered with ß2-1 fructan supplementation, with some studies reporting that ß2-1 fructan supplementation may alter T cell subpopulations in the blood (69), spleen (65, 80, 81) and thymus (65). However, other studies report no effect on these measurements in the blood (70), spleen (66, 67, 70, 80, 87, 88) and thymus (66).

Lymphocyte proliferation in the spleen appears not to be susceptible to modification by ß2-1 fructans (42, 67, 70, 87).

As in the GALT, systemic T cell cytokine production may be altered with ß2-1 fructan supplementation. In the spleen, some alterations in cytokine production have been observed (67, 72, 80) although several studies have reported no effect (67, 70, 81, 87). In the blood, serum and plasma, some alterations in cytokine concentrations have been observed (65, 84, 89) although several studies have reported no effect (84, 89, 90). Thus, the limited number of studies reporting T cell-derived cytokine production in animals receiving ß2-1 fructans suggest that some modification occurs. Why T cell cytokine production should be altered when T cell proliferation is not affected is not clear.

The delayed type hypersensitivity (DTH) response represents the summation of a cell-mediated immune response to an antigenic challenge, largely representing APC and T cell function. Therefore the observation that the DTH response to influenza vaccine was increased when GOS and IN, or GOS/ Raftiline-HP and acidic oligosaccharides, were supplemented to mice (42, 87) supports the findings of improved T cell cytokine production with prebiotics.

1.4.5.1.2 STUDIES IN HUMANS

Twenty one studies that included supplementation with ß2-1 fructans, either alone or in combination with other components, on the human immune system were identified; these have mainly measured aspects of the systemic immune system, via blood immune markers and immune cell responses, and are summarised in Appendix 1 (Lomax and Calder 2009(63)) and Table 5. Six of these studies investigated the effects of ß2-1 fructans alone (43, 91-95) and seven investigated supplements that contained ß2-1

fructans combined with antioxidants, vitamins, minerals, other prebiotics or fats (96-102). Thus, it is difficult to determine whether the effects that were observed were due to ß2-1 fructans, or to another component of the supplement, or to the specific combination. The remaining eight studies investigated synbiotics (103-111)), and are considered separately.

1.4.5.1.2.1 THE INNATE IMMUNE RESPONSE

A decrease in monocyte and granulocyte phagocytosis of *E. coli* was observed in elderly nursing home residents supplemented with short-chain FOS (43). This is in contrast to the effect observed in senior dogs and adult rats, where no modification of blood monocyte concentrations or phagocytosis was seen with ß2-1 fructans (70, 85) and to the findings of Seidel et al. (2007(98)) of no effect on phagocytosis of *E. coli* by granulocytes taken from young adult males consuming bread containing IN.

No effect of OF (43) or IN (98)) has been observed on NK cell numbers in human blood. There have been no reports of the effect of a ß2-1 fructan only supplement on human NK cell activity.

1.4.5.1.2.2 THE ADAPTIVE IMMUNE RESPONSE

There is consistency in the findings from human studies regarding an increase in B cell numbers following ß2-1 fructan supplementation (97, 98), but this is in contrast to observations in adult dogs, where B cell numbers in the blood were decreased (69).

A study in senior adults (92) found no effect of ß2-1 fructans upon concentrations of various classes of serum immunoglobulin, which is in agreement with several studies in laboratory animals which show no effect of ß2-1 fructans on these (42, 67, 72, 74, 76-78, 80, 85). However, a study in children found a decrease in total IgE, IgG1, IgG2 and IgG3 levels in plasma, but no effect on total plasma IgG4 levels (101)) Two studies in newborn infants supplemented with a mixture of GOS and IN in their formula showed an increase in faecal sIgA levels (99, 102), which fits with the findings from animal studies which show an enhancement of the antibody response in the GALT after ß2-1 fructan supplementation (67, 70, 72-75). No effect of Prebio1® (a mixture of OF and IN) was observed upon salivary sIgA levels in seniors (92).

The antibody response to vaccination is considered to be the gold standard for measuring the functioning of the immune system in vivo, based on its biological relevance, sensitivity and practical feasibility (10). Thus several human studies use this marker, but these have generated mixed results. A study in eight month old infants supplemented with Prebio1® reported an increase in post vaccination anti-measles IgG levels in the blood (91), but in Peruvian infants given OF-enriched cereal, and immunized against influenza,

there was no effect of OF upon post-vaccination antibody titres to *Haemophilus influenzae* type B (93). In healthy, free-living elderly adults, Prebio1® increased the antibody response to influenza B virus and *Streptococcus pneumoniae* after vaccination, yet this was also seen in the control group, and there was no effect of the supplementation upon antibody titres against influenza A virus (92). In elderly adults, FOS supplementation increased the number subjects with a four-fold or greater increase in serum antibody titre, and an antibody titre of 40 or more, to the A/Beijing component of the influenza vaccine six weeks after the vaccine was administered, but this was not seen to the other components of the vaccine (96). In elderly adults resident in long-term care facilities and vaccinated with the seasonal influenza vaccine, FOS supplementation did not alter the geometric mean antibody titre, but did increase the number of subjects with an antibody titre greater than 100 to the H1N1 component six weeks after the vaccine was given (97). Taken together, these studies suggest that ß2-1 fructans may increase the response to some vaccines or vaccine components but not all. This conclusion is consistent with that from animal studies (42), (67).

Some studies have reported alterations in various blood T cell populations upon ß2-1 fructan supplementation (43, 94, 97, 98), whilst no effects were observed on other populations (43, 97, 98). This mixed picture of effects of ß2-1 fructans on blood lymphocyte subsets is similar to that seen in laboratory animals (69, 70).

Lymphocyte proliferation to influenza vaccine components was increased in elderly adults supplemented with FOS (96), but in another study in elderly adults, there was no effect of Prebio1® upon vaccine stimulated lymphocyte proliferation (92).

Regarding cytokine expression, in elderly nursing home residents FOS supplementation decreased IL-6 mRNA expression in blood mononuclear cells (43), and in elderly adults resident in long-term care facilities, IL-6 production by stimulated blood mononuclear cells was decreased (97). IL-6 specific mRNA was decreased in blood mononuclear cells in elderly adults supplemented with FOS, although there was no effect on plasma sIL-6R levels (94). As an increase in IL-6 is associated with the pro-inflammatory state associated with aging (112), this could be considered a beneficial effect. In healthy free-living elderly adults, there was no effect of IN and OF upon IFN-γ and IL-4 secretion by cultured mononuclear cells (92). This is in contrast to a study in rats that showed an increase in blood IL-4 concentration (65). In patients with mild to moderate ulcerative colitis, Synergy1 supplementation had no effect on IL-8 levels in rectal dialysis fluid (95). A trend for a reduced IL-10 production from stimulated blood mononuclear cells was observed upon FOS supplementation in the elderly (97). TNF-α specific mRNA was decreased in blood mononuclear cells in elderly adults supplemented with FOS, but plasma TNF-α

levels were not affected and neither were plasma IL-2R levels, or IL-2, IL-1α or iNOS specific mRNA in blood mononuclear cells (94).

1.4.5.1.2.3 SYNBIOTICS AND THE IMMUNE SYSTEM IN HUMANS

In a study of adult colon cancer or polypectomized patients, Orafti® Synergy1 was given in combination with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12. The synbiotic prevented the increase in IL-2 secretion by mononuclear cells from polypectomized patients that was seen in control patients, and also increased IFN-γ production by mononuclear cells in colon cancer patients (104, 105). However, the synbiotic had no effect on several other immune markers in either cancer or polypectomized patients, including percentages of phagocytically active neutrophils and monocytes and their phagocytic intensity, percentage of neutrophils producing reactive oxygen species and the intensity of production, lytic-activity of NK cells or production of IL-10, IL-12 and TNF-α by activated blood mononuclear cells (105). In patients with active ulcerative colitis, supplementation with a synbiotic containing Orafti® Synergy1 reduced IL-1α and TNF-α mRNA levels in mucosal tissue, but had no effect on IL-10 mRNA levels (108). In patients undergoing elective abdominal surgery (107) or elective colorectal surgery (111), synbiotics containing OF had no effect on serum IL-6 levels. In adult multiple trauma victims in surgical intensive care units, supplementation with inulin plus other fibres and probiotics decreased serum IL-6 and TNF-α levels (109, 110). IN and OF in combination with *Lactobacillus paracasei* prevented the decrease in IL-2 production by mononuclear cells seen in controls after vaccination, and there was no effect upon TNF-α production by stimulated mononuclear cells before vaccination (103). Other findings from this study include an increase in NK activity and no effect on IFN-γ production with influenza virus antigen stimulation. There was no effect on IL-6 or IL-1 production by stimulated blood mononuclear cells, or on lymphocyte subpopulations (103). The decrease in numbers of T cells with NK activity that was seen in the control group was prevented with the supplementation (103)). After the vaccinations were given there was no effect of the synbiotic on the magnitude of the increase in anti-influenza vaccine or anti-pneumococcus vaccine antibodies, or on the DTH response (103). In children supplemented with IN and OF plus *L. acidophilus* and *L. casei*, there was no effect upon serum IgA, IgG IgE or IgM levels, or serum vaccine-specific antibody levels (106).

1.4.5.2 β2-1 FRUCTANS AND INFECTION

This section reviews studies in experimental animals and in humans that investigate the effects of increased consumption of ß2-1 fructans on infectious outcomes.

1.4.5.2.1 STUDIES IN LABORATORY ANIMALS

22 animal studies of infection were identified (four of which used synbiotics), and ß2-1 fructan supplementation generally appears to be beneficial in the models used. These studies are summarised in Appendix 1 (Lomax and Calder 2009a (63)) and Table 5.

A series of studies using piglets infected with *Oesophagostomum dentatum* or *Trichuris suis*, showed decreases in *Oesophagostomum dentatum* and *Trichuris suis* faecal egg counts, intestinal worm recovery, size of worms, and the female worm's ability to reproduce after IN supplementation (113-118). In models of *Salmonella typhimurium* infection, ß2-1 fructan supplementation appears to be beneficial (84, 119-123). ß 2-1 fructan supplementation increased survival in a hamster model of *Clostridium difficile* infection (124) and in murine models of *Listeria monocytogenes* (120). FOS decreased diarrhoea and increased survival rates in piglets infected with *E. coli* (125). These studies provide a consistent picture that ß2-1 fructans do improve host resistance to bacterial infections.

In contrast to the studies described above, a series of studies investigating OF or IN supplementation in calcium-deficient rats suggest increased *Salmonella typhimurium* colonisation and translocation and increased mucosal irritation (126-129). These findings may be explained by the calcium deficient state of the rats used, since a direct comparison of OF in rats fed calcium deficient and calcium sufficient diets showed different effects (128). While the calcium deficient animals displayed increased susceptibility to *S. typhimurium*, calcium sufficient animals did not. Thus, the relevance of the findings to animals or humans that are not calcium deficient is limited. However, a study of GOS and IN supplementation to newborn rats reported increased incidence of anaerobic and aerobic bacterial translocation towards the spleen, although several other measures of bacterial translocation were not altered (130), and a study in *S. typhimurium* infected mice fed IN or FOS found some indications that these supplements were not beneficial (88).

Four studies have investigated the use of synbiotics in animal models of infection. A study in mouse pups infected with rhesus rotavirus, demonstrated that OF in combination with *Bifidobacterium bifidum* and *Bifidobacterium infantis* reduced the duration of diarrhoea, although the synbiotic was no more effective than the probiotic alone (131). Rotavirus infects the enterocytes of the small intestine, but prebiotics and probiotics have their effects mainly in the large intestine. Thus, although improving the health of the large intestine is likely to be useful in diarrhoea, prebiotics may not be more helpful than a probiotic alone because of limited effects in the small intestine. Although piglets infected with *Salmonella typhimurium* were shown to have decreased shedding of *Salmonella*

typhimurium in faeces when supplemented with FOS, FOS given as part of a synbiotic had no effect on *Salmonella typhimurium* infection (121). In mice infected with *Salmonella*, IN given alone, in combination with *L. acidophilus* or *L. acidophilus* on its own all appeared to protect mice against salmonella induced liver damage, but the probiotic was the most effective, and there did not appear to be any synergistic effect between the prebiotic and the probiotic (123). In rats infected with *Toxoplasma gondii*, FOS plus *B. animalis* improved survival rates (90).

1.4.5.2.2 STUDIES IN HUMANS

1.4.5.2.2.1 INFANTS AND CHILDREN

Eleven studies have investigated the effect of FOS upon infectious outcomes in humans. Several studies have shown some benefit from ß2-1 fructans on common childhood diarrhoea in reducing severity (132, 133), and number of episodes (134), although some studies have shown no effect on the occurrence or prevalence of diarrhoea (93, 132, 133) or duration of diarrhoea (132, 133, 135). Duration (136) and incidence (137) of acute diarrhoea have both been reduced with ß2-1 fructan supplementation. Incidence of upper respiratory tract infections was shown to be non-significantly reduced, and antibiotic use was decreased (137). A study in healthy infants supplemented with short chain- GOS (sc-GOS) and IN for the first six months of life found benefits regarding different types of infections, including respiratory tract infections at six months and two years of age (138, 139)). Intestinal permeability has been shown to improve in one study with β2-1 fructans (140), but not in another (141). A study of preterm infants supplemented with a prebiotic mixture containing IN reported a trend for lower incidence and risk of infectious morbidity (142).

An additional four studies have investigated the effect of synbiotic supplements containing ß2-1 fructans on infectious outcomes in children, and have found improvements in *Helicobacter pylori* colonized children (143), and a non-significant trend for a reduction in respiratory tract infections (144). However in severely malnourished children, supplementation with a synbiotic containing IN seemed to increase the time with severe diarrhoea and vomiting in hospital, but tended to reduce the severity of diarrhoea once children were discharged (145). A synbiotic supplement containing IN and OF did not alter number of episodes of infection, or days of fever in children (106).

Taken together data from studies using ß2-1 fructans in infants and children are mixed, but are suggestive of a reduction in incidence, duration or severity of some infections.

1.4.5.2.2.2 ADULTS

Eight studies were identified which investigated the effect on ß2-1 fructans upon infectious outcomes in adults. Two studies showed a benefit regarding a decrease in relapse rate of in-patients with *Clostridium difficile* associated diarrhoea (146), and decreased upper respiratory tract infections in older adults post-influenza vaccine (96). Other studies report no benefit regarding antibiotic-associated diarrhoea in in-patients receiving broad-spectrum antibiotics (147), severity or duration of infections in burns patients (148), or travellers' diarrhoea in people holidaying to areas of medium/high risk of diarrhoea, although there was a non-significant decrease in diarrhoea and an increase in feelings of well-being (149).

In healthy adults, OF appears to have no effect upon faecal mucin excretion (150), and in men consuming a diet with limited calcium, intestinal permeability did not differ between the control and OF supplement periods, although faecal mucin excretion was increased with the addition of OF to the diet (151). In patients on an enteral diet, IN had no effect upon intestinal permeability (152).

Ten trials have been conducted to investigate the effect on synbiotics upon infection in adults, and most of these trials have been carried out in patients admitted to intensive care or surgery wards. Synbiotics containing ß2-1 fructans appear to exert some beneficial effects on infections in these patients (109-111, 153, 154). In studies where synbiotics containing IN have been compared with IN alone, synbiotics have been shown to be more beneficial in terms of reducing bacterial infections and length of antibiotic therapy in patients undergoing duodenal surgery (155) or liver transplantation (156). However, some of these studies report no effects of the synbiotics on other outcomes measured (110, 111, 153), and no benefits of synbiotics containing ß2-1 fructans have been observed in other studies (107, 157). Synbiotics containing ß2-1 fructans have produced several benefits on respiratory infections in healthy adults (158), and a study in healthy elderly adults found reduced infections in the synbiotic group following vaccination (103).

Overall, studies of ß2-1 fructans in adults are less convincing of a benefit with respect to infections compared with studies in infants and children. However it appears that ß2-1 fructans are useful in this regard in some situations (96, 146) and perhaps as a component of a synbiotic.

1.4.5.3 ß2-1 FRUCTANS IN INFLAMMATORY CONDITIONS

This section reviews studies in experimental animals and in humans that investigate the effects of increased consumption of ß2-1 fructans on inflammatory diseases.

1.4.5.3.1 STUDIES IN LABORATORY ANIMALS

14 studies were identified (two using synbiotics), with mostly consistent results, showing positive effects of FOS on inflammation in animal models. These results are summarised in Appendix 1 (Lomax and Calder 2009a (63)) and Table 5.

Considering the reported effects of ß2-1 fructans on T cell numbers and function in animal models described previously (65, 68-72, 80, 82, 83) it is not surprising that they are effective in colitis: six out of seven rodent studies of colitis report a decrease in inflammatory markers and the severity of disease when animals were supplemented with ß2-1 fructans (40, 44, 83, 159-161). Just one study showed no effect of sc-FOS on macroscopic or histological scores in a colitis model in male rats (162). ß2-1 fructans given in a synbiotic have also been shown to have beneficial effects in rat models of colitis (45, 163).

Neonatal necrotising enterocolitis may be in part caused by interactions between intestinal immaturity, inappropriate bacterial colonisation and infections (164). Thus, from the known beneficial microbiotia-stimulating effects, and previously described effects of ß2-1 fructans on the immune system, it is possible that ß2-1 fructans could be of benefit in this disease. Indeed, in a quail model of neonatal necrotising enterocolitis, OF decreased the occurrence and severity of intestinal lesions, although the clostridial species that was used to induce the colitis had an effect on the magnitude of this effect (41, 165).

In a rat model of allergic airway eosinophilia there was no benefit of FOS (166). The lack of effect of FOS on airways inflammation may be due to the distance of this compartment from the gastrointestinal tract. However, in mice sensitized to ovalbumin, short-chain GOS and IN decreased airway hyper-responsiveness, reduced the number of inflammatory cells in broncho-alveolar lavage fluid, and non-significantly decreased serum IgE levels (86). In an ovalbumin sensitized rat model of food allergy, some immunological markers were altered in the duodenum with FOS supplementation (167).

In pathogen free rats, a supplement of IN, sc-FOS and omega-3 fatty acids seemed to protect the rats against systemic inflammatory response induced by LPS (89).

Thus, animal studies provide strong evidence of a protective effect of ß2-1 fructans on colitis and necrotising enterocolitis. The consistent findings may relate to the action of prebiotics directly at the site of pathology. Effects of prebiotics on inflammatory processes distant from the intestinal tract (e.g. the lung) may be smaller in magnitude.

1.4.5.3.2 STUDIES IN HUMANS

Thirteen studies of ß2-1 fructans in human inflammatory conditions were identified (four where synbiotics were used), of which eleven were conducted in adults. The results are summarised in Appendix 1 (Lomax and Calder 2009a (63)) and Table 5.

In accordance with findings from animal experiments, ß2-1 fructan supplementation was shown to be beneficial in ulcerative colitis patients (95, 168). In patients with ileal pouch-anal anastomosis, IN did not produce any effects on clinical symptom scores, but there were reductions in total endoscopic and histological scores, mucous exudates and total Pouchitis Disease Activity Index (169). In patients with ileo-colonic Crohn's Disease, Prebio1® supplementation improved several outcomes (170).

Trials in Irritable Bowel Syndrome (IBS) do not report beneficial effects of ß2-1 fructans on symptom scores (171, 172), perhaps due to the nature of the disease regarding the relapse and remission pattern. However, a study in which patients with minor functional bowel disorders were supplemented with sc-FOS showed improvements in some symptoms (173).

In contrast to animal work which suggests that there is no benefit of ß2-1 fructans on allergic airway eosinophilia (166), two studies in infants at risk from atopy found a reduction in the development of atopic dermatitis (101) and in cumulative incidence of atopic dermatitis, recurrent wheezing and allergic urticaria at two years of age (138, 139). Several reasons could be given to explain this inconsistency in results. In the human studies, the composition of the formulas containing ß2-1 fructans and GOS was designed to closely resemble the composition of oligosaccharides of the mothers' milk, but as this was not the case in the animal study it may be that the amount of prebiotic given was not appropriate. Also, the rats were at a later stage of development than the infants in the human study were, and so as their immune systems would have been more developed, the prebiotics may have had less of an effect upon their immune system. Finally, the infants were at risk from allergy because of parental allergy (i.e. genetics was most likely an important factor), while in the animal model, the allergy was induced in the affected animals.

Synbiotic therapy for inflammatory bowel diseases produces mixed results. Orafti® Synergy1 in combination with *Bifidobacterium longum* improved markers of inflammation in patients with active ulcerative colitis (108). IN given in combination with other fermentable fibres and four lactic acid bacteria had no effect on relapse rates (either endoscopic or clinical) in Crohn's Disease patients undergoing resection (174). In a study of patients with acute pancreatitis, a synbiotic supplement containing IN and other fibres

was found to be more beneficial than when IN and other fibres were given alone regarding outcomes such as systemic inflammatory response and multi-organ failure (175). Regarding IBS, a synbiotic containing IN resulted in significant reductions in abdominal pain and distension, diarrhoea and constipation (176).

Table 5 : Studies investigating the effect of ß2-1 fructans upon immune function, infection and inflammatory conditions in laboratory animals and humans, published since Lomax and Calder 2009a (February 2008) and up to June 2010

Reference	Prebiotic(s) used	Animal studied (gender and age where given)	Findings
Milo (2004) (84)	FOS (7.5 g/L) or soy polysaccharides (SPS)	Piglets (newborn); infected with *S. typhimurium* after 7 d	• Animals consuming the control diet and infected with *S. typhimurium* had ↓ small intestinal lymphocyte migration in the T84 monolayer compared to animals consuming the control diet who were not infected, suggesting that the infection ↓ the ability of the small intestinal lymphocytes to migrate. However in animals consuming FOS or SPS, infection did not ↓ small intestinal lymphocyte migration, suggesting a protective effect of the fermentable fibres • ↑ Migration of neutrophils in SPS group compared to FOS or controls • ↓ IL-6 concentrations 2 hours after LPS challenge in plasma in FOS group compared to SPS or controls No effect on: • Pro-inflammatory cytokine (TNF-α, IL-1ß or IL-6) mRNA abundance in jejunum or ileum • IL-6 concentration at baseline, 6, 12 or 24 hours after addition of LPS to plasma in either group
Seidner (2005)(168)	FOS (approx 6.7 g/d for 6 m) in a nutritional formula which also contained proteins, fats, carbohydrates, fish oils (EPA and DHA), gum arabic, and antioxidant vitamins and minerals	Adults with active ulcerative colitis; n = 36 in test group, mean age = 45.5 y; n = 50 in placebo group, mean age = 42.7 y	• ↓ Dose of prednisone required to control clinical symptoms in the test group versus controls • Clinical improvements in both groups, (DAI, modified Baron's scale and total histology index scoring) with no difference between groups
Lara-Villoslada (2006) (160)	sc-FOS (5% of diet)	Rats (female); colitis induced by TNBS	• ↓ Impact of TNBS-induced colonic damage, with ↓ percentage of animals suffering from diarrhoea • ↓ Colonic damage score, with ↓ extent of colonic necrosis and/or inflammation, ↓ in colonic weight/length ratio (an index of colonic edema)

			• ↑ Recovery in intestinal architecture (↓ microscopic damage score) • ↓ Macroscopic damage score • ↓ MPO activity • Restoration in colonic glutathione levels • ↓ LTB4 • ↓ Colonic iNOS expression
Arslanoglu (2007)(138) and (2008 - two year follow-up)(139)	scGOS/ lcFOS (8g/l in a hypoallergenic formula for the first 6 m of life)	Healthy term infants with a parental history of atopic eczema, allergic rhinitis or asthma; n = 102 in test group; n = 104 in placebo group; (n = 66 in experimental group and n = 68 in placebo group completed the two year follow-up)	**During the first 6 m of life the test group had:** • ↓ Number of episodes of all types of infections combined • ↓ Cumulative incidence of having at least one episode of any infection, any recurrent infection, or recurring upper respiratory tract infection • ↓ Incidence of infections at 4 - 6 m • Trend for ↓ in number of upper respiratory tract infectious episodes and in number of infections requiring antibiotic treatment No significant effect on: • Number of episodes of otitis media, gastrointestinal infections of urinary tract infections • Cumulative incidence of recurrent otitis media or urinary tract infection • Incidence of infections at < 2 m, or 2 - 4 m **At the two year follow-up, the test group had:** • ↓ Number of overall infections (assessed by both physician-diagnosed infections and number of fever episodes) • ↓ Infections requiring antibiotic prescriptions • ↓ Episodes of upper respiratory tract infections • Trend for ↓ in urinary tract infections • No effect on episodes of lower respiratory tract infections, otitis media or gastrointestinal infections • ↓ Cumulative incidences of atopic dermatitis, recurrent wheezing and allergic urticaria

Colomé 2007(141)	GOS/FOS in infant formula (dose not given, duration not given)	Healthy infants, mean age = 72.5 d; Group 1 (breast fed), n = 11; Group 2 (prebiotic supplemented formula), n = 17; Group 3 (nucleotide supplemented formula), n = 9; Group 4 (lc-PUFA supplemented formula), n = 9; Group 5 (lc-PUFA and nucleotide supplemented formula), n = 11	No effect on intestinal permeability between the groups (as measured by lactulose/ mannitol ratio), although infants fed with a probiotic supplemented formula expressed higher lactulose/ creatinine and mannitol/ creatinine ratios (this could reflect changes in the paracellular area where lactulose permeates the intestinal mucosa)
Middelbos (2007)(77)	sc-FOS (0.9%, 1.2% or 1.5% of diet)	Dogs (female, adult)	No effect on: • Ileal IgA concentrations • Serum IgA, IgG or IgM concentrations • Counts of total white cells, neutrophils, lymphocytes, monocytes or esoinophils in blood
Pié (2007)(82)	IN (0.75% of diet) diet also contained other the fermentable carbohydrates sugarbeet pulp, lactulose and wheat starch)	Piglets (4 w old, weaned)	In the colon: • In the colon, ↑ IL-6 mRNA levels at d 4 post-weaning in test group, but not at d 10 • ↑ IL-1ß mRNA levels in both test and control groups at 4 d post-weaning, and returned to normal at d 10 • In the colon, ↓ TNF-α, IL-12p40 and IL-18 mRNA levels at d 10 in both test and control groups • No effect in either test or control groups in colonic IL-8 mRNA levels In the ileum: • ↑ IL-1ß mRNA levels on d 4 test and control group but not at d 10 • No effect on mRNA levels of IL-6, TNF-α, IL-12p40, IL-8 or IL-18

Schiffrin (2007)(94)	FOS (1.95 - 3.9g /d for 12 w, given in a nutritional supplement)	Elderly adults (aged < 70 y); 75 % female; community dwelling and nursing home residents; malnourished or at risk of malnutrition; n = 37 in test group; n = 37 in control group; mean age = 84 y	• ↓ IL-6 and TNF-α specific mRNA in peripheral blood mononuclear cells in test group • ↓ Serum sCD14 in test group • Tendency for ↑ in total lymphocyte counts and all subsets (CD3+, CD4+, CD8+, CD19+, CD16+CD56+ and CD4/CD8 ratio) No effect on: • Erythrocyte or white blood cell counts • Levels of circulating cytokines (IL-2R, TNF-α, sIL-6R) in plasma • iNOS, IL-2 or IL-1α specific mRNA in peripheral blood mononuclear cells
Spindler- Vesel (2007)(154)	IN (2.5 g/sachet, number of sachets / d not specified, duration not given) plus ß-glucan, pectin, resistant starch and probiotics	Patients with multiple injuries admitted to intensive care receiving enteral nutrition; n = 32 in Group A (glutamine), median age = 31 y; n = 29 in Group B (fermentable fibre; guar gum), median age = 36 y; n = 26 in Group C (peptide diet), median age = 41 y; n = 26 in Group D (synbiotic diet containing IN), median age = 48 y	• No effect on numbers of infections or rate of pneumonia between groups, but when Group D was compared with Groups A - C combined, rate of pneumonia and cumulative infection rates were decreased • ↑ Injury Severity Score, Acute Physiological Chronic Health Evaluation II scores, and gastric retention volume than in Group D compared to Group A • ↓ Procalcitonin value (blood levels rise in severe infections), and lactulose/ mannitol index (measure of intestinal permeability) in Group D versus group A or group C • Intestinal permeability ↑ in all groups until day 7, except group D where lactulose/ mannitol index ↓ on d 7 • No effect on mortality rates
Vos (2007a)(87)	GOS/Raftiline-HP® (9:1 ratio) at 2% of diet and given in different ratios to AOS (group 1 = 100% GOS/ Raftiline-HP®; group 2 = 100 % AOS; group 3 = GOS/Raftiline-HP®:AOS in	Mice (female, 6 w old); vaccinated with influenza vaccine; DTH response to influenza vaccine	• ↑ DTH response in AOS and GOS/Raftiline-HP groups, and this was ↑ in groups 3 and 4 who took the combined supplements, suggesting the supplements may interact No effect on: • Splenocyte proliferation • Spelenocyte cytokine production (IFN-γ, IL-2, IL-4, IL-5 or IL-10)

	a 2:3 ratio; group 4 = GOS/Raftiline-HP®:AOS in a 6:1 ratio)		• Splenocyte subpopulations: CD3+CD4+, CD3+CD8+, CD3+CD4+CD25+, CD3+CD4+CD25-, CD19+, NK1.1+ (only measured in control group and group 3)
Vos (2007)b(86)	sc-GOS and Raftiline-HP (1% of diet in a 9:1 ratio, or 1% of diet in a 8.3:1.7 ratio plus the rest as pectin derived AOS)	Mice (male, 5 - 8 w old); sensitized to ovalbumin	Both diets: • Non-significantly ↓ average IgE titres in serum • ↓ Airway hyperresponsiveness in the sensitized mice • ↓ Number of inflammatory cells in the broncho-alveolar lavage fluid, although airway inflammation was still present
Barrat (2008)(130)	IN (0.7g/L) plus GOS	Rats (male, newborn)	• ↑ Incidence of bacterial translocation (of both anaerobes and aerobes) towards spleen (at d 18, i.e after supplementation, but this effect did not persist at d 40, i.e. after weaning to solid foods not containing the supplement) No effect on: • Number of anaerobic and aerobic bacteria found in spleen • Incidence and intensity of bacterial translocation for lactic acid producing bacteria and enterobacteria • Colonic permeability • Expression of tight junction claudin-2 or claudin-3 mRNA, but ↓ ZO-1 mRNA expression
Cerezuela (2008)(177)	IN (0.5% or 1% of diet)	Gilthead seabream	No effect on: • Serum peroxidise • Complement activity • Phagocytic ability • Leucocyte peroxidise • ↓ Phagocytic activity of leucocytes in 1% inulin group • ↓ Respiratory burst of leucocytes in 0.5% inulin group

Pregliasco (2008)(158)	sc-FOS (given in a synbiotic, for 90 d over the winter season); group A consumed 3 g/d sc-FOS plus *L. plantarum, L. rhamnosus* and *B. lactis*; Group B consumed the same as A plus lactoferrin; Group C consumed 2.5 g / d of GOS plus 2 strains of *L. plantarum*, 2 strains of *L. rhamnosus* and *B. lactis*; Group D consumed 3 g / d of sc-FOS plus 2 strains of *L. plantarum*, 2 strains of *L rhamnosus* and *B. lactis*	Healthy volunteers; n = 721 altogether over 3 winter seasons; in Trial 1, n = 122 in Group A, mean age = 39.4 y; n = 115 in placebo group, mean age = 34.8 y; in Trial 2, n = 79 in Group A, mean age = 38.5 y; n = 79 in Group B, mean age = 36.9 y; n = 76 in placebo group, mean age = 38.1 y; in Trial 3, n = 84 in Group C, mean age = 42 y; n = 84 in Group D, mean age = 45 y; n = 82 in placebo group, mean age = 42 y	Trial 1 (Group A versus placebo): • Improved bowel functions • ↓ Overall number of acute respiratory tract infection episodes • ↓ Number of flu episodes • ↓ Overall number of days of illness • ↓ Number of days of illness/ individual subject • ↓ Number of overall acute respiratory infection episodes • ↓ Perceived severity for acute respiratory tract infectious episodes, upper respiratory tract infection episodes and for influenza like illness • ↓ Average length of overall acute respiratory infection episodes and in upper respiratory tract infection episodes • No significant effect on length of cold or influenza like illness infection episodes No effect on: • Number of cold or upper respiratory tract infection episodes • Perceived severity of colds Trial 2 (Group A versus Group B versus placebo): • Significantly improved bowel functions in Group A, and non- significantly improved in Group B • ↓ Overall number of days if illness and number of days of respiratory tract illnesses/ individual subject in groups A and B • ↓ Of overall number of episodes and number of overall acute respiratory infections per person • ↓ Total number of acute respiratory infections in both groups • ↓ Number of flu episodes in Group A • Non – significant ↓ in number of flu episodes in Group B

			• ↓ Average length of all acute respiratory infection events in both groups • ↓ Average length of upper respiratory tract infections and influenza like illness in Group A • ↓ Severity of total acute respiratory infections and influenza like illness in both groups No significant effect on: • Use of drug therapies • Severity of cold or upper respiratory tract infection episodes • Length of cold episodes in Group A or length of cold, upper respiratory tract infections or influenza like illness in Group B • Number of cold or upper respiratory tract infections in either group Trial 3 (Group C versus Group D versus placebo): • Improved bowel functions in Groups C and D • ↓ Number of overall acute respiratory infection episodes and flu episodes in both groups • ↓ Number of cold episodes in Group C • ↓ Severity of total acute respiratory infection episodes, cold episodes and flu episodes in both groups • ↓ Severity of cough episodes in Group D • ↓ Length of total acute respiratory infection episodes, cold episodes and cough episodes in both groups No significant effect on: • Use of drugs • Length of flu episodes in either group • Severity of cough episodes in Group C • Number of cold episodes in Group D or cough episodes in either group

Scholtens (2008)(102)	scGOS/lcFOS (6 g/l at a ratio of 9:1 in a milk formula, for the first 6 m of life)	Healthy term infants; n = 75 in scGOS/lcFOS milk group; n = 81 in placebo milk group; n = 31 in breast-fed group	• ↑ Faecal sIgA in test milk group compared to placebo milk group (the results from the subgroup of infants who were exclusively formula fed from birth were comparable to these results which considered the whole group, which included infants who may also have been breast-fed for some time)
Bruzzese (2009)(137)	GOS/ FOS (9:1 ratio, at a concentration of 0.4 g / 100 ml) in infant formula, (for 12 m)	Healthy term infants, aged between 15 - 120 d at start; had begun formula feeding before starting the study; n = 169 in test group; n = 173 in control group	• ↓ Rate of diarrhoeal episodes / child • ↓ Number of children with at least one episode of acute diarrhoea • Non-significant ↓ in number of episodes of upper respiratory tract infections (URTI) • No effect on number of children with at least one episode of URTI • Among children with at least one episode of URTI, the number of children with recurrent URTI (more than three episodes in 12 m) was non-significantly ↓ • ↓ Mean rate of antibiotic courses prescribed • ↓ % of children receiving two or more antibiotic courses / y
Frece (2009)(75)	IN (1 % of diet) plus *L. helveticus* M92 Also looked at synbiotics containing lactulose or raffinose instead of IN	Mice (4 m old, female, Swiss albino)	• ↑ Faecal sIgA in all groups (probiotic alone, probiotics alone, and all synbiotics, but was the highest levels were seen in the probiotics plus IN group) • ↑ Total serum IgA (probiotic alone and all synbiotics, but was the highest levels were seen in the probiotics plus IN group)
Janardhana (2009)(68)	OF (0.5% of diet)	Chickens (1 d old, male)	• ↓ Stimulation index of caecal tonsil lymphocytes when stimulated with ConA • ↓ % of B cell phenotypes in caecal tonsil • ↑ Plasma IgG and IgM titres No effect on: • Frequency of IgA-positive cells at the mucosal surfaces (duodenal) • mRNA expression of IFN-γ, IL-6 or IL-10 in caecal tonsil

			• Plasma IgA titres • % of IgM, IgG or IgA positive cells in caecal tonsil • % of T cell or macrophage phenotypes in caecal tonsil • Proportion of functional phagocytes in caecal tonsil
Kerac (2009)(145)	IN (approx. 2.5 g / d) given in Synbiotic 2000 Forte, which contained *Pediococcus pentosaceus, Leuconostoc mesenteriodes, L. paracasei* ssp *paracasei* and *L. plantarum*, (at 1 x 10^{10}/d in total) plus oat bran, pectin and resistant starch	Children in Malawi; age 5 – 168 m (median age = 22 m); with severe acute malnutrition;	• ↑ Time with severe diarrhoea and vomiting as inpatients in test group • Trend for less severe diarrhoea in outpatients in test group No effect on: • Use of non-routine outpatients medication (including antibiotics) • Rate of sepsis
Knight (2009)(157)	IN (5 g / d) given in Synbiotic 2000 Forte, which contained *Pediococcus pentosaceus, Leuconostoc mesenteriodes, L. paracasei* ssp *paracasei* and *L. plantarum* (at 2 x 10^{10} bacteria/d in total) plus betaglucan, pectin and resistant starch (until the earliest of either: 28 d after admission, death, or discharge from ICU)	Critically ill patients admitted to ICU, requiring mechanical ventilation for at least 48 h; n = 130 in test group, mean age = 49.5 y; n = 129 in control group, mean age = 50.0 y	No effect on: • Incidence of ventilator associated pneumonia • Median number of ventilator days • Ventilator associated pneumonia episodes per 1000 ventilator days • Length of ICU or hospital stay • Hospital or ICU mortality rates • Antibiotic administration

Oz (2009)(89)	IN and sc-FOS (0.52% of diet, for 6 d before LPS administration), diet also included EPA-DHA	Rats (male, specific pathogen-free, 4-6 w old); systemic inflammatory syndrome induced by LPS	• Rats on the EPA-DHA-FOS diet were protected against LPS-induced systemic inflammatory response; lost less weight, ↓ alanine aminotransferase (marker of hepatocyte injury) concentrations, protected against low hematocrit and hepatic pathology, ↑ blood IL-10 levels, less prominent ↓ in intrahepatic-reduced GSH • No effect on release of inflammatory cytokines (TNF-α, IFN-γ or IL-6) in blood
Petersen (2009)(88)	IN (10%) or FOS (10%)	Mice (BALB/c); infected with *S. typhimurium* SL1344	• ↑ Numbers of *S. typhimurium* in liver, spleen and mesenteric lymph nodes of FOS group compared to control diet • ↑ Serum haptoglobin (a good marker for translocation of *S. typhimurium*) in FOS group • Non-significant ↑ in faecal and ileal counts of *S. typhimurium* in FOS group • No effect of inulin on *S. typhimurium* counts in liver, spleen, mesenteric lymph nodes, ileum or faeces • No effect on spleen immune cell percentages (CD4+, CD8+, NK, NKT, B or dendritic cells) and % of neutrophils was ↑ in infected animals compared to non-infected animals
Rishi (2009)(123)	IN (2 mg/d) alone, in combination with *L. acidophlus* or *L. acidophilus* alone	Mice (BALB/c); infected with *Salmonella enterica*	• ↓ Bacterial load in liver • ↓ Serum aspartate aminotransferase and alainine aminotransferase (liver damage markers) levels before animals were challenged with Salmonella • ↓ Histological damage in liver • ↓ Levels of oxidants (malondialdehyde levels) in liver • ↓ Levels of nitrite in liver • ↑ Levels of antioxidants (superoxide dismutase and reduced glutathione) in liver • These findings were most marked in the probiotic group, and there was no synergistic effect of the probiotic and prebiotic observed. Experiments were also carried out using FOS instead of IN, no synergistic effect was seen here either
Ryz (2009)(80)	IN (5% of diet)	Rats (female, 3 weeks old)	• In the spleen, total cell number was ↓, proportion of TCRαβ+CD4+ cells were ↑, proportion and numbers of TCRαβ+CD8+ cells were ↓, numbers of TCRγδ+ cells were ↓, and there was no effect on

			TCRαβ+, TCRγδ+ -CD4+, TCRγδ+CD8+ or OX62+TCRγδ- cell numbers or proportions • In the mesenteric lymph nodes, total cell numbers were ↓, proportion and number of TCRαβ+CD8+ cells were ↓, number of CD45RA+ were ↓, and there were no differences in TCRγδ+CD4+ or OX62+TCRγδ- cell numbers or proportions, or on TCRαβ+, TCRαβ+CD4+, TCRγδ+ or TCRγδ+CD8+ cell numbers • In the Peyer's Patches, there was no difference between groups in any of the immune cell phenotypes measured, except that the proportion of TCRαβ+CD8 was ↓, and OX62+TCRγδ- cell numbers and proportion were ↑ • ↑ ConA stimulated ex vivo secretion of IL-2, IL-10 and IFN-γ in spleen and mesenteric lymph nodes • No effect on serum IgG1 or IgG2a
Westerbeek (2009)(142)	Prebiotic mixture containing 80 % short-chain GOS/lc-FOS: 20 % AOS (increased up to a maximum dose of 1.5 g/kg/d during d 3 - 30 of life, administered in breast milk or preterm formula)	Preterm infants (gestational age < 32 w and / or birth weight < 1500 g);	• No significant reduction in risk of serious infectious morbidity • Trend for lower incidence of serious infectious morbidity especially for infections with endogenous bacteria
Arribas (2010)(83)	FOS (100 mg/ml, average intake 1 g/d)	Rats (female); colitis induced by TNBS	Compared to TNBS induced control group, the FOS group had: • Faster weight recovery after colitis induction • ↓ Colonic damage score • ↓ Extent of colonic necrosis and / or inflammation • ↓ Colonic weight / length ratio (which was increased due to the inflammatory process) • Recovery of intestinal architecture, with the presence of mucin - replenished goblet cells • ↓ Colonic MPO activity

			• ↑ in colonic GSH content (which was depleted in control animals) • ↓ colonic TNF-α concentration • Non-significant ↓ in IL-1β concentration • No effect on colonic iNOS expression
Perez (2010)(106)	IN (240 mg/d) and OF (950 mg/d), in combination with *L. acidophilus* strain CRL730 (95 x 10^6 cfu/d) and *L. casei* strain CRL431 (95 x 10^6 cfu/d) for at least four m; both test and control products contained *S. thermophilus* at 95 x 10^8 cfu/d	Children of a "low socio-economic status", so living in a less hygienic environment; aged 9 m - 10 y; n = 70 in test group (median age = 46.5 m); n = 70 in control group (median age = 45.5 m); received tetanus or pneumococcal vaccine depending upon age	No effect (post-vaccination) on: • Serum IgG, IgA, IgM, IgE or isoagglutinin levels • Serum vaccine-specific antibody levels post-vaccination • Days of fever • Number of episodes of infection (upper respiratory tract infections, gastroenteritis, varicela, pneumonia)
Ribeiro (2010)(90)	FOS (50 mg/d) plus *B. animalis*	Rats; female; specific pathogen free; dexamethasone treated and infected with *Toxoplasma gondii*	• All dexamethasone treated, *T. Gondii* infected, synbiotic supplemented rats survived until the end of the experiment (21 d), but dexamethasone treated, *T. Gondii* infected rats who did not receive the synbiotic died between d 5 - 8 • ↓ Serum IFN-γ levels in dexamethasone treated, *T. Gondii* infected, synbiotic supplemented rats at d 21 compared to rats who were not treated with dexamethasone • No effect on serum IL-10 levels at d 15 or 21 between groups, but decreased progressively during the study in all groups

d, day; h, hour; m, month; w, week; y, year ; OF, oligofructose; IN, inulin; FOS, fructooligosaccharides; sc-FOS, short chain fructooligosaccharides; lc-FOS, long chain fructooligosaccharides; GOS, galactooligosaccharides; sc- GOS, short-chain galactooligosaccharides; AOS, acidic oligosaccharides; SPS, soy polysaccharides; EPA, eicosapentanoic acid; DHA, docosahexaenoic acid; lc-PUFA, long chain polyunsaturated fatty acids; LPS, lipopolysaccharide; URTI, upper respiratory tract infection; ICU, intensive care unit; DAI, disease activity index; TNBS, trinitrobenzene sulphonic acid; MPO, myeloperoxidase; GSH, glutathione peroxidase; LTB4, leukotriene B4

1.5 EFFECTS OF PROBIOTICS ON IMMUNE FUNCTION, INFECTION AND INFLAMMATORY CONDITIONS

1.5.1 INTRODUCTION

Probiotics have been defined as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (Food and Agriculture Organisation (178)). The most common probiotics are the bacteria bifidobacteria and lactobacilli, but probiotics also include other bacteria such as enterococcus, bacillus and escherichia and the yeast *Saccharomyces boulardii*. Research on the potential health benefits of probiotics has occurred over a long period of time but has grown over the last 15 years or so, with a recent interest in the effects on the immune system, the host's ability to fight infection, and inflammatory processes and conditions. All studies investigating the influence of probiotics on immunity, host defence and inflammatory processes and conditions in humans have been collected and collated. The tables generated are found in Appendix 2 (Lomax and Calder 2009b (179)), and a table summarising studies investigating the effect of ß2-1 fructans on these outcomes that have been published since this review article was prepared (June 2008) and up to June 2010 is included at the end of this section (Table 6). This section will summarise the results from all available human studies investigating the effect of probiotics on these outcomes, and is a summary and update of a comprehensive review article published elsewhere (Lomax and Calder 2009b (179)). Studies in model systems including experimental animals are not considered here. Studies using the traditional yoghurt cultures *Streptococcus thermophilus* and *Lactobacillus delbruekii* sp. *bulgaricus* are not included here, as there is disagreement as to whether these organisms are considered to be probiotic (180, 181).

1.5.2 OVERVIEW OF THE MECHANISM OF ACTION OF PROBIOTICS

In order to be effective, a probiotic must travel through the gastrointestinal tract, resisting hydrolysis and digestion, and reach the colon. Here they must adhere to the intestinal cells (182) and colonise the intestine, thus modulating the composition and activity of the intestinal flora, and increasing the numbers of the beneficial bacteria in the colon. This may have several beneficial effects, as summarized in Figure 15 and below:

1. Increased competition with pathogenic bacteria for nutrients and adhesion sites and therefore reduced survival of pathogenic bacteria (183)
2. Production of antimicrobial substances such as bacteriocins that are harmful to pathogenic bacteria (50)
3. Production of SCFAs by fermentation of carbohydrates. This may:

a) reduce the pH of (i.e. acidify) the colon which is detrimental to pathogenic bacteria (183);

b) provide nutrition to colonocytes (184);

c) alter epithelial cell gene expression (mainly an effect of butyrate), for example for IL-8 and MCP-1, which in turn alters the signalling of the epithelial cell to the mucosal immune system (57);

4. Reduced intestinal permeability (185);

5. Modulate immune function through direct interaction with the mucosal immune system (48).

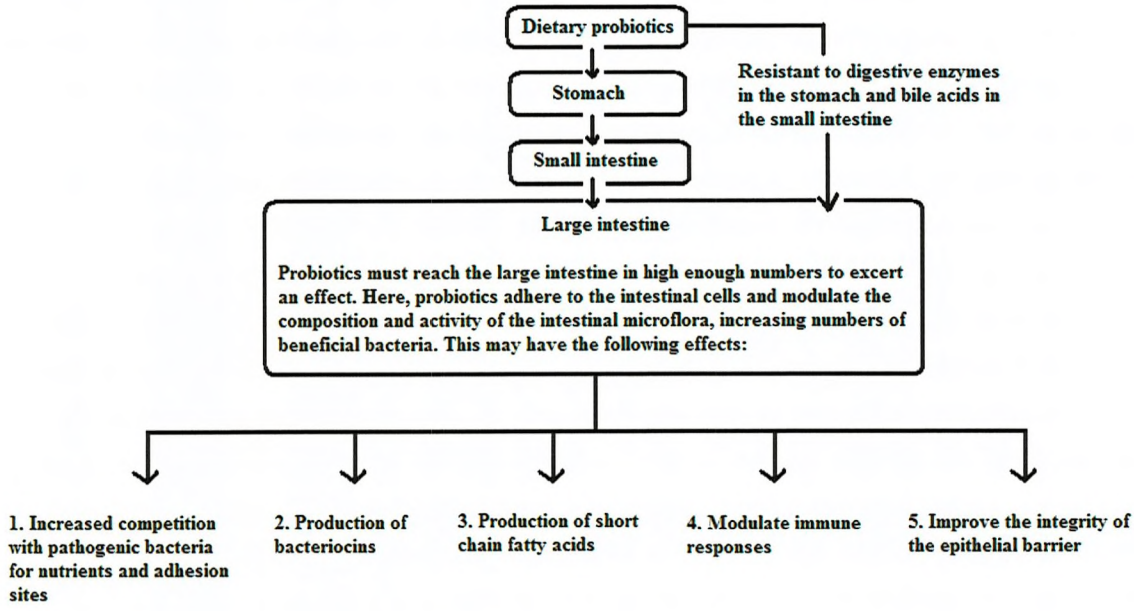


Figure 15 : Mechanisms of action of probiotics (taken from Lomax and Calder 2009b(179))

1.5.3 SUMMARY OF THE EFFECT OF PROBIOTICS ON IMMUNE FUNCTION IN HUMANS

A large number of studies have been performed examining the influence of various probiotic organisms, either alone or in combination, on immune parameters, and these are summarised in Appendix 2 (Lomax and Calder 2009b (179)) and Table 6. The majority of studies investigating phagocytosis have shown an enhancing effect, although several studies showed no effect. NK cell activity has been enhanced by most probiotic bacteria studied. Little effect of probiotics has been observed on lymphocyte subpopulations in the blood or the GALT, although there may be specific local effects in the latter. Studies indicate a limited effect of probiotics on lymphocyte proliferation in infants and adults, and no effect has been observed regarding T cell activation. Regarding cytokine production and levels in the bloodstream, probiotic supplementation has resulted in mixed effects, although there appears to be very little effect on the majority of cytokines measured. Faecal and salivary IgA levels have been increased by probiotics in a number of

studies in infants, but few effects have been seen in adults. There appears to be little influence of probiotics upon intestinal production of other antibodies. Systemic levels of antibodies are also little affected by probiotics. Antibody responses to vaccination have been increased by probiotics in some, but not all, studies. Thus, the picture that emerges from studies of probiotics on immune outcomes in humans is mixed, with some studies showing improvements in some parameters but others showing no effect on these same parameters. There appear to be species and strain differences in the effects seen. Other reasons for differences in immune effects seen will include dose of probiotic organism used, duration of supplementation, characteristics of the subjects studied (age, background diet, healthy vs. diseased etc.), sample size, and technical differences in how the measurements were made.

1.5.4 SUMMARY OF THE EFFECT OF PROBIOTICS ON INFECTIONS IN HUMANS

Studies describing the effect of probiotics upon infections in humans are summarised in Appendix 2 (Lomax and Calder 2009b (179)) and Table 6.

In pre-term infants in intensive care, probiotics may have benefit on some clinical outcomes. A range of probiotics administered to children with acute diarrhoea appear to exert beneficial effects with regards to reducing duration. Results of studies investigating the effects of probiotic supplementation on common childhood diarrhoea are mixed, although most studies show some benefit. Most studies have shown that probiotic supplementation, including certain lactobacilli and bifidobacteria, can reduce the risk of developing antibiotic-associated diarrhoea, but mixed effects have been reported on duration, and no effect is seen regarding severity. Results of studies investigating the effect of probiotics on *H. pylori* infection and hospital-acquired diarrhoea in children have reported mixed results. Several other studies have looked at different types of infection in children, but as they are so varied it is difficult to draw conclusions.

Probiotic supplementation has been shown to reduce the incidence, risk, or frequency of travellers' diarrhoea in adults, but has not shown an effect on duration. In radiation-induced diarrhoea or bowel symptoms, probiotics have shown mixed results. In patients with chronic diarrhoea probiotics have produced several beneficial effects. Probiotic supplementation in hospitalised patients seems to produce largely beneficial results regarding infectious outcomes, but these patients are often weak and susceptible to infection, so this should be considered. In patients with acute diarrhoea, mixed results are reported regarding probiotic treatment. Little effect has been reported of probiotics on common diarrhoea. The use of probiotics for antibiotic-associated diarrhoea has produced mixed results; this seems to depend on what condition the patients were receiving the antibiotics for. Mixed effects are reported regarding the effect of probiotics

to eradicate *H. pylori* infection in adults, but most studies have shown an improvement in symptoms or side effects. Probiotic supplementation in preventing occurrence, severity and duration of common respiratory illnesses has produced mixed results.

1.5.5 SUMMARY OF THE EFFECTS OF PROBIOTICS ON INFLAMMATORY CONDITIONS IN HUMANS

Studies describing the effect of probiotics upon inflammatory diseases in humans are summarised in Appendix 2 (Lomax and Calder 2009b (179)) and Table 6.

Studies investigating the effect of probiotics in children and adults with IBS have produced mixed results, with some showing clinical benefit. In children and adults with ulcerative colitis, probiotics have been shown to be beneficial, although results from studies in adult patients who had previously undergone surgery for this condition are less conclusive. Probiotic supplementation in children and adults with Crohn's Disease has produced mixed results, and in children probiotics may be more effective if given when the disease is active, rather than when the patients are in remission. Probiotics have little, if any, effect in children and adults with arthritis. Probiotic supplementation to mothers during pregnancy and/or lactation may reduce the risk of their infants becoming allergic, although results of studies are mixed. When probiotics are given to infants at risk of allergy, or to infants who had hay-fever, results are also mixed. In infants and children with allergic dermatitis and/or cows' milk allergy, again results are mixed but there are a number of positive studies using lactobacilli and bifidobacteria supplementation. It may be that allergic or food sensitized children are more receptive to probiotic therapy. In adults, probiotic therapy to those with nasal allergies produces mixed results regarding clinical outcomes, and probiotics may modify some cytokines in these subjects, although mixed effects are seen on eosinophil numbers and eosinophil cationic protein, and little effect is seen on IgE levels. In adults with food allergy or with several different allergies, probiotic supplementation may be beneficial, and in adults with atopic dermatitis, mixed results were observed with probiotic supplementation.

Table 6 : Studies investigating the effect of probiotics upon immune function, infection and inflammatory conditions in humans, published since Lomax and Calder 20009b (179) (June 2008) and up to June 2010

Study	Probiotic used (strain, dose and duration)	Subjects studied (gender, age, numbers, conditions)	Findings
Oláh (2002)(186)	*L. plantarum* 299 (2 x 10^9 bacteria/d for at least 7 d) plus 20 g oat fibre/d	Patients with acute pancreatitis, receiving enteral nutrition; n = 23 in control group (heat-killed *L. plantarum* 299), mean age = 46.5 y; n = 22 in test group (live *L. plantarum* 299), mean age = 44.1 y	• ↓ Pancreatic sepsis • ↓ Number of surgical interventions (for a septic complication; abscess or infected necrosis) No effect on: • Length of hospital stay (longer in control group but not significant) • Systemic inflammatory response syndrome • Multiple organ failure • Positive blood culture • Pancreatic abscess • Infected necrosis • Chest infection • Antibiotic therapy • Death
Rayes (2002a)(187)	*Lactobacillus plantarum* 299 (2 x 10^9 bacteria/d for 4 d post-surgery) Plus oat fibre	Adult patients undergoing major abdominal surgery; n = 30 in Group A (conventional parenteral/ fibre-free enteral nutrition diet), mean age = 62 y; n = 30 in Group B (enteral nutrition + oat fibre + living *Lactobacilli* diet), mean age = 60 y; n = 30 in Group C	• ↑ Infections in Group A compared to Groups B and C (pneumonia was the most frequent infection and enterococci were the predominant bacteria isolated) • ↑ Length of antibiotic therapy in Groups A and C compared to Group B No effect on: • Leukocyte counts, CRP levels, serum urea nitrogen, haemoglobin, bilirubin or albumin concentrations • There were significant differences between groups regarding CD3+, CD4+, CD8+, or NK cell counts, CD4:CD8 ratio, and % of CD45RA+or CD45RO+ cells, but all immune parameters remained within normal ranges • Highest incidence of postoperative complications was found in Group A, then Group C, then Group B, but the differences were not significant

		(enteral nutrition + oat fibre + heat-killed *Lactobacilli* diet), mean age = 61 y	• Length of stay in intensive care or total length of hospital stay (although this was longer in Group A compared to Groups B and C) • No benefits of living *lactobacilli* compared to heat-killed *lactobacilli*, but benefits were observed in patients with gastric and pancreas resections
Rayes (2002b)(188)	*L. plantarum* 299 (2×10^9 bacteria/d for 12 d post-surgery) Plus oat fibre	Adult patients undergoing liver transplantation; n = 32 in Group 1 (selective bowel decontamination therapy), mean age = 47 y; n = 31 in Group 2 (enteral nutrition + live *L. plantarum* + oat fibre), mean age = 50 y; n = 32 in Group 3 (enteral nutrition + heat-killed *L. plantarum* + oat fibre), mean age = 50 y	• More infections in Group 1 compared to Group 2 (also ↑ infections in Group 1 compared to Group 3 but this was not significant) and cholangitis and pneumonia were the most frequent infections, and enterococci were the predominant bacteria isolated • ↑ Length of antibiotic therapy in Groups 1 and 3 compared to Group 2 (not significant) No effect on: • Leukocyte counts (although lower in Group 2 compared to Groups 1 and 3) • CD4:CD8 ratio (although higher in Group 2 compared to Groups 1 and 3) • Length of stay in intensive care unit or total length of hospital stay (although length of both stays were longest in Group 1) • Blood CRP or IgA levels • CD3+, CD4+, CD8+, CD19+, NK cell or CD45RA/CD45RO cell counts
Tursi (2004)(189)	VSL#3 (9×10^{11} bacteria/d, for 8 w)	Patients with newly diagnosed or recently relapsed, mild to moderate ulcerative colitis; n = 30 in Group A (Basalazide + VSL#3), mean age = 41 y; n = 30 in Group B (Basalazide), mean age = 38 y, n = 30 in Group C (Mesalazine),mean age=44y	• ↑ Number of patients who obtained remission in Group A compared to other groups • Remission was achieved faster in Group A compared to other groups • Group A was better than other groups in all parameters measured (improved well-being, bowel frequency, endoscopic scores and histological scores)

Galpin (2005)(190)	*Lactobacillus* GG (10^{10} bacteria/d for 30 d)	Children in Malawi; age 36 - 60 m; healthy, not malnourished; n = 81 in test group, mean age = 45.7 m; n = 83 in control group, mean age = 47 m	• No effect on mannitol, lactulose or sucrose excretion, or urinary sucrose: lactulose ratio (measure of gastric damage) or lactose: mannitol ratio (measure of intestinal damage) following sugar-absorption tests, therefore no effect on intestinal integrity • No effect on incidence of fever, cough or diarrhoea
Kanazawa (2005)(191)	*B. breve* strain Yakult (3 x 10^8 bacteria/d) and *L. casei* strain *Shirota* (3 x 10^8 bacteria/d)plus GOS (12 g/d) via enteral feeding from postoperative d 1 - d 14	Patients with biliary cancer scheduled to undergo combined liver and extrahepatic bile duct resection with hepaticojejunostomy; n = 21 in test group, mean age = 62.5 y; n = 23 in control group, mean age = 64.9 y	• ↓ WBC counts on postoperative d 10 • ↓ Serum C-reactive protein on postoperative d 10 • ↓ Incidence of postoperative infectious complications • Non-significant ↓ in post-operative hospital stay • Non-significant ↓ in cumulative incidence of antibiotic use and % of patients using antibiotics for therapeutic reasons No effect on: • Postoperative changes in lactulose / mannitol ratio • Postoperative changes in serum diamine oxidase activity
Matsuzaki (2005)(192)	L. casei strain Shirota (8 x 10^{10} bacteria/d for 4 w)	Patients with clinical T-cell lymphotrophic virus type-1 - associated myelopathy/ tropical spastic paraparesis; age 34 - 62 y; n = 10; no control group	• No effect on frequency or absolute number of any T cell phenotypes measured ($CD4^+$, $CD8^{high+}$, naïve in $CD8^{high+}$, memory in $CD8^{high+}$, effector in $CD8^{high+}$, effector / memory in $CD8^{high+}$, $CXCR3^+$, $CXCR3^+$ in $CD4^+$, $\delta\gamma T^+$, $NKG2A^+$ or $CD16^+CD56^+/CD3^-$) • ↑ NK cell activity
Shen (2005)(193)	VSL#3 (6g/d, number of bacteria not given, the trial was designed to last for 8 m, and the primary	Ulcerative colitis patients with antibiotic- dependent pouchitis; treated with standard therapy for 2 w	• At the end of 8 m, 23 patients had discontinued VSL#3 because of recurrent symptoms and 2 because of adverse effects • Only 6 patients remained on VSL#3 at the end of the 8 m; they were in symptomatic remission, but had evidence of mild or moderate endoscopic pouch inflammation which was not significantly lower from baseline values

	outcome was the number of patients who still remained on VSL#3 at this time)	before supplementation began to induce remission; n = 31; no control group	
Sugawara 2006(194)	Preoperative treatment (Group B): *L. casei* strain *Shirota* (4×10^{10} bacteria/d) and *B. breve* strain *Yakult* (1×10^{10} bacteria / d) plus GOS (15 g/d) for 2 w before surgery Postoperative treatment (Groups A & B): *L. casei* strain *Shirota* (3×10^{8} bacteria/d) and *B. breve* strain *Yakult* (3×10^{8} bacteria/d) and GOS (15 g/d) via enteral feeding from postoperative d 1 - d 14	Patients with biliary cancer who were undergoing combined liver and extrahepatic bile duct resection with hepaticojejunostomy; n = 40 in Group A, mean age = 63.2 y; n = 41 in Group B, mean age = 63.1 y	• ↑ Peripheral blood NK cell one d before surgery in Group B compared to Group A, but not significantly. Following surgery, changes in activity were similar in both groups (decreased followed by an increase to preoperative levels) • ↑ Lymphocyte counts one d before surgery in Group B compared to Group A, but not significantly. Following surgery, changes in lymphocyte counts were similar between groups (decreased followed by an increase to preoperative levels) • ↓ IL-6 one d before surgery in Group B compared to Group A, and at postoperative d 2, 7 and 21. • ↓ WBC counts in Group B at postoperative d 2 and 7 • ↓ Serum C-reactive protein in Group B at postoperative d 7 and 21 • ↓ Postoperative infectious complications in Group B • ↓ Postoperative hospital stay in Group B • ↓ Cumulative duration of antibiotic therapy in Group B No difference between groups regarding: • Perioperative changes in lactulose/ mannitol ratio • Perioperative changes in serum diamine oxidase activity
Tursi (2006)(195)	*L. casei* subsp. *casei* DG (16×10^{9} bacteria/ d for 15 d of each m, for 12 m)	Patients with symptomatic uncomplicated diverticular disease of the colon; mean age = 67.5 y (range 39 - 84	• 76.7 % of Group M, 76.7% of Group L and 96% of Group LM were symptom-free at the end of follow-up, there were statistically more of Group LM who were symptom-free compared to the other two groups

		y); remission was induced with rifaximin and mesalazine, so all patients were asymptomatic at the time of enrolment; n = 90, split into in Group M (mesalazine), Group L (*L. casei*) and Group LM (mesalazine + *L. casei*)	
Zocco (2006)(196)	*Lactobacillus* GG (1.8×10^{10} bacteria/d for 12 m)	Ulcerative colitis patients in remission; n = 65 in probiotic group (Group 1), mean age = 34 y; n = 60 in mesalazine group (Group 2), mean age = 33 y; n = 62 in probiotic plus mesalazine group (Group 3), mean age = 33 y	• Treatment with LGG alone or in combination with mesalazine was more effective than mesalazine alone, in prolonging relapse-free time, and there was no advantage from combining LGG with mesalazine as compared to LGG alone No difference between groups regarding: • Clinical or endoscopic relapse rates at 6 and 12 m, suggesting that all three treatments had equivalent efficacy • Clinical, endoscopic or histological scores at 6 and 12 m among the three groups
Abrahamsson (2007)(197) and Böttcher (2008)(198)	*L. reuteri* (1×10^8 cfu/d; mothers took this for 4 w before delivery and after birth the baby continued with the supplement until 12 m of age)	Infants at risk of allergy (one or more family members with eczema, asthma, gastrointestinal allergy, allergic urticaria or allergic rhinoconjunctivitis); n = 95 in test group; n = 93 in placebo group	• ↑ % of children who were prescribed antibiotics during the first y of life (acute otitis media was the indication for 70% of prescriptions) • Tendency for ↑ acute otitis media in the test group • ↓ Circulating IgE to egg white at 2 y of age • ↓ Ig-E associated eczema during the second y of life • Tendency for ↓ in cumulative incidence of any positive skin-prick test • Tendency for ↓number of sensitized infants at 2 y of age (either a positive skin prick test and/or circulating IgE to food allergens)

		In a sub-group, breast milk was analysed for several immune components and these findings were related to the development of eczema and sensitization in the infants; n = 54 in test group, mean age of mothers = 29 y; n = 55 in placebo group, mean age of mothers = 31 y	• The effect of the treatment was also assessed depending upon the parenteral atopic status. If only infants whose mothers had allergic disease were included, then the effect of the treatment was more pronounced, with the effect on IgE-associated eczema (at 6 -12, 12 -24 and 0 -24 m), skin-prick test reactivity (at 24 m and 0 - 24 m) and sensitization (at 24 m and 0 - 24 m) becoming significant No effect on: • Circulating IgE to other food allergens • Number of infections up to 2 y of age • Cumulative incidence of eczema • Circulating IgE to food allergens (except egg white) In the subgroup: • ↓ TGF-ß2 levels in colostrum in test group • ↑ IL-10 levels in colostrum in test group • No effect on levels of TGF-ß1, TNF, sCD14, total IgA or SigA in colostrum • No effect on levels of TGF-ß1, TGF-ß2, TNF, IL-10, sCD14, total IgA or SigA in mature milk (1 m after delivery) • Low levels of TGF-ß2 in colostrum were associated with ↓ sensitization (positive skin prick test and/or circulating allergen-specific IgE) during infancy, which was significant at 24 m of age, and a similar trend was associated with TGF-ß2 levels in mature milk • Tendency for TGF-ß2 levels in colostrum to be ↑ in mothers of babies with IgE-associated eczema, and similar trends were observed for TGF-ß2 levels in mature milk
Gionchetti (2007)(199)	VSL#3 (3.6×10^{12} bacteria/ d for 4 w, then if patients were in remission they received	Patients with active mild pouchitis; n = 23; mean age = 31.8 y; no control group	• 69% of patients went into remission by the end of w 4, and all remained in remission at 6 m • ↓ Median bowel frequency • ↓ Median total Pouchitis Disease Activity Index score (due to ↓ in clinical, endoscopic and histological scores of

	1.8×10^{12} bacteria/ d for 6 m as maintenance therapy)		the Pouchitis Disease Activity Index) at both 4 w and 6 m • Improved Median Irritable Bowel Disease Questionnaire score at the end of the study compared to baseline
Hickson (2007)(200)	*L. casei* DN-114 001 (1.94×10^9 cfu/d, begun within 48 h of starting antibiotics, and continued until 1 w after stopping antibiotics) also contained *S. thermophilus* (1.94×10^9 cfu/d) and *L. bulgaricus* (1.94×10^8 cfu / d)	Hospitalized patients receiving antibiotics, aged over 50 y; n = 69 in test group, mean age = 73.7 y; n = 66 in placebo group, mean age = 73.9 y	• ↓ Incidence of antibiotic associated diarrhoea • ↓ Incidence of *C. Difficile* associated diarrhoea
Kukkonen (2007)(201)	*L. rhamnosus* GG ATCC 53103 (1×10^{10} cfu/d), *L. rhamnosus* LC705 DSM 7061 (1×10^{10} cfu/ d), *B. breve* Bb99 DSM 13692 (4×10^8 cfu/d) and *Propionibacterium freudenreichii* ssp. *shermani* JS DSM 7076 (4×10^9 cfu/ d) the mothers consumed this for 2 - 4 w before delivery and the	Pregnant mothers carrying infants at increased risk of allergy (a least one parent with a physician-diagnosed allergic disease); n = 461 in test group; n = 464 in placebo group	• ↓ Cumulative incidence of IgE-associated (atopic) diseases at 2 y • ↓ Cumulative incidence of eczema at 2 y • ↓ Cumulative incidence of atopic dermatitis at 2 y • ↓ Antibiotic prescriptions during the 6 m intervention • ↓ Frequency of respiratory infections from 6 - 24 m of age No effect on: • Cumulative incidence of sensitization at 6 m or 2 y • Cumulative incidence of allergic diseases as a whole (eczema, food allergy, asthma or allergic rhinitis) • Occurrence of gastroenteritis or middle ear infections, or antibiotic prescriptions during 6 - 24 m of age • Occurrence (at least once) of respiratory infections, middle ear infections or gastroenteritis during the 6 m intervention

	infants received half this dose for 6 m after birth, along with 0.8g GOS		In a sub-group of 237 infants, faecal analysis was performed: • Non-significant ↑ in faecal IgA and TNF-α at 3 m, not a 6 m (antibiotic treatment abolished this effect) • ↑ Faecal α1-antitrypsin at 3 m, not at 6 m (antibiotic treatment abolished this effect) • ↑ Faecal calprotectin at 6 m, when adjusted for antibiotic use
Matsumoto (2007)(202)	*B. animalis* subsp. *lactis* LKM512 (5.2×10^9 cfu / d for 4 w) also given with *L. delbruekii* subsp. *bulgaricus* and *S. thermophilus* which were also present in the placebo supplement	Adults with moderate atopic dermatitis; n = 10, average age =22.1y; crossover study	• ↑ Serum IFN-ɤ after consumption of test (by six-fold) and placebo (by three-fold) supplements • Improvement in itch in 4/10 in test and 1/10 in placebo (therefore tendency for greater improvement in test group) • Improvement in burning in 3/8 in test and 2/8 in placebo (therefore tendency for greater improvement in test group) • Improvement in pain in 2/6 in test and 3/6 in placebo • Improvement of irritation in 1/8 in test and placebo • Improvement in visual status in 3/10 in test and placebo • No effect on serum IL-4, IL-5 or IL-12 concentrations after either supplement, and IL-10 was below the levels of detection
Myllyluoma (2007)(203)	*L. rhamnosus* GG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *B. lactis* Bb12 (2.5×10^9 cfu of each/d for 8 w)	Patients referred for diagnostic upper endoscopy; majority female; n = 7 *H. pylori* positive subjects, mean age - 51 y; n = 6 *H. pylori* negative subjects, mean age = 48 y	• ↓ Serum gastrin-17 in *H. pylori* infected subjects (suggests a beneficial effect of probiotics on gastric function at the antrum level) • ↓ ^{13}C- urea breath test values (non-significant) No effect on: • Serum Helicobacter IgG antibody titres • Chronic inflammation in antrum and corpus in *H. pylori* positive subjects, as measured by histology • Serum Helicobacter IgG antibody titres • Gastric serum PGI, PGII and PGI/PGII ratio

Taylor (2007)(204) and Prescott (2008)(205)	*L. acidophilus* LAVRI-A1 (3 x 10^9 bacteria/d for 6 m)	Infants born to allergic mothers (asthma, allergic rhinitis or eczema plus positive skin prick test to at least one common allergen); began supplementation within 48 h of delivery; n = 89 in test group; n = 89 in control group	• ↑ Proportion of children with atopic dermatitis and positive skin prick test at 12 m • ↑ Rate of sensitization to common allergens at 12 m • ↑ Rate of wheezing at 12 m • Non-significant ↑ in ear infections at 6 and 12 m • Non-significant ↑ in likelihood of being prescribed and antibiotic during the supplementation period No effect on: • Rate of atopic dermatitis at 6 m of age • Other clinical outcomes (upper respiratory tract infection, cough without cold, wheeze, bronchiolitis, chest infection or gastrointestinal infections) at 6 m of age • Rate or severity of atopic dermatitis at 12 m of age • Rate of symptomatic (IgE-mediated) food allergy at 12 m • Upper respiratory tract infections, cough without cold, bronchiolitis, chest infections or gastrointestinal infections at 12 m • No association between detection of Lactobacillus species, Bifidobacteria species or coliform species in stools at 1 m of age and frequency of atopic dermatitis, sensitization, or clinical food allergy at 1 y in either group • No relationship between colonization at 6 m and subsequent clinical outcomes, except that children with culturable Lactobacilli at 6 m were more likely to develop a positive skin prick test to milk • No evidence that culturable *Lactobacilli* in stools at 1 m of age protected from infections during the first y of life A follow-up study was performed when the infants were 2.5 y of age (n = 77 in test group, n = 76 in control group): • No difference between groups regarding the rate of allergic disease (food allergy, dermatitis, asthma) • Trend for ↓ sensitization to cows' milk in test group • In children who were sensitized at 1 y, the test group were ↓ likely to still have milk allergy at 2.5 y, and also there was a trend for less of the test group to have grass pollen allergy at 2.5 y

			• In children who were not sensitized at 1 y, the test group were less likely to have house dust mite allergy at 2.5 y • Rates of sensitization, positive skin prick test/atopic dermatitis, and wheezing were all ↑ in the test group at 1 y of age, but this was no longer evident at 2.5 y of age • ↓ GI infections in the test group in the from 12 - 30 m of age • 2.5 y summary: probiotic supplementation did not have any long-term effects on allergic outcomes
Xiao (2007)(206)	*B. longum* BB536 (10×10^{10} / d for 4 w)	Adults with clinical history of Japanese cedar pollinosis; n = 24; age 25 - 56 y; crossover trial; exposed to Japanese cedar pollen for 4 h, in a controlled environment, at the end of each supplementation period	During exposure: • Ocular symptom scores were improved starting from 30 m after the beginning of exposure • No effect on scores for nasal symptom and disruption of normal activities • No effect on scores for each nasal symptom: sneezing, nose blowing, nasal blockage, but scores for eye itching and watery eyes were reduced After exposure: • No effect on nasal and ocular symptom scores • Throat symptoms tended to be lower the day after exposure • Scores for disruption of normal activities tended to be lower on the two days after exposure • No effect on number of subjects taking medications for relieving symptoms, but ↓ prevalence for total counts of the number of days for oral medication and eye drop usage and total medication No effect on total and Japanese cedar pollen- specific IgE in serum
Forestier (2008)(207)	*L. casei rhamnosus* strain 35 (2×10^9 cfu/ d from third day after admission to intensive care until discharge or death)	Adults in intensive care (mostly due to trauma, respiratory distress or admitted after surgery); fed by nasogastric feeding tube; n = 102 in test group, mean median age = 60 y; n = 106 in placebo group,	• ↑ Time to acquisition of respiratory tract infection with *P. aeruginosa* • ↓ Number of patients with *P. aeruginosa* infection when patients with *P. aeruginosa* in one or both isolates (gastric or respiratory) were considered together • After multivariate Cox proportional hazards modelling, the absence of probiotic treatment ↑ risk for *P. aeruginosa* colonization in the respiratory tract • Non-signficant ↓ on ventilator-associated pneumonia due to *P. aeruginosa* No effect on:

		median age = 57 y	• Ventilator-associated pneumonia due to *Enterobacteriaceae* or *Staphylococcus aureus* • Isolation of *Candida* spp. from the respiratory tract • Frequencies of urinary tract, catheter-related on bloodstream infections • Number of patients with gastric infection with *P. aeruginosa* • Time to acquisition of gastric infection with *P. aeruginosa* • Number of patients with respiratory tract infection with *P. aeruginosa*
Huurre (2008)(208)	*L. rhamnosus* GG and *B. Lactis* Bb12 (1×10^{10} cfu / d of each from the first trimester of pregnancy until the end of exclusive breastfeeding)	Pregnant women; n = 72 in test group; n = 68 in placebo group	• No effect on infant sensitization (assessed by skin prick test) in test versus placebo groups as a whole (antigens tested: cows' milk, egg white, wheat and rice flour, cod, soya bean, birch, six grasses, cat, dog, Der p1, latex, potato, carrot, banana) at 12 m, but in the subgroup of infants who had a high hereditary risk due to maternal sensitization (mother was SPT positive), probiotic supplementation had a protective effect against sensitization • Non-significant ↑ in TGF-B2 in colostrum of mothers from test group, but this disappeared by 1 m after birth (the same trend was seen when allergic mothers were analysed separately) No effect on: • Concentrations of sCD14, IFN-γ, TNF-α, IL-10, IL-6, IL-4 or IL-2 in colostrum or breast milk • Incidence of atopic eczema
Ivory (2008)(209)	*Lactobacillus casei Shirota* (6.5×10^9 bacteria/ d for 5 m)	Adults aged 18 – 45 y with seasonal allergic rhinitis with detectable levels of pollen-specific IgE antibodies in the blood before the start of the pollen season; n = 10 in test group; n = 10 in placebo group	• Early pollen antigen-specific plasma IgE levels were ↓ in the test group • Early pollen antigen-specific plasma IgG levels were ↑ in the test group • ↓ Levels of IL-5 in un-stimulated and early and late pollen antigen stimulated cultures • Trend for ↓ IL-10 in probiotic group from baseline No effect on: • IL-6 levels in unchallenged cultures, but ↓in early and late pollen antigen stimulated cultures • IFN-γ levels in un-stimulated cultures, but ↓in early and late pollen antigen stimulated cultures • IL-2, IL-4, IL-8 or IL-12p70, IL-1α or TNF-α in supernatant of mononuclear cells • Total plasma IgE or IgG levels, or on late pollen antigen- specific plasma IgE or IgG levels

Klarin (2008)(210)	*L. plantarum* 299v (16 x 10^{10} cfu/d for first 3 d of stay in intensive care, then 8 x 10^{10} cfu/d for the remainder of the stay)	Patients in intensive care receiving enteral nutrition; n = 22 in test group, median age = 65.5 y; n = 22 in placebo group, median age = 64 y	• ↓ IL-10 at d 8 • ↓ Colonisation with *C. difficile* (measured in faeces) • ↓ White blood cell counts on d 7 - 9 and d 12 - 14 • ↓ (improved) lactulose / rhamnose ratio on d 3 or 4 No effect on: • CRP, TNF-α, IL-1ß, IL-6 in blood • Length of stay in intensive care • Number of days on ventilator • Intensive care and in-hospital deaths
Kopp (2008)(211)	*L. rhamnosus* GG ATCC 53103 (1 x 10^{10} cfu/ d taken by the mother for 4 - 6 w before delivery. If the mothers breast-fed, they took the LGG for a further 3 m postnatally, and then their infants took the LGG for a further 3 m (therefore 6 m postnatally altogether). If the mothers did not breast-feed the infants received LGG for 6 m	Pregnant women and their infants (who had a family history of atopic disease [at least one of their mother, father or sibling had atopic dermatitis, allergic rhinitis or asthma and allergic sensitization against an inhalant allergen]); n = 50 in test group, mean age of mothers = 32.9 y; n = 44 in placebo group, mean age of mothers = 33.8 y In a subgroup, cord blood	• ↑ Frequency of children with recurrent episodes of wheezing bronchitis (≥ 5 episodes) during the first 2 y of life No effect on: • Number of children who experienced upper respiratory infections, number of fever episodes or use of antibiotics at 1 y of age • Total IgE concentrations or number of children with specific sensitization to inhalant allergens • Rate of atopic dermatitis at 2 y of age • Cumulative incidence of atopic symptoms at 2 y of age • Severity of atopic dermatitis in affected children • Number of children who experienced upper respiratory infections, number of fever episodes or use of antibiotics at 1 y of age In the subgroup analysis: • No effect of LGG on proliferative capacity of cord blood mononuclear cells or maternal peripheral blood mononuclear cells in response to stimulation with IL-2, LGG or ß-lactoglobulin

		and maternal blood was collected at birth; n = 40 in test group, mean age of mothers = 33.2 y; n = 28 in placebo group, mean age of mothers = 33.9y	• No difference in cytokine levels (IL-10, IL-13 or IFN-γ) in supernatants of stimulated cord blood mononuclear cells and maternal peripheral blood mononuclear cells between test and placebo groups. However, in contrast to un-stimulated controls, in vitro stimulation of cord blood mononuclear cells with LGG induced an ↑ release of IL-10 and IFN-γ (but not IL-13), but this was seen in test and placebo groups
Kukkonen (2008)(212)	*L. rhamnosus* GG ATCC 53103 (1×10^{10} cfu/ d), *L. rhamnosus* LC705 DSM 7061 (1×10^{10} cfu/ d), *B. breve* Bb99 DSM 13692 (4×10^{8} cfu/ d) and *Propionibacterium freudenreichii* ssp. *shermani* JS DSM 7076 (4×10^{9} cfu/ d) the mothers consumed this for 2 - 4 w before delivery and the infants received half this dose for 6 m after birth, along with 0.8g GOS	Pregnant mothers carrying infants at increased risk of allergy (a least one parent with a physician-diagnosed allergic disease); n = 461 in test group; n = 464 in placebo group	In a sub-group of 237 infants, faecal analysis was performed: • Non-significant ↑ in faecal IgA and TNF-α at 3 m, not a 6 m (antibiotic treatment abolished this effect) • ↑ Faecal α1-antitrypsin at 3 m, not at 6 m (antibiotic treatment abolished this effect) • ↑ Faecal calprotectin at 6 m, when adjusted for antibiotic use
Narayanappa (2008)(213)	BifilacTM; *Streptococcus faecalis* JPC (9×10^{7}/d), *Clostridium butyricum* (6×10^{6}/d), *Bacillus*	Children hospitalised with acute rotaviral diarrhoea; received rehydration therapy; age 3 m - 3 y; n = 40 in test group, n = 40 in	• ↓ Frequency of diarrhoea • ↓ Duration of diarrhoea • ↓ Degree of dehydration • ↓ Volume of intra venous fluid (IVF) administration

	mesentericus JPC (3 x 10^6/d), *L. sporogenes* (1.5 x 10^8/d) plus vegetable fibres (for up to 14 d)	control group	• ↓ Duration of IVF administration • ↓ Volume of oral rehydration salts (ORS) administration • ↓ Duration of ORS administration • ↓ Number of patients with rotaviral shedding at the time of discharge
Ouwehand (2008)(214)	Group 1 consumed *B. longum* 2C and *B. longum* 46 (10^9 cfu/d of each, for 6 m); Group 3 consumed *B. animalis* ssp. *lactis* Bb-12 (10^9 cfu/d, for 6 m)	Elderly; resident in nursing homes; average age = 84.3 y; majority female; n = 56 in Group 1; n = 67 in Group 2 (placebo); n = 86 in Group 3	• ↓ Serum IL-10 levels at the start and end of the intervention in Group 3 compared to Group 2 • Strong trend for ↓ serum IL-10 levels in Group 1 after 6 months compared to Group 2 • No change in serum IL-10 in any treatment group during the study • ↑ Serum TNF-α levels in Group 1 compared to Group 3 at 0, 3 and 6 months • ↑ Serum TNF-α levels in Group 2 compared to Group 3 at 0 and 6 months • No change in serum TNF-α in any treatment group during the study • No difference in serum TGF-ß1 levels between groups, but ↑ in all groups between the start and end of the study (significantly in Groups 2 and 3)
Paineau (2008)(215)	Group 1cosumed *B. lactis* Bi-07; Group 2 consumed *B. lactis* Bl-04; Group 3 consumed *L. acidophilus* La-14; Group 4 consumed *L. acidophilus* NCFM®; Group 5 consumed *L. plantarum* Lp-115; Group 6 consumed *L. paracasei* Lpc-37; Group 7 consumed *L. salivarius* Ls-33 (2 x 10^{10} cfu/ d for	Healthy adults aged 18 - 62 y; vaccinated with oral cholera vaccine at d 7 and 14; n = 9 in Group 1, mean age 35.3 y; n = 9 in Group 2, mean age = 38.0 y; n = 9 in Group 3, mean age = 34.5 y; n = 9 in Group 4, mean age = 40.6 y; n = 9 in Group 5, mean age =35.0 y; n = 9 in Group 6, mean age = 44.5 y; n = 9 in Group 7, mean age = 35.5 y; n = 20	• During d 0 - 21 serum IgG tended to increase during probiotic consumption, this was significant in Groups 2 and 3 compared to placebo, and in Group 2 compared to values at d 0 • During d 0 - 21 there was no change in serum IgA or IgM in any group • During d 0 - 21 there was a smaller change in salivary IgA in Group 7 than in the placebo group • During d 21- 28 there was no change in serum IgG, IgA or IgM in any probiotic group compared to placebo, but serum IgA and IgM increased in Group 4 • During d 21 - 28 salivary IgA increased in the placebo group and Group 3 • Overall vaccination titres (serum IgA, IgM and IgG, and salivary IgA) did not change during the study, although Group 5 showed significantly lower levels of serum IgM

	21 d)	in Group 8 (placebo), mean age = 34.5 y	
Prescott (2008)(216)	*L. rhamnosus* HN001 (6 x 10^9 cfu/ d) or *B. lactis* HN019 (9 x 10^9 cfu/d) for 2 - 5 w before delivery, and for 6 m after in lactating women, and for 2 y in the infant	Pregnant women and their infants (who were at risk of allergy - at least one parent had been treated for asthma, allergic rhinitis or eczema); n = 34 in *L. rhamnosus* group; n = 35 in *B. lactis* group; n = 36 in placebo group	• ↑ IFN- γ levels in cord blood in mothers who had consumed a probiotic (significant for both groups combined and the *L. rhamnosus* group, but not the *B. lactis* group) • No effect on cord blood levels of IL-13, IL-10, IL-6, IL-5, TGF-ß1 or TNF-α • ↓ sCD14 levels in cord blood in *B. lactis* group (significant) and in both groups combined (non-significant) • ↑ Likelihood of mothers having detectable levels of IgA in breast milk 1 w after birth in both probiotic groups • ↑ Likelihood of mothers having detectable levels of IgA in breast milk 3 m after birth in the combined group and *B. lactis* group, but not the *L. rhamnosus* group • ↑ Likelihood of mothers having detectable levels of IgA in breast milk at 6 m after birth (in the combined group, but not in the *B. lactis* and *L. rhamnosus* groups considered separately) • ↑ Absolute IgA levels in breast milk at 1 w after birth in the *B. lactis* and *L. rhamnosus* groups • ↑ Absolute IgA levels in breast milk at 3 m after birth in the *L. rhamnosus* group (significant) and the *B. lactis* group (non-significant) • ↑ TGF- ß1 levels in breast milk at 1 w after birth in both groups combined and the *B. lactis* group (significant) and in the *L. rhamnosus* group (non-significant) • ↑ Number of women with detectable levels of IL-6 in breast milk 1 w after birth in the combined probiotics group, and there was a trend for this in the *B. lactis* group • Trend for lower prevalence of atopic dermatitis in the children at 2 y of age who received *L. rhamnosus* compared to controls and those who received *B. lactis* • No effect on levels of IL-10, IL-5, IL-13, IFN- γ or TNF- α in breast milk at 1 week after birth • No effect on levels of IL-10, IL-5, IL-13, IFN- γ, TNF- α, IL-6 or TGF- ß1 at 3 m or 6 m after birth • No effect on levels of sCD14 levels in breast milk at any time point

Pronio (2008)(217)	VSL#3 (9 x 10^9 bacteria/ d for 12 m)	Patients who had recently undergone ileal pouch-anal anastomosis for ulcerative colitis, who had no active disease or chronic pouchitis, and who were not taking medication; n = 16 in test group, mean age = 37 y; n = 12 in control group, mean age = 35 y	• ↓ Pouchitis Disease Activity Index scores after 3, 6 and 12 months in the test group • ↓ Expression of mRNA for IL-1ß in mucosal biopsies from the ileal pouch in the test group compared to controls • ↑ % of CD4+CD25^{high} lamina propria mononuclear cells at 3 and 6 months in the test group • ↑ % of CD4+LAP+ T cells in lamina propria mononuclear cells at 3 months in the test group • ↑ Foxp3 mRNA content in mucosal biopsies from the ileal pouch in the test group compared to controls No effect on: • % of CD4+CD25^{low} lamina propria mononuclear cells • % of CD4+CD25^{high} or CD4+CD25low peripheral blood mononuclear cells • CD4+LAP+ T cells in peripheral blood mononuclear cells
Scaccianoce (2008)(218)	Consumed either Reuflor® (*L. reuteri* ATCC 55730 at 2 x 10^8 cfu/d) or Probinul (*L. plantarum* at 10 x 10^9 cfu/d, *L. reuteri* at 4 x 10^9 cfu/d, *L. casei* subsp. *rhamnosus* at 4 x 10^9 cfu/d, *B. infantis* at 4 x 10^9 cfu/d, *B longum* at 4 x 10^9 cfu/d, *L. salivarius* at 2 x 10^9 cfu/d, *L. acidophilus* at 2 x 10^9 cfu/d, *Streptococcus thermophilus* 10 x 10^9	Dyspeptic adults with *H. pylori* infection who had not previously been treated for *H. pylori* infection; Group A received standard triple therapy for 7 d, n = 16, mean age = 48 y; Group B received standard triple therapy for 7 d plus Reuflor®, n = 17, mean age = 51 y; Group C received standard triple therapy for 7 d plus Probinu1®, n = 15, mean age = 50 y; Group D received standard triple therapy for 14 d plus	• No statistically significant differences in eradication rates (measured by ^{13}C-urea breath tests at 4-6 w after treatment), and no treatment achieved a > 80 % eradication rate. There was there was a tendency for a higher eradication rate in group D • No significant difference in incidence of side effects, but the lowest incidence was seen in group B and the highest in group D

	cfu/d, *L. sporogenes* 2 x 10^9 cfu/d	Probinu1® n = 17, mean age = 52 y	
Soo (2008)(219)	VSL#3 (1.8 x 10^{12}/ d for 5 w)	Adults with ulcerative colitis; n = 15; mean age = 52 y; no control group	• ↓ Mean ulcerative colitis disease activity index scores • ↑ Mucosal alkaline sphingomyelinase activity (↓ levels are found in ulcerative colitis)
West (2008)(220) and (2009)(221)	*L. paracasei* subsp. *paracasei* strain F19 (LF19) (1 x 10^8 CFU/serving of cereal, consumed on average just less than one serving/d, from 4 - 13 m of age)	Healthy, full term infants; immunized with DTaP (diphtheria, tetanus toxoid and acellular pertussis), polio and Hib-conjugate vaccines at 3, 5.5 and 12 m; n = 84 in test group; n = 87 in control group	• ↓ Cumulative incidence of eczema at 13 m of age (also seen in the sub-group of infants who were at high risk of allergy) • No effect on % of children diagnosed with asthma or allergic rhino-conjunctivitis (nor in the sub-group of infants at high risk of allergy) • No effect on median serum total IgE or frequency of sensitization to cow's milk, egg white, cat or dog, in the whole group or in the sub-group of high-risk infants • At 5.5 m of age, no effect on IFN-α or IL-4 mRNA levels, or IFN-α / IL-4 mRNA ratio, from PBMCs stimulated in vitro with anti-CD4 and anti-CD28 • At 13 m of age, ↑ IFN-α / IL-4 mRNA ratio in test group (in the high-risk sub-group of infants, this ratio was also higher but non-significantly) • No effect on IFN-α / IL-4 mRNA ratio from unstimulated cells at 5.5 or 13 m of age
Wickens (2008)(222)	*L. rhamnosus* HN001 (6 x 10^9 cfu/ d) or *B. animalis* subsp *lactis* HN019 (9 x 10^9 cfu/ d) mothers took the supplement from 35 w gestation until 6 m if breast- feeding, and the infants took the supplement from birth until 2 y	Pregnant women and their infants (who were at risk of allergic disease; i.e. their mother or father had either asthma, eczema or hay-fever); n = 144 in *L. rhamnosus* group; n = 152 in *B. animalis* group; n = 150 in placebo group	• ↓ Risk of developing eczema by 2 y in *L. rhamnosus* group (hazard ratio was similar for those with IgE-associated eczema [significant], and non-IgE-associated eczema [non-significant]) • ↓ Risk of developing SCORAD ≥ 10 by 2 y in *L. rhamnosus* group No effect of: • *B. animalis* on risk of developing eczema • *B. animalis* on risk of developing SCORAD ≥ 10 by 2 y • *L. rhamnosus* or *B. animalis* on the likelihood of having a positive skin prick test result for any allergen or food allergen at 2 y

Boge 2009(223)	*L. casei* DN-114 001 (20 x 10^{10} cfu/d for 7 w in pilot study, and for 13 w in confirmatory study)	Healthy elderly adults (aged ≥ 70 y) living in nursing home; vaccinated against influenza after 4 w consumption of the product Pilot study: n = 44 in test group, mean age = 82.36 y; n = 42 in control group, mean age = 84.98 y Confirmatory study: n = 113 in test group, mean age = 85.01 y; n = 109 in control group, mean age = 84.25 y	In the pilot study, compared to the control group, the test group had: • Trend for a better antibody response to influenza vaccination, especially for the H3N2 strain, 3 w post - vaccination • Trend for a higher seroprotection rate (the percentage of subjects achieving an influenza antibody titre of ≥ 40 in HI assay) for all vaccine strains, 3 w post - vaccination • Trend for a higher seroconversion rate (the percentage of subjects achieving at least a four-fold increase in antibody titre following vaccination) for all strains of the vaccine, 3 w post - vaccination In the confirmatory study, compared to the control group, the test group had: • ↑ Antibody levels for all three strains 3, 6 and 9 w post - vaccination, but only significantly so for the B strain of the vaccine • Many subjects were already seroprotected at baseline. If these subjects were removed from the analysis, there was an ↑ rate of seroprotection against the H1N1 strain in the test group 3 w post - vaccination. The rate was also ↑ for the H3N2 and B strains, but was not significant • No effect on seroconversion rates to the H1N1 and H3N2 strains 3, 6 and 9 w post -vaccination. There was a trend for an ↑ in seroconversion rates to the B strain at 3 w, and this was significant at 6 w and 9 w post - vaccination. 5 m after vaccination, seroconversion rates to H3N2 and B strains were still ↑ in the test group.
Kawase (2009)(224)	*Lactobacillus* GG (15.4 x 10^9 cfu / d) and *L. gasseri* TMC0356 (11 x 10^8 cfu / d) for 10 w, given in a fermented milk with *S. thermophilus* and *L. delbruekii* subsp. *bulgaricus*	Adults with clinical history of Japanese cedar pollinosis; n = 21 in test group, mean age = 36.9 y; n = 19 in placebo group, mean age = 36.8 y	• ↓ Mean symptom score (subjective symptom score) for nasal blockage at 9 w, and non-significantly at 10 w • ↓ Mean symptom - medication scores (sum of subjective symptom score and medication use score) for nasal blockage at 9 w and 10 w No effect on: • Symptom scores for sneezing, rhinorrhoea and itching • Blood eosinophil counts • Blood anti-Japanese cedar pollen IgE, total IgE, CRP concentration or Th1/Th2 ratio • Nasal symptoms (swelling and colour of inferior turbinate, and the amount and appearance of nasal discharge)

Kuitunen (2009)(225)	*L. rhamnosus* GG (10×10^9 cfu / d), *L. rhamnosus* LC705 (10×10^9 cfu/d), *B. Breve* Bb99 (4×10^8 cfu/d) and *Propionibacterium freudenreichii* ssp *shermani* JS (4×10^9 cfu/d) (supplemented to mothers from w 36 of gestation until birth, then the infants received half this dose plus 0.8 g GOS/d for 6 m)	Pregnant women and their infants (who were at risk of allergic disease; i.e. their mother and / or father had either asthma, allergic rhinitis, or atopic eczema); n = 445 in test group, n = 446 in placebo group	At 5 y of age: • In caesarean - delivered children, the test group had ↓ IgE-associated allergic diseases (particularly eczema) and less IgE sensitization. In vaginally delivered children there were no significant differences between groups. No effect on: • Percentage of children with allergic or IgE-associated disease • Frequency of eczema, IgE-associated (atopic) eczema, asthma, allergic rhinitis or atopic sensitization
Leyer (2009)(226)	*L. acidophilus* NCFM (1×10^{10} cfu/d for 6 m) or *L. acidophilus* NCFM and *B. animalis* subsp *lactis* Bi-07 (both 5×10^9 cfu/d for 6 m)	Healthy children, aged 3- 5 y; n = 110 in *L. acidophilus* group, mean age = 3.7 y; n = 112 in mixed group, mean age = 3.8 y; n = 104 in placebo group, mean age = 4.1 y	• ↓ Number of children who were absent for at least one day from their day care, in both test groups • ↓ Number of days absent in both test groups • ↓ Incidence and odds of having fever, cough and antibiotic use in *L. acidophilus* group • ↓ Incidence and odds of having fever, cough, rhinorrhoea and antibiotic use in the mixed group • ↓ Symptom duration (once corrected for age) in both test groups No effect on number of visits to a physician
Miele (2009)(227)	VSL#3 (4.5×10^{11} to 1.8×10^{12} bacteria/d depending upon age, for 1 y or until relapse)	Children with newly diagnosed ulcerative colitis; mean age = 9.8 y (range 1.7 - 16.1 y); received concomitant steroid	• ↑ Number of patients who achieved remission following the steroid induction treatment (measured by Lichtiger Colitis Activity Index) • ↓ Number of patients who relapsed within one year • ↓ Endoscopic and histological scores at 6 m, 12 m or at time of relapse

		induction treatment and oral mesalazine maintenance treatment; n = 14 in test group; n = 15 in placebo group	• No effect on duration of steroid exposure
Ouwehand (2009a)(228)	*L. acidophilus* NCFM (2 x 10^{10} cfu/d for 2 w) plus lactitol (prebiotic)	Adults aged over 65 y and regularly using non-steroidal anti-inflammatory drugs; n = 24 in test group, mean age = 70.3 y; n = 23 in placebo group, mean age = 71.7 y	• ↑ Faecal PGE2 concentrations • Trend for ↑ faecal calprotectin concentrations No effect on: • Faecal TNF-α concentrations • No effect on faecal IgA concentrations
Ouwehand (2009b)(229)	*L. acidophilus* NCFM (1.25 x 10^9 cfu/d) and *B. lactis* B1-04 (3.75 x 10^9 cfu/d), from March to June (the birch pollen season is during May and June)	Children with clinical and immunological birch pollen allergy; n = 24 in test group, mean age = 9.0 y; n = 23 in placebo group, mean age = 8.9 y	In the test group, compared to placebo group: • ↑ Number of children with infiltration of eosinophils in nasal mucosa in the placebo group in April / May, and this was prevented in the test group • ↑ Faecal IgA in the placebo group during the pollen season which was prevented in the test group • ↓ Serum IL-6 and TNF-a at the beginning of the study (March) but not differences in April/May • Non-significant ↓ in number of children with runny nose in May • Non-significant ↓ in number of children with nasal blocking in June • Non-significant ↑ in days with eye symptoms in May No effect on: • Serum birch pollen-specific IgE, IL-4, IL-5, IL-10, TGF-ß2, CD14, or blood eosinophil concentrations • Respiratory difficulty, coughing, or skin symptoms during the study • Faecal calprotectin concentrations • Serum birch pollen-specific IgE, IL-4, IL-5, IL-10, TGF-ß2, CD14, or blood eosinophil concentrations

Rautava (2009)(230)	*L. rhamnosus* GG 53103 and *B. lactis* Bb12 (1 x 10^{10} cfu of each/d until the age of 12 m)	Healthy infants who began to receive formula feeding before 2 m of age; mean age at start of study in test group = 38 d, and in control group = 35 d (range 2 - 65 d)	• ↓ Incidence of early acute otitis media during first 7 m of life • ↓ Need for antibiotic treatment during first 7 m of life • Incidence recurrent (3 or more episodes) respiratory infections during the first 12 m of life • ↓ Incidence of tympanostomy during first 12 m of life No effect on: • Incidence of respiratory or gastrointestinal infections during first 7 m of life • Incidence of recurrent acute otitis media or antibiotic use during the first 12 m of life
Scalabrin (2009)(231)	*L. rhamnosus* GG (1 x 10^{8} cfu/g, from 14 - 120 d of age)	Full-term formula-fed infants, 14 d of age; n = 70 in control group (extensively hydrolysed formula); n = 63 in EHF-LGG group (extensively hydrolysed formula plus LGG); n = 77 in PHF-LGG group (partially hydrolysed formula plus LGG)	No effect on: • Total serum IgE • Serum cows milk protein specific-IgE • Serum diphtheria, tetanus, *Hemophilus influenzae* type b, or poliovirus 1, 2 or 3 specific-IgG
Shimizu (2009)(232)	*B. breve* (3 x 10^{8}/d) and *L. casei Shirota* (3 x 10^{8}/d) plus GOS (10 g /d) from the day of admission into hospital until oral intake was initiated	Patients with severe systemic inflammatory response syndrome; n = 29 in test group, mean age = 55 y; n = 26 in control group, mean age = 55 y	• ↓ Incidence of infectious complications (enteritis, pneumonia, bacteremia) • Non-significant ↓ in mortality due to multiple organ dysfunction syndrome No effect on: • Number of patients staying for more than 21 d • Incidence of urinary tract infections • Ventilation-free days, or intensive care unit-free days at d 28

Gianotti (2010)(233)	*L. johnsonii* La1 and *B. longum* BB536 (in a 1:1 ratio; 2 x 10^7 cfu / d in low dose group; 2 x 10^9 cfu / d in high dose group; for 3 d before scheduled surgery, and resumed on d 2 after surgery until d 4 after surgery)	Adults undergoing elective colorectal resection for cancer; n = 10 in high dose group, mean age = 62.7 y; n = 11 in low dose group, mean age = 64.7 y; n = 10 in control group, mean age = 63.3 y	• No effect on ex-vivo analysis of intestinal DC phenotypes (% positive CD11c, HLA-DR, CD123, CD1a, CD83, CD83-123, CD83-11c, CD83-HLADR) • ↓ % positive HLA-DR, CD83, CD83-123, CD83-11c, CD83-HLADR when DC stimulated ex-vivo with LPS, and non-significantly ↓ % positive CD11c, CD123 or CD1a, in both low and high dose groups • Significant inverse correlation between the log number of the DC subsets CD83-123, CD83-11c,CD83-HLADR, and the total Log number of enteric bacteria (including the probiotics given) in stools • Subjects who were colonised with La1 had a ↓ proliferation of CD83-123, CD83-11c and CD83-HLA-DR compared to those who were not colonised with La1 • Ex vivo analysis of lymphocytes suggested probiotics ↑ non-specific proliferation of CD3, CD4, CD8, CD45RO and CD45RA positive cells, but this was not significant, and there was no effect on B cells (CD19) • No effect of LPS stimulation on lymphocyte (CD3, CD4, CD8, CD19, CD45RO, CD45RA positive cells) proliferation
Guillemard (2010)(234)	*L. casei* DN-114 001 (20 x 10^{10} cfu/d for 3 m)	Free living healthy elderly adults (aged ≥ 70 y); n = 537 in test group, median age = 76 y; n = 535 in control group, median age = 76 y	• ↓ Duration of common infectious disease episodes (during product consumption and whole study phase (product consumption phase and one month follow-up) • ↓ Cumulative duration of common infectious diseases (during product consumption and whole study phase) • ↓ Episode duration and cumulative duration of upper respiratory tract infections and rhinopharyngitis (during product consumption and whole study phase) No effect on: • Oxidative burst activity of blood monocytes • Cytolytic activity and count of blood NK cells • IL-1, IL-6, IFN-α, IFN-ß, IL-12, IL-10, TNF-α and IL-8 production in serum • Cumulative number of all common infectious diseases (during product consumption and whole study phase) • Episode duration or cumulative duration of lower respiratory tract infections or gastrointestinal infections • Number of upper respiratory tract infections, lower respiratory tract infections, gastrointestinal infections or each

			type of common infectious disease • Occurrence of common infectious diseases (the number of subjects having at least one common infectious disease) • Time to the first event or the severity of common infectious disease, intensity or duration of fever, or common infectious disease-associated medication • Quality of life scores
Hojsak (2010)(235)	*L. rhamnosus* GG (10^9 cfu/d for the duration of hospitalisation)	Hospitalized children older than 12 m; n = 376 in test group, mean age = 9.9 y, n = 366 in placebo group, mean age = 10.6 y	• ↓ Risk of gastrointestinal infections • ↓ Risk of respiratory tract infections • ↓ Risk of vomiting episodes • ↓ Risk of diarrhoeal episodes • ↓ Risk of episodes of gastrointestinal infections that lasted > 2 d • ↓ Risk of episodes of respiratory tract infections that lasted > 3 d • No effect on hospitalization duration
Luoto (2010)(236)	*Lactobacillus rhamnosus* GG (6 x 10^9 cfu / d given prophylactically from birth until discharge) or probiotics given "on demand" for gastrointestinal problems (strain and dose not given)	Very low birth weight enterally fed infants in neonatal intensive care units; n = 418 in prophylactic LGG group, n = 1024 in "probiotics on demand "group; n = 1900 in "no probiotics" group	This was a retrospective study, establishing the incidence of neonatal necrotising enterocolitis (NEC) in all very low birth weight infants or infants with gestational age < 30 w in five hospitals in Finland: one hospital gave LGG prophylactically to these infants, three hospitals gave probiotics "on demand" to infants with gastrointestinal problems, and the other hospital did not use probiotics. They found: • Highest incidence of NEC in the hospital who used LGG prophylactically (4.6%), and lowest in the hospitals who used probiotics on demand (1.8%). In the hospital that used no probiotics, incidence of NEC was 3.2%. The difference between groups was significant • LGG had no effect on the clinical course of NEC (age at onset of disease, need for surgery, incidence of bowel perforation with NEC, focal intestinal perforation with or without other clinical signs of NEC, or death cause by NEC)

*VSL#3 is a compound containing *Lactobacillus casei, L. plantarum, L. acidophilus, L. delbruekii* subsp *bulgaricus, Bifidobacteria longum, B. breve, B. infantis* and *Streptococcus salivarius* subsp *thermophilus* d, day; hr, hour; m, month; w, week; y, year; CRP, C-reactive protein; PGE2, prostaglandin E2; LPS, lipopolysaccharide; GI, gastrointestinal

1.6 CONCLUSIONS

This chapter has presented a description of the immune system and how its activity can be measured, and has summarized the results from studies that have investigated the effect that prebiotics and probiotics may have upon the functioning of the immune system, and subsequently upon infection and inflammatory diseases. Overall the results indicate that ß2-1 fructans are able to modulate some aspects of immune function, to improve the host's ability to respond successfully to certain intestinal infections, and to modify some inflammatory conditions. Overall, the picture that emerges from studies of probiotics on immune, infectious and inflammatory outcomes in humans is mixed and there appear to be large species and strain differences in effects seen. There are large differences between the studies using both prebiotics and probiotics, regarding the supplement given, the dose, duration of supplementation, characteristics of the subjects used, sample size, differences in the outcomes measured, and technical differences in how the measurements were made. It is important that future studies build upon the evidence reported so far, and that the gaps in the existing literature are addressed. The remainder of this thesis will describe a study that investigated the effect of Orafti® Synergy1 on the functioning of the immune system in healthy human middle-aged adults. This is an important area to investigate, because although several studies in older adults (43, 92, 94, 96, 97, 103) and one study in younger adults (98) have reported mixed effects of prebiotics on the adaptive immune system, this will be the first study of this type in middle-aged adults (45 - 65 years old). Orafti® Synergy1 was used as it contains both inulin and oligofructose. Inulin and oligofructose each promote the growth of different types of bacteria that may be beneficial to health and they appear to be active at different locations in the gut. Therefore to use the combination of inulin and oligofructose may be superior than to use either inulin or oligofructose individually.

CHAPTER 2

METHODS

In this chapter the following methods are described:

1. The processing of samples collected as part of experimental protocols involving human subjects
2. The analysis of blood immune cell phenotypes
3. The analysis of leukocyte phagocytic activity (Phagotest) and oxidative burst (Phagoburst)
4. Cell culture assays:
    a. Resuscitation of cryopreserved blood mononuclear cells
    b. Lymphocyte activation
    c. Lymphocyte cytokine production
    d. Lymphocyte proliferation (including development of the CFSE dilution assay)
5. The analysis of serum and salivary immunoglobulin concentrations
6. The analysis of influenza vaccine strain-specific immunoglobulin concentrations
7. The analysis of influenza vaccine-specific, antibody class-specific immunoglobulin concentrations

2.1 SAMPLE PROCESSING

2.1.1 PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION AND CRYOPRESERVATION

Blood (35 ml) was collected from subjects in the fasting state; blood was collected into heparin tubes and kept at room temperature until processing (which was within 8 hours of blood collection). Whole blood was layered onto an equal volume of Histopaque, density 1.077 g/ml (Sigma), and centrifuged (2000 rpm, 15 minutes, room temperature). Plasma was removed from the top layer and frozen at -80°C. Peripheral blood mononuclear cells (PBMCs) were removed from the interface and washed with RPMI-1640 medium (PAA) containing 0.5 ml penicillin and streptomycin (Sigma), and 2 ml of 200 mM glutamine (Sigma) per 500 ml. The PBMCs were then re-suspended in RPMI plus 2% fetal calf serum (FCS; PAA) and washed twice (1200 rpm, 10 minutes, room temperature). The PBMCs were then re-suspended in 1ml of RPMI plus 2% FCS, counted using a haemocytometer (Leica Galen III) and adjusted to the appropriate cell concentration for cryopreservation. An equal volume of freezing medium (FCS plus 15% dimethyl sulphoxide [DMSO, Fischer] at 4°C) was added drop-wise to the cell suspension on ice to make a final concentration of between 10×10^6 to 25×10^6 cells/ml. Cells were then stored at -196°C in a liquid nitrogen tank until use in further experiments.

2.1.2 SERUM PREPARATION

Blood (5 ml) was collected from subjects in the fasting state; blood was collected into serum tubes and kept at room temperature until processing. The serum tubes were centrifuged (2000 rpm, 10 minutes, 4°C), and serum was removed and stored at -80°C until analysis.

2.1.3 SALIVA PREPARATION

Saliva samples were collected by asking volunteers to chew on a dental roll for approximately 30 seconds; the dental rolls were then kept on ice until processing. Saliva was collected by pressure: by pushing the dental roll through a 5ml syringe. Samples were then centrifuged (2000 rpm, 10 minutes) to remove debris, and stored at -80°C until analysis.

2.2 BLOOD IMMUNE CELL PHENOTYPES

To enumerate different immune cell phenotypes within heparinised whole blood, cells were "stained" with various antibodies and analysed by flow cytometry. 100 µl of heparinised whole blood was added to tubes that contained various fluorescently conjugated antibodies to cell surface structures (Table 7), vortexed, and incubated at 4°C for 30 minutes. All antibodies were purchased from AbD Serotec.

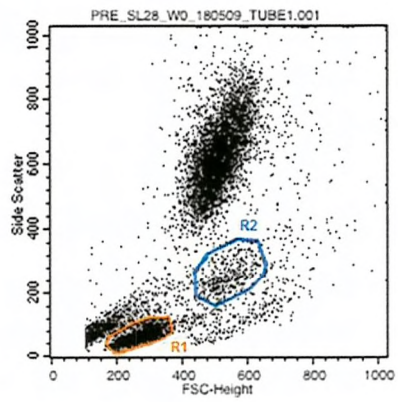
Tube	Stain	Cell type	Phenotype	Volume (µl)
1	None	Negative control (no stain)		-
2	Anti-CD3(FITC)/anti-CD4(RPE)	Th/inducer cells	$CD3^+CD4^+$	10
3	Anti-CD3(FITC)/anti-CD8(RPE)	Tc/suppressor cells	$CD3^+CD8^+$	10
4	Anti-CD3(FITC)/anti-CD16(RPE)	NK cells	$CD3^-CD16^+$	10
5	Anti-CD3(FITC)/anti-CD19(RPE)	B cells	$CD3^-CD19^+$	10
6	Anti-CD14 (FITC)	Monocytes	$CD14^+$	10
7	Anti-CD4(FITC)/anti-CD25(PECy5)/anti-CD127(PE)	T reg cells	$CD4^+CD25^+CD127^{lo}$	10+5+20
8	Anti-CD3(FITC)/ IgG1(RPE)	CD3(FITC) and PE dual stain controls		10+10
9	IgG2a(FITC)	CD14+ control		10
10	Anti-CD4(FITC)/ IgG1(RPECy5)/ IgG1(RPE)	T reg control		10+5+20
11	Anti-CD4(FITC)/anti-CD25(PECy5)/ IgG1(PE)	T reg control		10+5+20

Table 7 : Staining for blood immune cell phenotype analysis (fluorescein isothiosyanate [FITC], phycoerythrin [PE] and PE-cyanine 5 [PECy5] indicate the fluorochrome conjugated to the antibody)

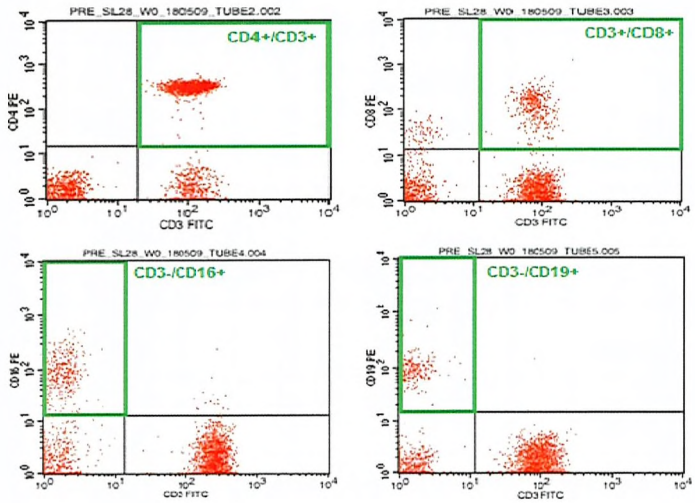
Then, cells were lysed (cell lysis buffer, BD) and incubated for a further 10 minutes at 4°C, protected from light. Samples were then centrifuged (1000 rpm, 7 minutes), the supernatant removed, and the cells washed (cell wash solution, BD) at 1000 rpm for 7 minutes. Supernatant was again removed, and cells fixed in 200 µl cell fix solution (BD), and stored at 4°C until analysis by flow cytometry within 48 hours (*Figure 16*) on a Becton Dickinson FACSCalibur fluorescence-activated cell sorter. Results analysed using Cellquest software.

Figure 16 : Flow cytometric analysis of cell phenotypes

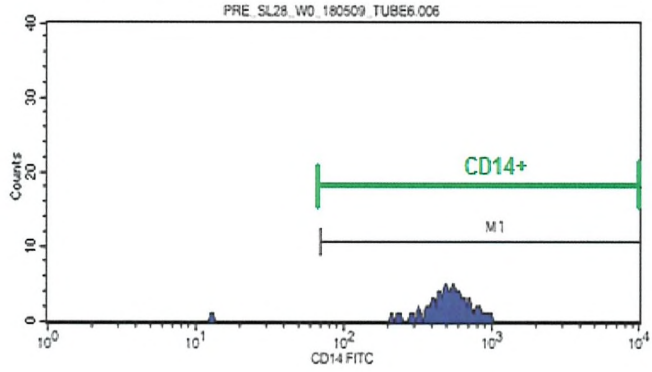
i) Gates were set around lymphocytes (R1) and monocytes (R2), and 10 000 total "events" were collected from each tube.



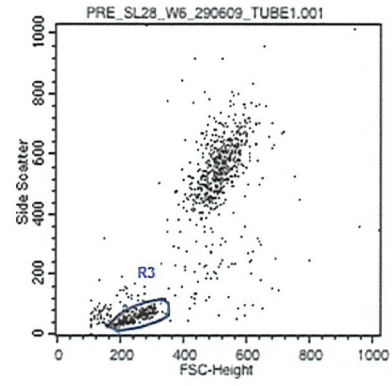
ii) Cells gated in R1 were then displayed in dot plots showing FITC against PE. Quadrants were drawn using the control tube CD3 (FITC) / IgG1 (PE), and the quadrants left in the same position while the stained tubes were run. $CD3^+/CD4^+$ and $CD3^+/CD8^+$ cells are reported as the percentage of cells in the upper right hand quadrant, and $CD3^-/CD16^+$ and $CD3^-/CD19^+$ cells are reported as the percentage of cells in the upper left hand quadrant.

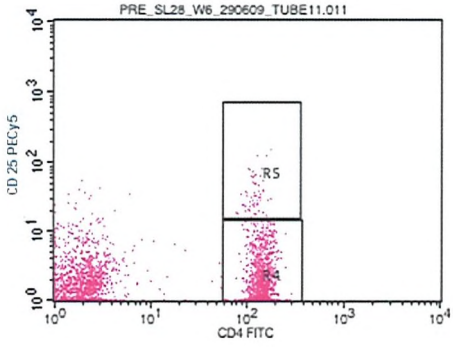


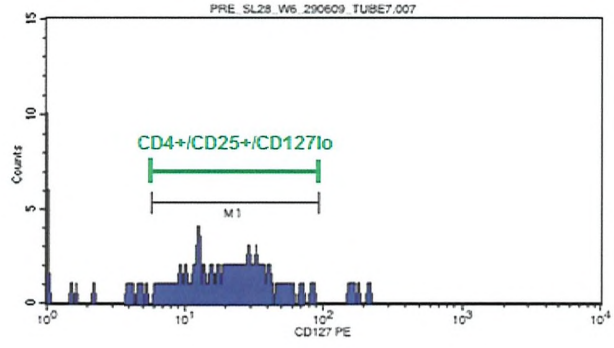
iii) Cells gated in R2 were then displayed in a histogram of CD14 FITC. The IgG2a (FITC) control tube was used to set the marker (M1), and the marker left in this position while the tube stained with CD14 FITC was run. The percentage of cells falling into M1 are reported as $CD14^+$ monocytes.



iv) T_{reg} cells were defined as $CD4^+/CD25^+$ cells with low staining for CD127. Lymphocytes were gated in R3 (2000 "events" in R3 were collected), and these cells displayed in a dot plot of CD4 (FITC) CD25 (PECy5), and a marker set around the cells staining positive for both (R5). Cells in R5 were then displayed in a histogram of CD127 (PE), and cells in the $CD127^{lo}$ section were reported as T_{reg} cells.







2.3 MEASUREMENT OF LEUKOCYTE PHAGOCYTOSIS (PHAGOTEST) AND OXIDATIVE BURST (PHAGOBURST)

Phagotest and Phagoburst kits (supplied by Orpegen Pharma) were used. Use of both kits was co-ordinated (timings of incubations/ washes) so that both assays could be done on the same day with several samples. Although the bacterial peptide N-formyl-Met-Leu-Phe (fMLP) was included in the Phagoburst test, as a negative control, use of this agent was excluded as there was little difference between the results seen with cells exposed to fMLP or the wash solution (negative control).

A sample of heparinised whole blood was stored at room temperature until analysis (within 8 hours of collection). Approximately 600 µl of fresh blood/subject was vortexed, aliquoted into 5 x 100 µl samples, and cooled on ice for 10 minutes.

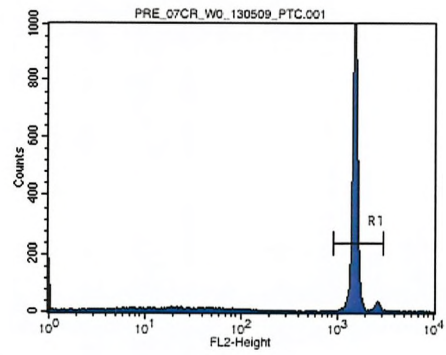
2.3.1 PHAGOTEST

20 µl of FITC-opsonized *E. coli* was added to two tubes, each containing 100 µl whole blood, and mixed. The test sample was incubated in a preheated water bath at 37°C for 10 minutes, while the control sample was incubated on ice for this time. Samples were then placed on ice to stop phagocytosis, and were quenched, to allow discrimination between internalised and surface-bound FITC-labelled *E. coli*. Samples were then washed twice, lysed, washed again, and finally the DNA stained. After incubation for 10 minutes on ice, samples were analysed by flow cytometry on a Becton Dickinson FACSCalibur fluorescence-activated cell sorter. Data were collected for 10,000 cells, and results analysed using Cellquest software. The percentage of cells performing phagocytosis (gated neutrophils and monocytes) and the number of ingested bacteria per cell (mean fluorescence intensity) were determined. An example of a typical flow cytometry profile is shown in Figure 17, along with a description of the method for gating and identifying the active cells and their phagocytic activity.

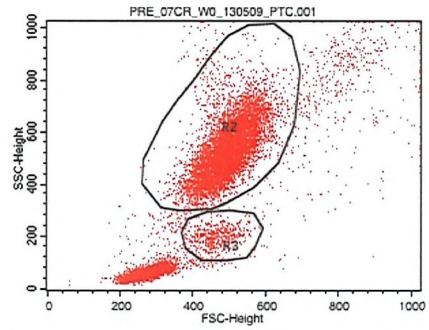
Figure 17 : Typical profiles obtained when measuring leukocyte phagocytic activity (Phagotest)

a) Control sample (whole blood incubated with stimulant at 0°C)

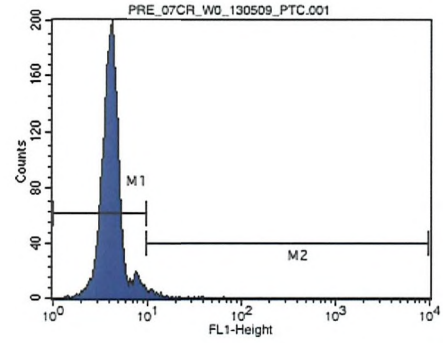
i) The profile from each sample was displayed in a histogram, so that leukocytes could be gated (R1), excluding any other material in the sample and any bacteria which had not been ingested. Diploid cells were stained with propidium iodide, which fluoresces in the FL-2 channel.



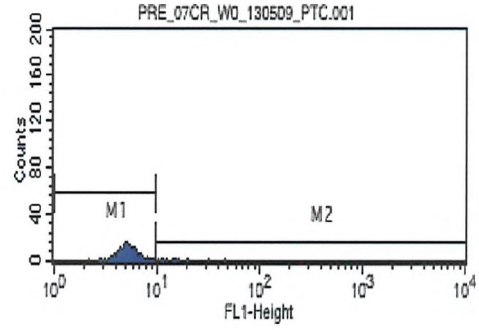
ii) The gated leukocytes were displayed in a dot plot, and grouped by size (forward scatter; FSC) and granularity (side scatter; SSC). Different cell populations were then identified and neutrophils (R2) and monocytes (R3) gated.



iii) Neutrophils (R2) from the control sample were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Markers M1 and M2 were set so that the number of cells which have undergone phagocytosis (M2) was set at ≤ 3 % for the cells gated in R2.

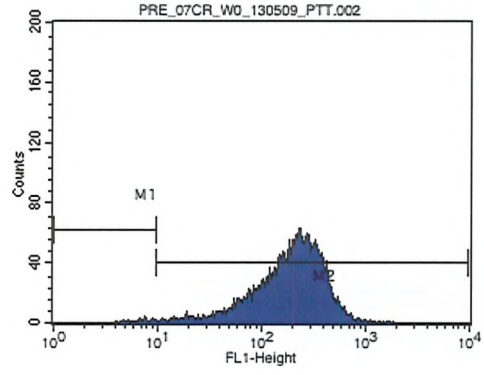


iv) Monocytes (R3) from the control sample were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Markers M1 and M2 were set so that the number of cells which have undergone phagocytosis (M2) was set at ≤ 3 % for the cells gated in R3.

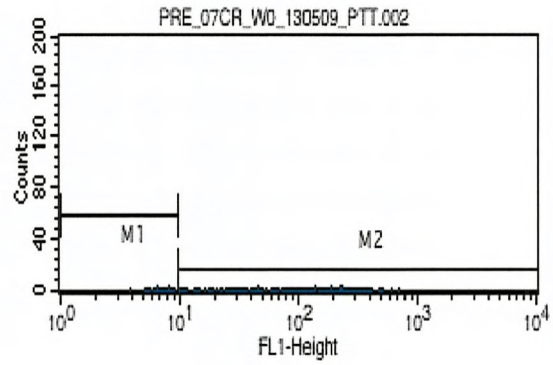


b) Test sample (whole blood incubated with stimulant at 37°C)

i) Neutrophils (R2) from the test sample were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Keeping the markers at the same position as in the control sample allows the number of cells which have undergone phagocytosis to be established (cells in M2), and the quantity of bacteria engulfed per neutrophil is determined by the mean fluorescence of cells in M2.



ii) Monocytes (R3) from the test samples were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Keeping the markers at the same position as in the control sample allows the number of cells which have undergone phagocytosis to be established (cells in M2), and the quantity of bacteria engulfed per monocyte is determined by the mean fluorescence of cells in M2.



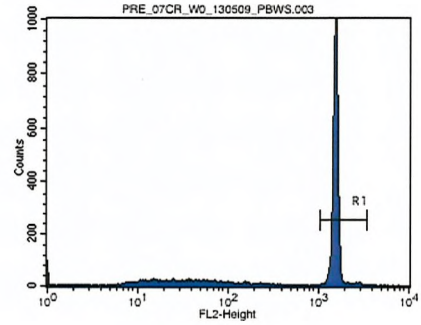
2.3.2 PHAGOBURST

20 µl of *E. coli,* or phorbol myristic acetate (PMA) or wash solution (control) was added to 3 x 100 µl whole blood, vortexed, and incubated in a preheated water bath at 37°C for 10 minutes. 20 µl of substrate solution was added to each tube, and the samples incubated again at 37°C for 10 minutes. Samples were lysed, washed, and the DNA stained. After incubation for 10 minutes on ice, the samples were analysed by flow cytometry on a Becton Dickinson FACSCalibur fluorescence activated cell sorter. Data were collected on 10,000 cells, and results analysed using Cellquest software. Neutrophils and monocytes were analysed separately, and the percentage neutrophils/monocytes producing reactive oxygen metabolites, and the mean fluorescence intensity were determined. An example of a typical flow cytometry profile is shown in *Figure 18*, along with a description of the method for gating and identifying the active cells and their oxidative activity.

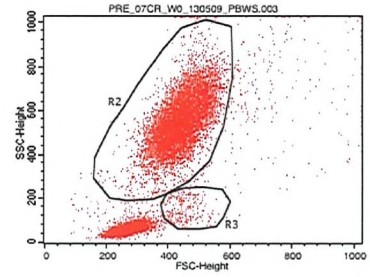
Figure 18 : Typical profiles obtained when measuring leukocyte oxidative burst (Phagoburst)

a) Control sample (whole blood incubated without stimulant at 37°C)

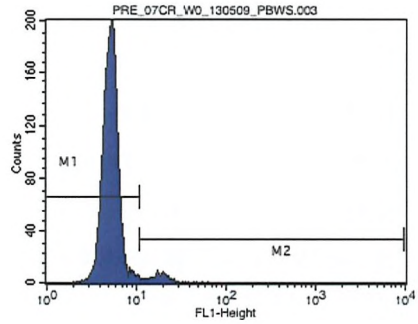
i) The profile from each sample was displayed in a histogram, so that leukocytes could be gated (R1), excluding any other material in the sample and any bacteria which have not been ingested. Diploid cells were stained with propidium iodide, which fluoresces in the FL-2 channel.



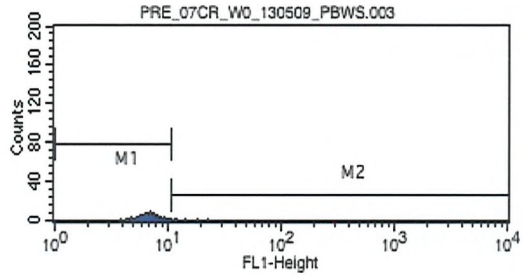
ii) The gated leukocytes were displayed in a dot plot, and grouped by size (forward scatter; FSC) and granularity (side scatter; FSC). Different cell populations were then identified and neutrophils (R2) and monocytes (R3) gated.



iii) Neutrophils (R2) from the control sample were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Markers M1 and M2 were set so that the number of cells undergoing oxidative burst (M2) was set at ≤ 3 % for the cells gated in R2.

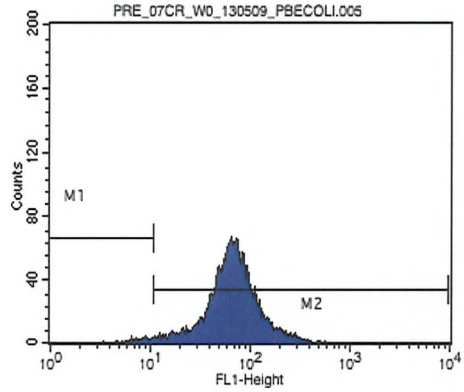


iv) Monocytes (R3) from the control sample were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Markers M1 and M2 were set so that the number of cells undergoing oxidative burst (M2) was set at ≤ 3 % for the cells gated in R3.

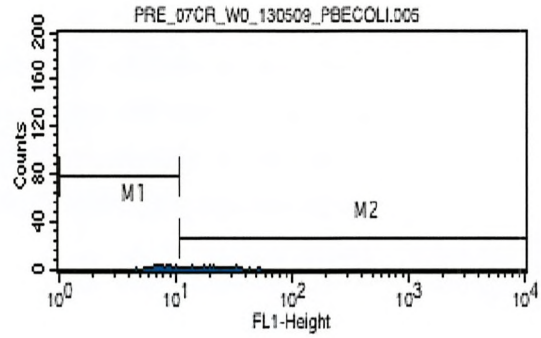


b) Test sample (whole blood incubated with E. Coli at 37°C)

i) Neutrophils (R2) from the test sample (incubated with *E. Coli*) were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Keeping the markers at the same position as in the control sample allows the number of cells which have undergone phagocytosis to be established (cells in (M2), and the quantity of bacteria engulfed per neutrophil is determined by the mean fluorescence of cells in M2.

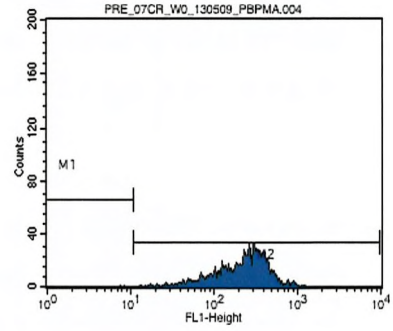


ii) Monocytes (R3) from the test samples were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Keeping the markers at the same position as in the control sample allows the number of cells which have undergone phagocytosis to be established (cells in M2), and the quantity of bacteria engulfed per monocyte is determined by the mean fluorescence of cells in M2.

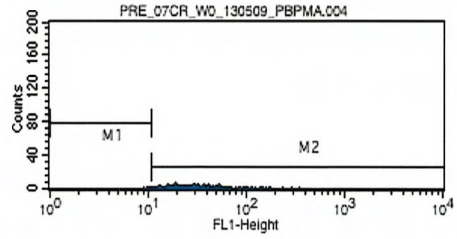


c) Positive control sample (whole blood incubated with PMA at 37°C)

i) Neutrophils (R2) from the positive control sample (PMA) were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Keeping the markers at the same position as in the control sample allows the number of cells which have undergone phagocytosis to be established (cells in M2), and the quantity of bacteria engulfed per neutrophil is determined by the mean fluorescence of cells in M2.



ii) Monocytes (R3) from the positive control sample (PMA) were then displayed in a histogram, showing the absolute cell numbers against FITC-staining (which is fluorescent in the FL-1 channel). Keeping the markers at the same position as in the control sample allows the number of cells which have produced ROS to be established (cells in M2), and the enzymatic activity per monocyte is determined by the mean fluorescence of cells in M2.



2.4 RESUSCITATION OF CRYOPRESERVED PBMCs FOR USE IN BATCH CULTURE

Cryopreserved cells in cryovials were removed from a liquid nitrogen storage tank (Statebourne, Biostar 5) and stored in a thermos in liquid nitrogen until resuscitation. Each cryovial was thawed rapidly in a water bath at 37°C. Cells were then placed on ice, and ice cold RPMI was added drop-wise for 1 minute and the volume then topped up to 10 ml. Cells were centrifuged (1200 rpm, 7 minutes, room temperature), then re-suspended to 1 ml and counted. Cells were then re-suspended to 1 x 10^6/ml in RPMI containing 10% FCS.

2.5 MEASUREMENT OF T CELL ACTIVATION BY CD69 EXPRESSION

Cryopreserved PBMCs were resuscitated as described in section 2.4, and re-suspended to 1 x 10^6cells/ml in RPMI medium plus 10% FCS. For the pilot study (Chapter 3), cells were cultured in triplicate (where possible) with ConA (Sigma, 5 µg/ml), PHA (Sigma, 5 µg/ml), non-dialysed vaccine (1/10, 1/100, 1/1000, 1/10 000, 1/100 000 dilution) and dialysed vaccine (1/10, 1/100, 1/1000, 1/10 000, 1/100 000 dilution). Unstimulated cultures were also performed.

For the main study (Chapters 5 and 6), cells were cultured in duplicate with ConA (5 µg/ml) and non-dialysed vaccine (1/10 dilution in RPMI); unstimulated cultures were also performed.

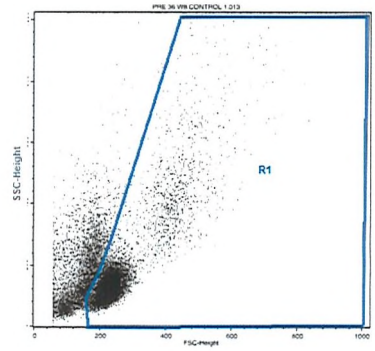
Cells were cultured in 96 well plates, for 24 hours at 37°C, 5% CO_2. Total culture volume was 200 µl (2 x 10^5 cells/well), consisting of RPMI medium supplemented with glutamine, antibiotics (penicillin and streptomycin) and 10% FCS, and the appropriate stimulant (see above). After 24 hours of culture, plates were centrifuged (1000 rpm, 10 minutes), and cells were removed and transferred to FACS tubes, centrifuged (1000 rpm, 7 minutes), and re-suspended in cell wash solution (BD). 100 µl of this cell suspension was added to each FACS tube containing the appropriate "stain":

1. 10 µl of anti-CD3(FITC)/anti-CD4(RPE) + 10 µl IgG2a(PECy5) (only used on cells from control cultures)
2. 10 µl of anti-CD3(FITC)/anti-CD4(RPE) + 5 µl anti-CD69(PECy5)

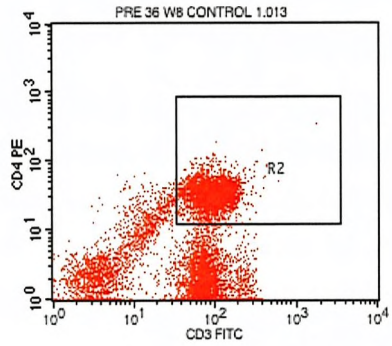
Cells were incubated for 20 to 30 minutes at 4°C, washed, and re-suspended in 200 µl Cell Fix Solution (BD), and then kept at 4°C until analysis by flow cytometry. A typical flow cytometry profile is shown in Figure 19, along with a description of the method for gating and identifying the appropriate cells.

Figure 19 : Analysis of T cell activation using the flow cytometer

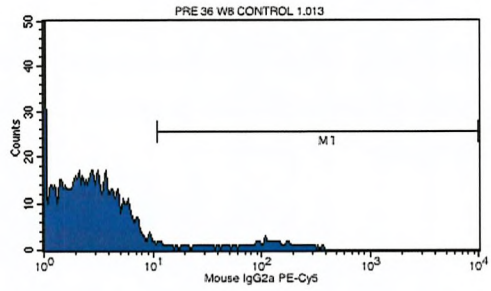
i) The profile of cells from the control sample (isotype control) was first displayed in a dot plot, so that they could be grouped by size (forward scatter; FSC) and granularity (side scatter; SSC). Cells were gated to isolate lymphocytes (R1).



ii) The gated lymphocytes were then displayed in a dot plot, showing CD3 FITC fluorescence against CD4 PE fluorescence, and a marker set around cells staining positive for both.

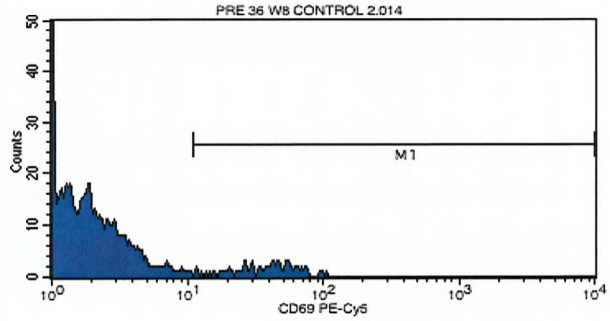


iii) Cells gated in R2 were then displayed in a histogram. A marker (M1) was then drawn to exclude cells with non-specific staining for PECy5. The marker was then left in the same position, and cells from the control, ConA and vaccine stimulated cultures (stained with anti-CD3[FITC]/anti-CD4[RPE] + anti-CD69[PECy5] were then run. The percentage of cells in M1 is reported as the percentage of cells which had undergone activation. The MFI (geometric mean) of cells in M1 was also reported.

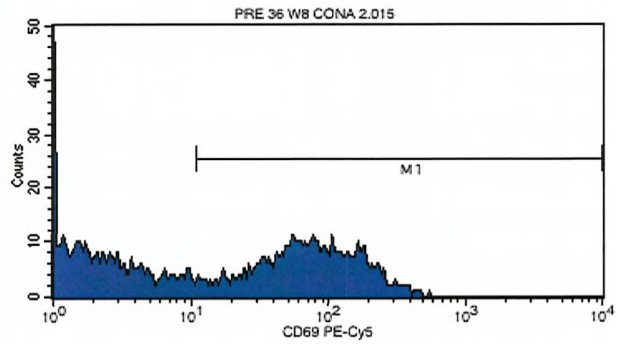


Typical profiles of cells cultured with varying stimulants:

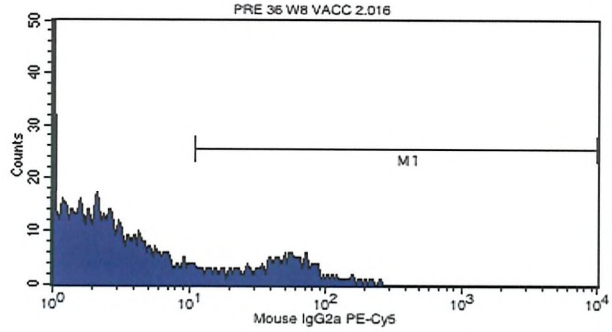
Control tube



ConA tube



Vaccine tube



2.6 MEASURMENT OF LYMPHOCYTE CYTOKINE PRODUCTION

Cryopreserved PBMCs were resuscitated as described in section 2.4, and re-suspended to 1 x 10^6 cells/ml in RPMI medium plus 10% FCS.

For the pilot study, cells were cultured in triplicate if possible (this was reduced to duplicate or singlet where total cell counts were low) in 96 well plates, for 24, 48 or 72 hours at 37°C, 5% CO_2, with the stimulants as described in section 2.5.

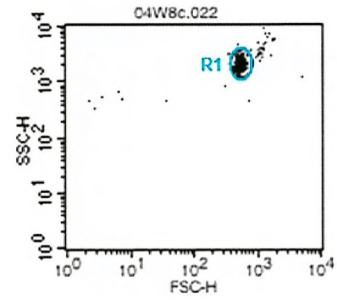
For the main study, cells were cultured in 96 well plates, for 24 hours at 37°C, 5% CO_2, with the same stimulants as described in section 2.5.

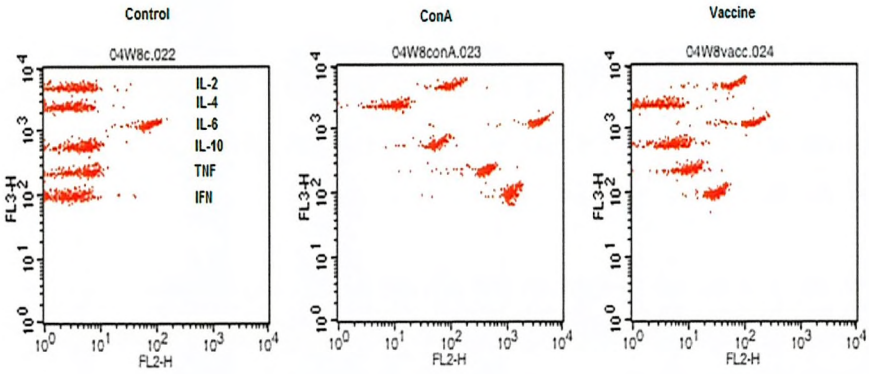
Total culture volume was 200 µl (2 x 10^5 cells/well), consisting of RPMI medium supplemented with glutamine, antibiotics (penicillin and streptomycin) and 10% FCS, and the appropriate stimulant. Following culture, plates were centrifuged at 1000 rpm for 10 minutes, and the supernatants removed and stored at -20 °C until analysis.

The Human Th1/Th2 Cytokine Kit II (supplied by BD) was used to analyse the concentrations of IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ in the culture supernatants. 25 µl of each sample were added to tubes containing 25 µl of the cytokine capture beads and 25 µl of detection reagent. Samples were incubated for 3 hours, then washed and re-suspended. Data was collected on a Becton Dickinson FACSCalibur fluorescence-activated cell sorter using CellQuest software. Cytokine standards from a concentration of 0 pg/ml up to 20 000 pg/ml were run. Analysis was performed with FCAP Array software. Standards were used to generate a standard curve, and from the standard curve, the concentrations of the different cytokines in each sample were determined.

Figure 20 : Lymphocyte cytokine production

Cytokine capture beads were gated in R1. These beads were then displayed in dots plots of FL2 against FL3, as shown below. For control cultures, concentrations of all cytokines (excluding IL-6 which has high background readings) are low. When cells are stimulated, with, for example, ConA, concentrations of all cytokines are increased.





2.7 DETERMINATION OF LYMPHOCYTE PROLIFERATION

2.7.1 USING ^{3}H-THYMIDINE INCORPORATION (FROM PILOT STUDY SAMPLES)

Cryopreserved PBMCs from pilot study samples were resuscitated as described in section 2.4, re-suspended to 1 x 10^{6}/ml, and stimulated as described in section 2.5. Cells were cultured in triplicate in 96 well plates, for 48 hours at 37°C, 5% CO_2. Total culture volume was 200 µl (2 x 10^{5} cells/ well), consisting of RPMI medium supplemented with glutamine, antibiotics (penicillin and streptomycin) and 10% FCS, and the appropriate stimulant. After 48 hours in culture (for mitogen stimulated and control wells) and after 120 hours in culture (for vaccine stimulated cultures), 20 µl ^{3}H-thymidine was added to each well (0.2 µCi/well) and cells were incubated for a further 18 hours. Plates were then stored at -20 °C until harvesting, or harvested immediately. Cells were harvested onto glass fibre filters and washed and dried using a Skaton Cell Harvester. Radioactive thymidine incorporation was determined by liquid scintillation counting for one minute. Data are expressed as peak thymidine incorporation and stimulation index (thymidine incorporation of stimulated cells /thymidine incorporation of unstimulated cells).

2.7.2 CARBOXYFLUORESCEIN SUCCINIMIDYL ESTER (CFSE) DILUTION

2.7.2.1 INTRODUCTION

The principles of this method are described in section 1.3.2.2.2. The method was adapted from the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Molecular Probes™). The final method is described first, followed by descriptions of the experiments which lead to this adapted method.

2.7.2.2 FINAL METHOD

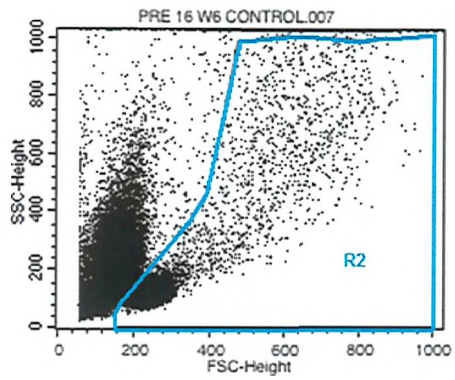
Cryopreserved PBMCs were resuscitated as described in section 2.4, and re-suspended at 1 x 10^6/ml. 1mM CFSE stock solution (Molecular Probes™), was added to the cell suspension at a 1/100 dilution (a 10 μM CFSE working solution), and incubated for 37°C, 5% CO_2 for 10 minutes. Staining was quenched by adding five volumes of ice cold media, on ice, and incubating for 5 minutes on ice. Cells were then washed and re-suspended to 1 x 10^6/ml in RPMI plus 10% FCS. Cells were cultured in duplicate in 96 well plates:

- For the pilot study, for 96 hours at 37°C, 5% CO_2.
- For the main study, for 168 hours at 37°C, 5% CO_2.

Total culture volume was 200 μl (2 x 10^5 cells/ well), consisting of RPMI medium supplemented with glutamine, antibiotics (penicillin and streptomycin) and 10% FCS, and the appropriate stimulant. After culture, cells were transferred to FACS tubes and kept on ice until analysis using the flow cytometer. An example of a typical flow cytometry profile is shown in Figure 21, along with a description of the method for gating and identifying the appropriate cells.

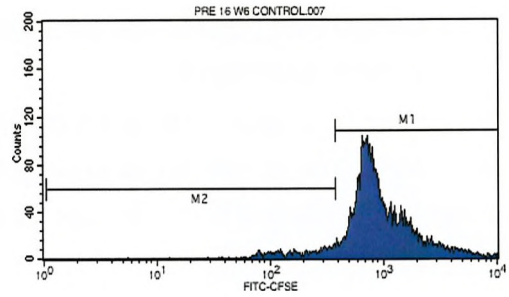
Figure 21 : Typical analysis of lymphocyte proliferation by CFSE staining (after 168 hours culture)

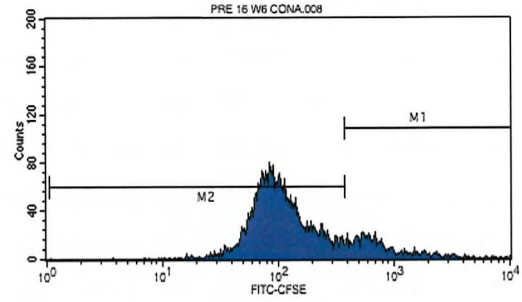
1. The profile of cells from the control (no stimulation) sample, stained with CFSE is first displayed as a dot plot, so that they can be grouped by size (forward scatter; FCS) and granularity (side scatter; SSC). Lymphocytes were then gated (R2).



2. Gated lymphocytes were displayed as a histogram of FITC-CFSE staining against cell count. As can be seen, the fluorescence of cells stained with CFSE, but not stimulated (controls) lies at around 10^3, and remains in this position during the period of culture (96 / 168 hours).

3. For cells stained with CFSE and stimulated with ConA, fluorescence decreases progressively as the cells divide.





FlowJo software was used for analysis. A marker was set on the control sample, around the peak of undivided cells (M1). The marker was kept in this position when analysing the stimulated samples, so that those cells which have proliferated will move out of M1 and into M2. The percentage of cells that have proliferated (M2) is reported. The mean fluorescence intensity of the sample changes as the cells divide, this is also reported. Software programmes such as Weasel (used for pilot study) or FlowJo can be used to determine the number of peaks, and therefore divisions, that have occurred.

2.7.2.3 MODIFICATIONS MADE TO THE ORIGINAL METHOD (USING PILOT STUDY SAMPLES)

2.7.2.3.1 RESUCITATION OF CRYOPRESERVED PBMCs

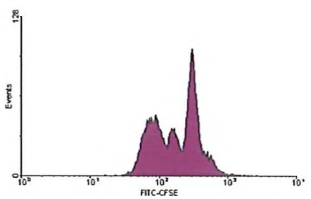
Cryopreserved PBMCs were first resuscitated and re-suspended in phosphate buffered saline (PBS), but upon counting on the hemocytometer, the majority of cells were found to be dead. Thus, this was repeated, this time resuscitating the cells in RPMI plus FCS. Upon counting, there was found to be a good viability (approximately 90%) and thus from this point forward, RPMI plus FCS was used in this stage.

2.7.2.3.2 DETERMINATION OF THE OPTIMUM CONCENTRATION OF CFSE

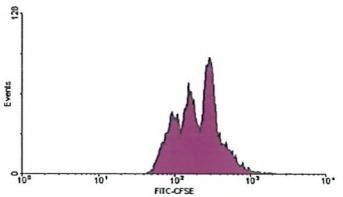
The method was followed as described above, but cells were stained with 1 μM, 10 μM, or 25 μM CFSE and cultured for 72 hours. From the results (Figure 22), it can be seen that both 1 μM and 10 μM produce good traces, and that using 25 μM results in staining that is too bright.

Figure 22 : Determination of optimum concentration of CFSE for use in cell proliferation assays (cells stained with varying concentrations of CFSE and stimulated with PHA or ConA or not stimulated (control), and cultured for 72 hours)

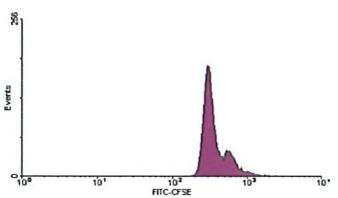
ConA 1 µM CFSE



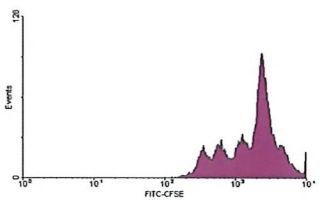
PHA 1 µM CFSE



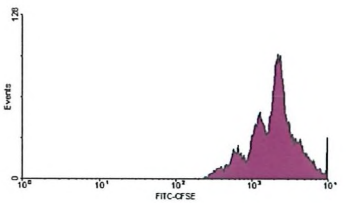
Control sample (no stimulation) 1 µM CFSE



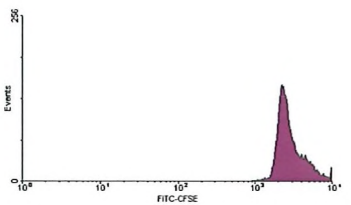
ConA 10 µM CFSE



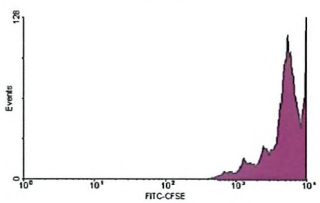
PHA 10 µM CFSE



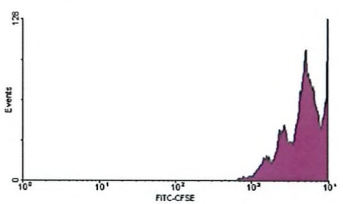
Control sample (no stimulation) 10 µM CFSE



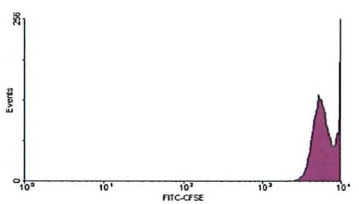
ConA 25 µM CFSE



PHA 25 µM CFSE



Control sample (no stimulation) 25 µM CFSE



2.7.2.3.3 DETERMINATION OF THE OPTIMUM CONCENTRATION OF PHA AND ConA

^{3}H-thymidine incorporation was used to determine the optimum concentration of PHA and ConA to stimulate the cells. Cryopreserved PBMCs from three subjects were resuscitated in RPMI medium plus FCS, washed, and made up to 1 x 10^6/ml in RPMI plus 8% FCS. PBMCs were cultured in triplicate at 37°C, 5% CO_2 in a 96 well plate at 2 x 10^5 cells/well. The total culture volume was 200 µl, consisting of RPMI medium supplemented with glutamine plus antibiotics (penicillin and streptomycin) and 8% FCS. Cells were stimulated with PHA or ConA at concentrations of 0, 0.5, 1, 5, 10 and 50 µg/ml. After 48 hours in culture, 20 µl of ^{3}H-thymidine was added to each well (0.2 µCi/well) and the cells cultured for a further 18 hours. Cells were harvested onto glass fibre filters and washed and dried using the Skatron Cell Harvester. Radioactive thymidine incorporation was determined by liquid scintillation counting for one minute. Data were expressed as peak thymidine incorporation, and the optimal concentration of both PHA and ConA was determined to be 5 µg/ml, and PHA was seen to generate a greater proliferative response (Figure 23 and Figure 24).

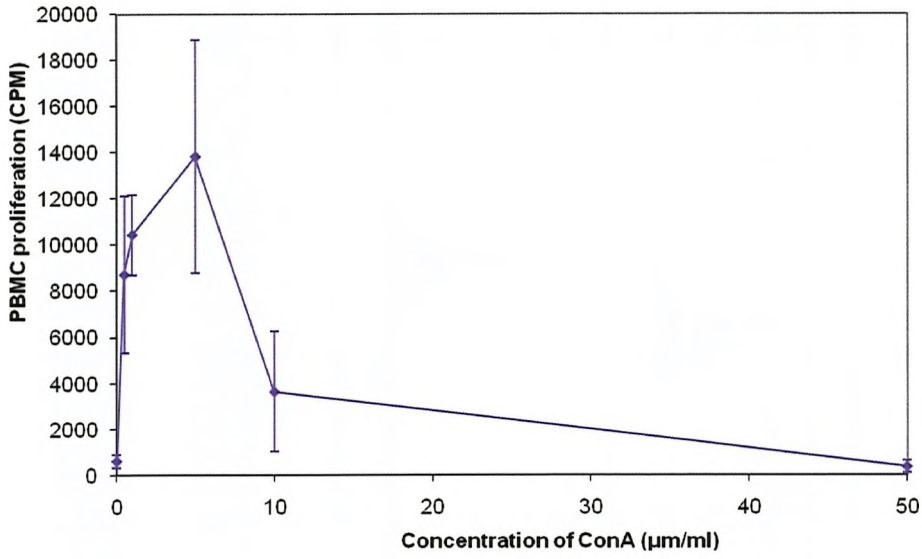


Figure 23 : Relationship between concentration of ConA and magnitude of lymphocyte proliferation, as measured by ^{3}H-thymidine incorporation

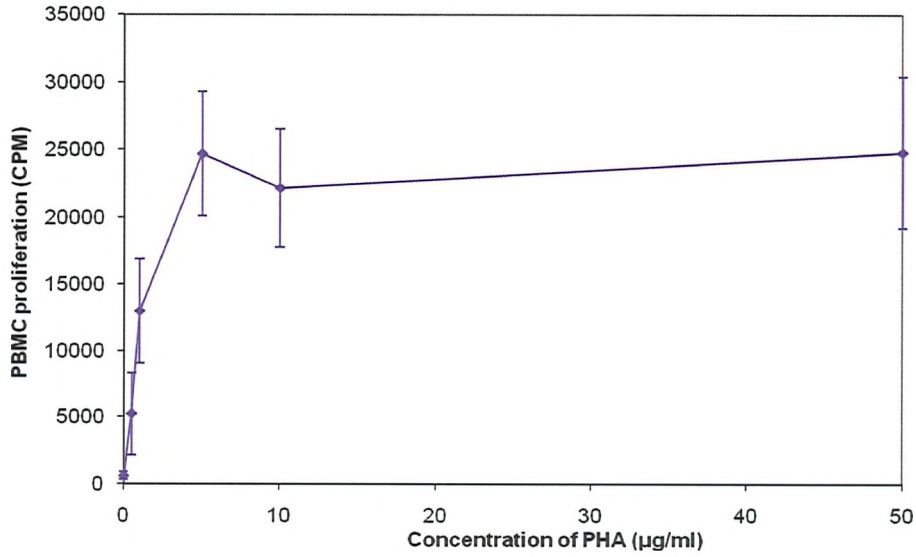
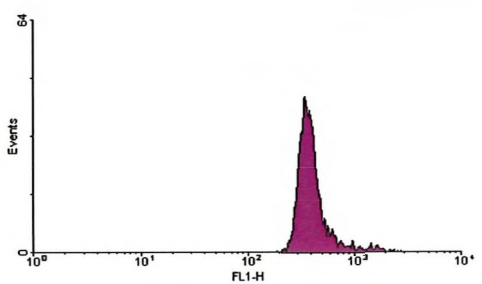


Figure 24 : Relationship between concentration of PHA and magnitude of lymphocyte proliferation, as measured by H-thymidine incorporation

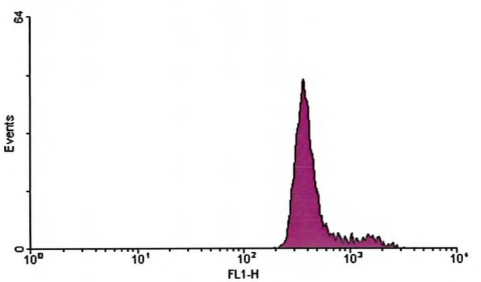
A concentration of 5 µg/ml of PHA and ConA was then used to stimulate cells in a CFSE assay. As shown in Figure 25, cells were shown to proliferate, and the comparison of using 1 µg/ml of stimulant or 5 µg/ml of stimulant is shown.

Figure 25 : Comparison of using 1 µg/ml and 5 µg/ml PHA and ConA for stimulation of lymphocyte proliferation, measured by CFSE staining

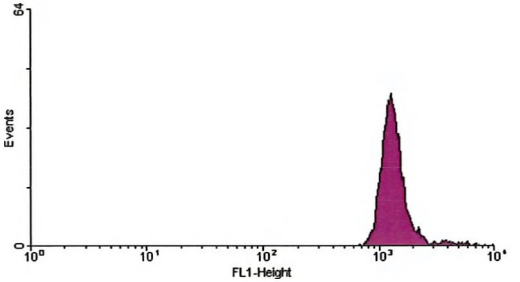
PHA
1 µg/ml



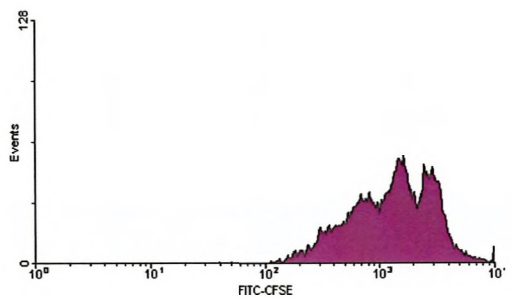
ConA
1 µg/ml



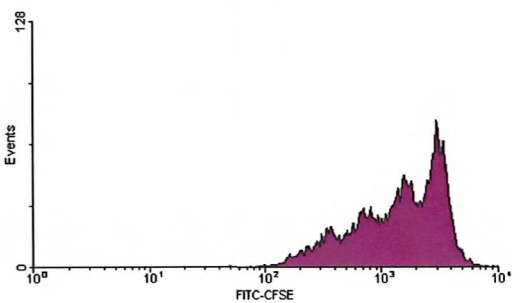
Control



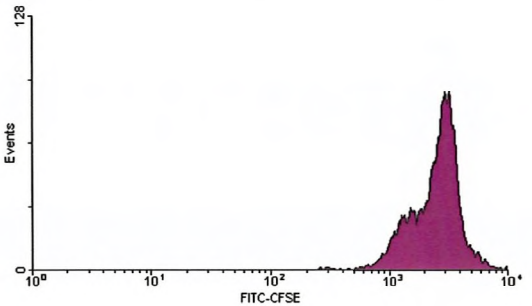
PHA
5 µg/ml



ConA
5 µg/ml



Control

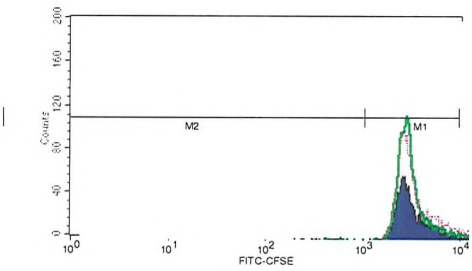


2.7.2.3.4 DETERMINATION OF THE OPTIMUM INCUBATION TIME WITH STIMULATION WITH MITOGENS

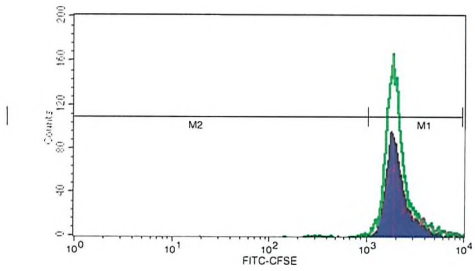
The method was followed as above, but cells were cultured for 24, 48, 72 or 96 hours. From the results, shown in Figure 26, it can be seen that 96 hours is the optimal time for culture in order to see maximum proliferation (purple peak = control sample, green peak = ConA stimulated sample, pink peak = PHA stimulated sample)

Figure 26 : Determination of the optimum culture time to measure lymphocyte proliferation by CFSE staining with stimulation with mitogens

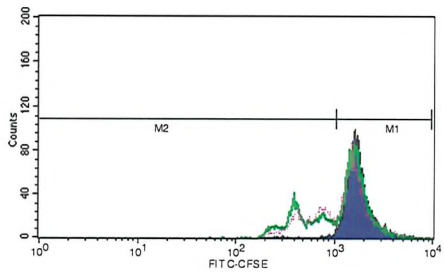
Day 1



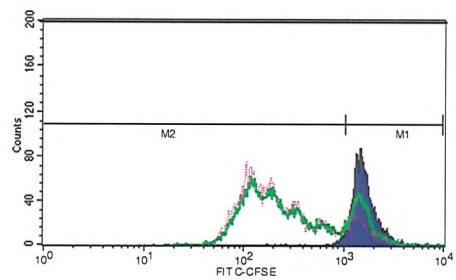
Day 2



Day 3



Day 4



For the pilot study, vaccine stimulated cultures were cultured for 96 hours before assessing lymphocyte proliferation by CFSE dilution, as there are a small number of vaccine- specific T cells, and thus it will take longer to see a proliferative response, compared to mitogen stimulated cultures. Upon analysis of the pilot study data, it was apparent that even 96 hours is not long enough to see a good vaccine-specific proliferative response, and so for the main study, cells were cultured for 168 hours (7 days), for both mitogen and vaccine stimulated cultures.

2.7.2.3.5 TRIPLICATE AND SINGLE WELLS

Unlike ^{3}H-thymidine incorporation, there is little variation in CFSE dilution between triplicate samples, as shown in Table 8. Therefore, singlet or duplicate wells can be used and this will allow cells to be used in other experiments.

	Control	ConA	PHA
D1	325.3	359.4	381.7
	309.6	339.8	383.3
	313.9	346.6	405.6
St Dev	8.1	10.0	13.3
Coefficient of variation	2.6%	2.9%	3.4%
D2	234.4	281.9	277.3
	242.4	268.3	282.1
	238.9	297.1	259.8
St Dev	4.0	14.4	11.8
Coefficient of variation	1.7%	5.1%	4.3%

Table 8 : Intra-variability of triplicate samples of CFSE staining

2.7.2.3.6 DAY 0 READING

It was anticipated that Day 0 readings of both unlabelled and labelled cells would be made so as to establish settings on the flow cytometer. However it was found that the readings for day 0 are always very bright, due to the CFSE having just been added. Therefore, it became clear that day 1 readings may be used to establish the settings, as the brightness diminished somewhat by this point.

2.7.2.3.7 PROPIDIUM IODIDE (PI)

1 µl of PI (25 µl of a 1 mg/ml solution into 75 µl PBS) was added to the samples, in order to exclude the dead cells. This was decided to be unnecessary, as there are very few dead cells in the gated population being analysed (R1 in *Figure 27*). There are virtually no cells appearing in the area of Plot 2 (gated on R1), where dead cells stained with PI would be expected to appear. Therefore, staining with PI to exclude dead cells was deemed unnecessary, and was not included in the final protocol.

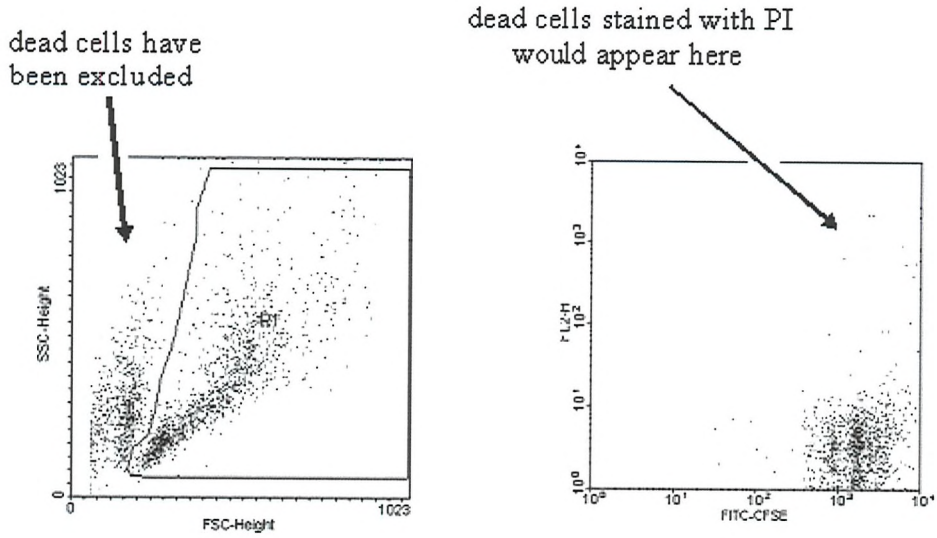


Figure 27 : Exclusion of dead cells by propidium iodide

2.8 MEASUREMENT OF TOTAL ANTIBODY CONCENTRATIONS

2.8.1 SERUM IgA

Human IgA ELISA kit (Zeptometrik, catalogue # 0801197) was used to analyse the IgA concentration of serum samples. Samples were analysed in singlet. Serum samples were diluted 1:40,000 in assay diluent, and 200 µl of each sample were added to separate wells. Samples were incubated for 30 minutes at 37°C, and wells were then washed four times. 100 µl of Detector Antibody (conjugated goat anti-human peroxidase) was added to each well, and samples were incubated for a further 30 minutes at 37°C. Wells were washed again four times, and 100 µl of Substrate (tetramethyl benzidine) was added to each well, and samples were incubated for a further 30 minutes at room temperature. 100 µl of Stop Solution was added to each well and the optical density of each well at 450 nm was measured using a microtitre plate reader (Thermo Labsystems, Original Multiskan).

2.8.2 SERUM IgM

Human IgM ELISA kit (Zeptometrik, catalogue # 0801183) was used to analyse the IgM concentration of serum samples. Samples were analysed in singlet. Serum samples were diluted 1:20,000 in assay diluent, and 200 µl of each sample were added to separate wells. Samples were incubated for 30 minutes at 37°C, and wells were then washed four times. 100 µl of Detector Antibody (conjugated goat anti-human IgM peroxidase) was added to each well, and samples were incubated for a further 30 minutes at 37°C. Wells were washed again four times, and 100 µl of Substrate (tetramethyl benzidine) was added to each well, and samples were incubated for a further 30 minutes at room temperature.

100 µl of Stop Solution was added to each well and the optical density of each well at 450 nm was measured using a microtitre plate reader (Thermo Labsystems, Original Multiskan).

2.8.3 SERUM IgG

IgG ELISA kit (Immunodiagnostic, catalogue # K6510) was used to analyse the IgG concentration of serum samples. Samples were analysed in singlet. Serum samples were diluted 1:10,000 in 0.9 % NaCl. 10 µl of each sample was added to separate wells containing 200 µl of NaCl, and samples were incubated for 60 minutes at room temperature on a horizontal mixer. Wells were washed five times, and 200 µl conjugate (rabbit-anti-IgG, peroxidise labelled) added to each well. Samples were incubated for 60 minutes at room temperature on a horizontal mixer. Wells were washed five times, and 200µl of substrate solution (tetramethyl benzidine) added to each well. Samples were incubated for 10 - 15 minutes at room temperature, and 50 µl of stop solution added to each well, and mixed shortly. The optical density of each well at 450 nm was measured using a microtitre plate reader (Thermo Labsystems, Original Multiskan).

2.8.4 SALIVARY sIgA

Secretory IgA ELISA (Demeditec, catalogue # DEXK276) was used to analyse the sIgA concentration of saliva samples. Samples were analysed in singlet. Saliva samples were diluted 1:2000 in buffer. 100 µl of each sample was added to separate wells, mixed shortly, and incubated for 90 minutes at 37°C. Wells were washed three times, and 100 µl of conjugate (second antibodies directed towards the alpha-chain of sIgA and labelled with peroxidise enzyme) was added to each well. Samples were incubated for 30 minutes at 37°C. Wells were washed five times and 100 µl of substrate (chromagen substrate mixture) was added to each well. Samples were incubated for 15 minutes at 20 - 25 °C. 100 µl of stop solution was added to each well and the optical density of each well at 450 nm was measured using a microtitre plate reader (Thermo Labsystems, Original Multiskan).

Total salivary protein content was measured using a Bradford assay. Samples were analysed in singlet, and were diluted (1:10 for pilot study samples and 1:5 for main study samples) in de-ionised water. 250 µl of Bradford's reagent (Sigma) and 10µl of each sample were added to wells in 96 well plates, and mixed. The optical density of each well at 620 nm was measured using a microtitre plate reader (Thermo Labsystems, Original Multiskan). Bovine serum albumin (Sigma Aldrich) was used as the standard.

2.9 SERUM VACCINE-SPECIFIC ANTIBOBIES BY HEMAGGLUTINATION INHIBITION ASSAY

Vaccine-specific antibodies in serum were measured externally by ViroClinics B. V. (The Netherlands), using a hemagglutination inhibition (HI) assay. This is based on the principle that influenza viruses agglutinate erythrocytes of some avian species (including turkeys), and incubation of the virus with virus-specific antibodies (present in serum of subjects vaccinated) prior to this agglutination reaction will inhibit the agglutination.

Briefly, the method is as follows. Serum samples were pre-treated with cholera to remove non-specific anti-hemagglutinin activity (serum was incubated with cholera filtrate for 16 hours at 37°C and then for a further 1 hour at 56°C to inactivate the cholera filtrate). Turkey erythrocytes were collected and stored overnight at 4°C, and then prepared by washing three times in PBS at 600 x g for 10 minutes at room temperature, and leukocytes were removed after each wash. Turkey red blood cells were then diluted in PBS to make a 1% solution. Virus antigen was then titrated (by incubating two-fold serial dilutions of the virus with turkey erythrocytes and reading hemagglutination patterns) and the working dilution of the virus antigens was determined. Pre-treated serum was then serially diluted two-fold (from a 1:20 dilution to a 1:20480 dilution in rows in microtitre plates) and 100 µl of serum was incubated with 25 µl of the working dilution of the virus for 30 minutes at 37°C. 25 µl of turkey erythrocytes were then added, the solution mixed and incubated for 1 hour at 4°C. Plates were then scored for inhibition of hemagglutination as shown by the sedimentation of erythrocytes. In each row the highest well number that showed complete inhibition was scored, starting with well number one for the 1:20 dilution. If wells showed half or incomplete inhibition, the previous well number was scored with the addition of "++". The scored well numbers correspond to HI titres which are read from Table 9, below. The antibody titre therefore, is the maximum dilution of the serum able to inhibit agglutination of the turkey erythrocytes with the influenza viruses under the above conditions.

Score	Inhibition of agglutination	Corresponding titres
0	Not present	<10
++	Half at dilution 1:20	10
1	Complete at dilution 1:20	20
1++	Half at dilution 1:40	30
2	Complete at dilution 1:40	40
2++	Half at dilution 1:80	60
3	Complete at dilution 1:80	80
3++	Half at dilution 1:160	120
4	Complete at dilution 1:160	160
4++	Half at dilution 1:320	240
5	Complete at dilution 1:320	320
5++	Half at dilution 1:640	480
6	Complete at dilution 1:640	640
6++	Half at dilution 1:1280	960
7	Complete at dilution 1:1280	1280
7++	Half at dilution 1:2560	1920
8	Complete at dilution 1:2560	2560
8++	Half at dilution 1:5120	3840
9	Complete at dilution 1:5120	5120
9++	Half at dilution 1:10240	7680
10	Complete at dilution 1:10240	10240
10++	Half at dilution 1:20480	15360
11	Complete at dilution 1:20480	20480 or higher

Table 9 : Scoring of serum samples for inhibition of agglutination, and corresponding antibody titres

2.10 MEASUREMENT OF ANTIBODY CLASS - SPECIFIC, VACCINE - SPECIFIC ANTIBODIES BY ELISA

This method was developed based on the method described in Olivares et al. 2007 (237). A 96 well Maxisorb ELISA plate (Fischer Scientific) was coated with a 500 ng/ml solution of vaccine (Solvay Biologicals) in coating buffer (0.5 M Na_2CO_3 [Sigma] in dH_20), 100 µl/well, and incubated overnight at 4°C. Plates were then washed three times with 250 µl wash solution/well (wash solution: 50 mM TRIS (Aldrich), 0.14 M NaCl (Fischer), 1% BSA (Sigma), 0.2% Tween-20 (Sigma), in dH_20). 100 µl block buffer (5 % BSA in PBS) was added to each well and incubated at 37°C for 1 hour. Plates were then washed three times with 250 µl wash solution/well. 100 µl plasma was added to each well (neat for IgA, IgD, IgM,

and diluted 1:100 in PBS for IgG1), and incubated at room temperature for 1 hour. Plates were then washed three times with 250 µl wash solution/well. 100 µl antibody (mouse anti-human IgA, IgD, IgG1, IgM; 0.5 mg/ml; AbD Serotec) was added to each well, and incubated at room temperature for 1 hour. Plates were then washed three times with 250 µl wash solution/well. 100 µl goat anti-mouse IgG (H/L):horseradish peroxidise (AbD Serotec; diluted 1:10,000 in PBS) was added to each well, and incubated at room temperature for 1 hour. Plates were then washed three times with 250 µl wash solution/well. Staining was performed by adding 100 µl of 3,3',5,5' tetramethylbenzidine (Sigma) to each well and incubating at room temperature in the dark for 20 minutes. 100 µl of stop solution (Sigma) was added to each well, and plates read on a plate reader (Thermo Labsystems, Original Multiskan) at 450 nm.

CHAPTER 3

PILOT STUDY OF SEASONAL INFLUENZA VACCINATION

3.1 INTRODUCTION

The main aim of the research described in this thesis is to investigate the effects of a prebiotic supplement (Orafti® Synergy1) on the functioning of the immune system in healthy human adults with the main outcome being the response to seasonal influenza vaccination. There are numerous methods available for assessing the human immune response (section 1.3). These have been evaluated by a panel of European experts (10). Based on its biological relevance, sensitivity and practical feasibility, response to vaccination was identified by this panel as the gold standard for measuring the functioning of the immune system in vivo (10). However, although response to vaccination is regarded as the "gold standard" method to measure the response of immune system in vivo, and response to vaccination has been used numerous times as a functional outcome (96, 97, 238-240), there are no standard protocols to follow for this method. Therefore it was necessary to develop a standardised protocol. Thus, a pilot study, involving a small number of subjects, was conducted to establish a seasonal influenza vaccination protocol and the conditions to study ex vivo immune responses to the vaccine. This chapter describes the design, methodology, and results of this pilot study, and the conclusions drawn from it.

3.2 SUBJECTS, STUDY DESIGN AND METHODS

3.2.1 SUBJECTS

The study was approved by the Southampton and South West Hampshire Local Research Ethics Committee (08/H0504/6) and clinical governance was provided by the Southampton University Hospitals NHS Trust R&D. Healthy male and female volunteers, aged 45 to 65 years, with a body mass index (BMI) between 20 and 32 kg/m^2 and not consuming prebiotic or probiotic supplements, were recruited. Subjects who were diabetic, using any prescribed medications that could affect their participation in the study, suffering from any infectious illness, or who had chronic gastrointestinal problems, had recently donated blood or participated in another clinical trial, or had been vaccinated with the current influenza vaccine were excluded. A total of six participants were recruited, three male and three female, with ages ranging from 47 to 62 years.

3.2.2 STUDY DESIGN

The study outline is shown in Figure 28. Briefly, at the first appointment, subjects in the fasting state gave a 30 ml blood sample (20 ml into heparin coated tubes and 10 ml into a serum tube) and a saliva sample (obtained by chewing on dental roll for approximately 30 seconds, with saliva collected using a 5 ml syringe; see section 2.1.3). Subjects then received the currently available influenza vaccination (2007/2008 Influvac® vaccine;

Solvay). One, two, four and six weeks later, subjects returned to give another fasting blood and saliva sample, as shown in Figure 28.

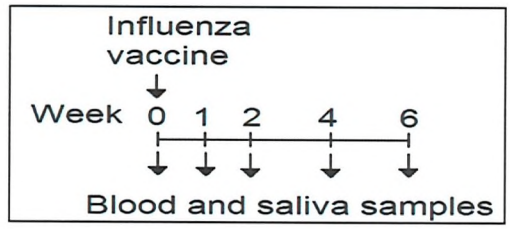


Figure 28 : Pilot study outline

3.2.3 METHODS

3.2.3.1 LABORATORY METHODS

Samples were processed and mononuclear cells isolated from blood and cryopreserved for later batch culture. The analyses performed are outlined in Figure 29, and details of the methods used are described in Chapter 2.

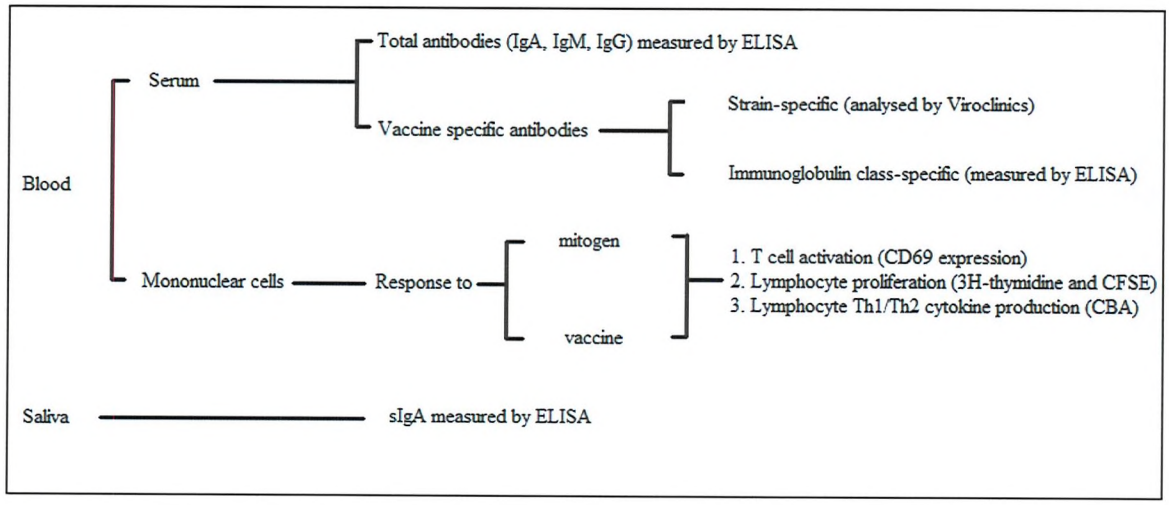


Figure 29 : Outline of sample analysis

Complete sets of samples were collected for all subjects. Due to a limitation on the number of peripheral blood mononuclear cells (PBMCs) available for each sample, samples from three subjects were used to measure T cell activation (CD69 expression) and Th1 and Th2 cytokine production (cytometric bead array), and samples from the three remaining subjects were used to measure T cell proliferation (CFSE dilution and ^{3}H-thymidine incorporation). Total serum antibody (IgA, IgG and IgM) and salivary sIgA concentrations for all subjects were determined by ELISA. Total vaccine-specific antibodies were analysed at Viroclinics (The Netherlands). All methods are described in Chapter 2.

3.2.3.2 STATISTICAL ANALYSIS

As this study involved a small number of subjects, and had the primary aim of identifying experimental conditions to use in a later larger supplementation study, statistical analysis was not carried out for most of the data. However, one-way ANOVA (fixed factor = time) was carried out on antibody data, to explore the changes over time more thoroughly. All analyses were performed using SPSS version 17.0.

3.3 RESULTS

3.3.1 SERUM VACCINE STRAIN-SPECIFIC ANTIBODY CONCENTRATIONS

Vaccine-specific antibodies were measured in serum from all subjects ($n = 6$). Data were log transformed and analysed by one-way ANOVA (fixed factor: time), and results are shown in Figure 30. The maximum vaccine-specific antibody response to all three strains of the virus was seen at two weeks post-vaccination. Although there was no significant difference between the response of HAH3_WI-specific antibodies at the different time points (one way ANOVA, effect of time; $p = 0.112$), the response of HAH1_SO- and HAB_MA-specific antibodies was significantly different between the different time points ($p < 0.001$ and $p < 0.001$ respectively, fixed factor: time, the effect of time on outcome). There was a large variation in antibody responses between subjects; this large variation and the small sample size most likely explain why there was no significant effect on HAH3_WI-specific antibodies despite the obvious large increases seen.

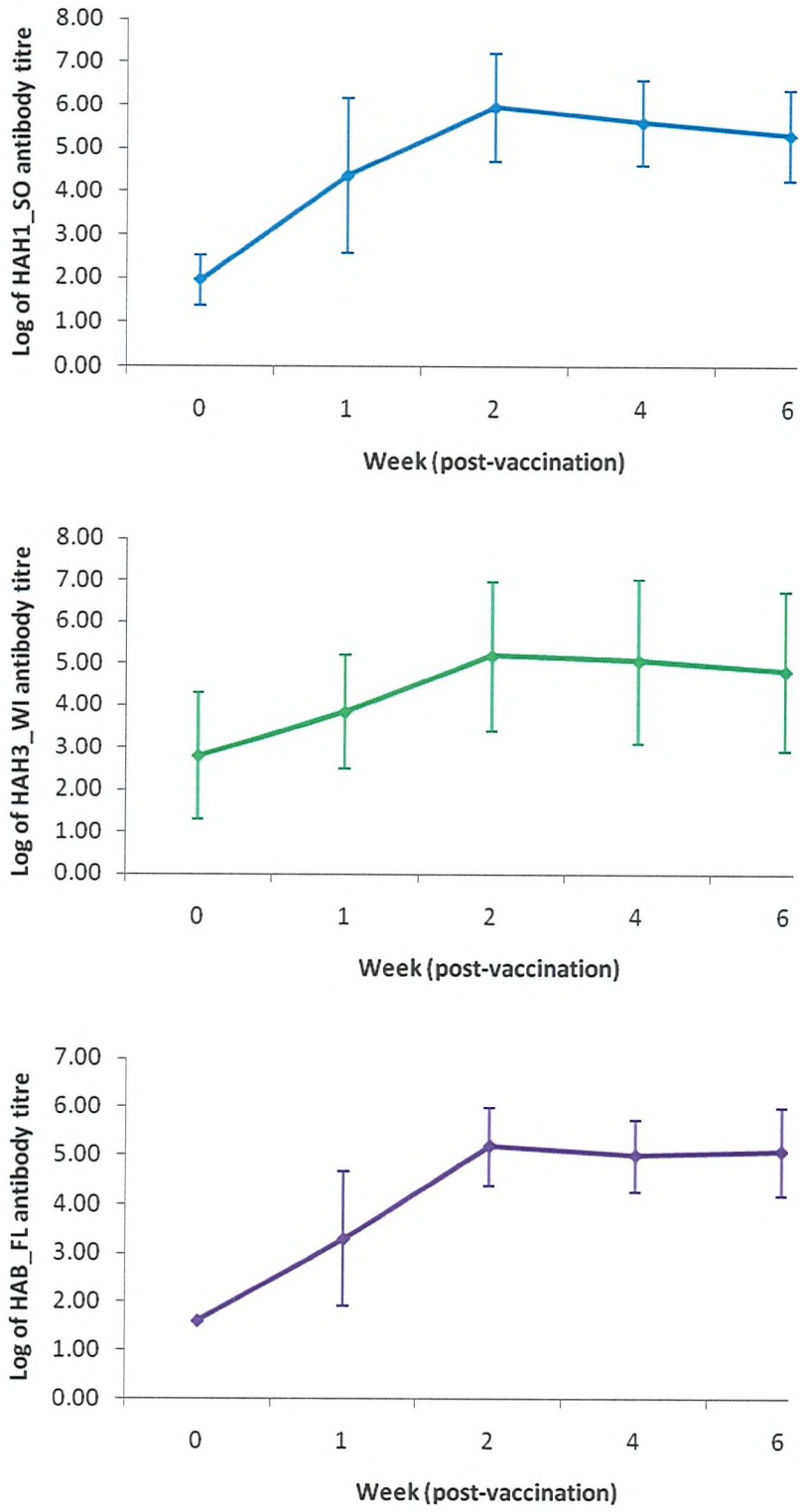


Figure 30 : Log of vaccine-specific antibody response (to the three virus strains in the vaccine: a = HAH1_SO, b = HAH3_W1, c = HAB_MA) post-vaccination. Data are mean of all subjects (n = 6); error bars indicate =/- 1 standard deviation

3.3.2 SERUM IMMUNOGLOBULIN CLASS-SPECIFIC VACCINE-SPECIFIC ANTIBODY CONCENTRATIONS

Serum samples from all subjects (n = 6) were analysed by ELISA to determine vaccine-specific concentrations according to immunoglobulin class (Figure 31).

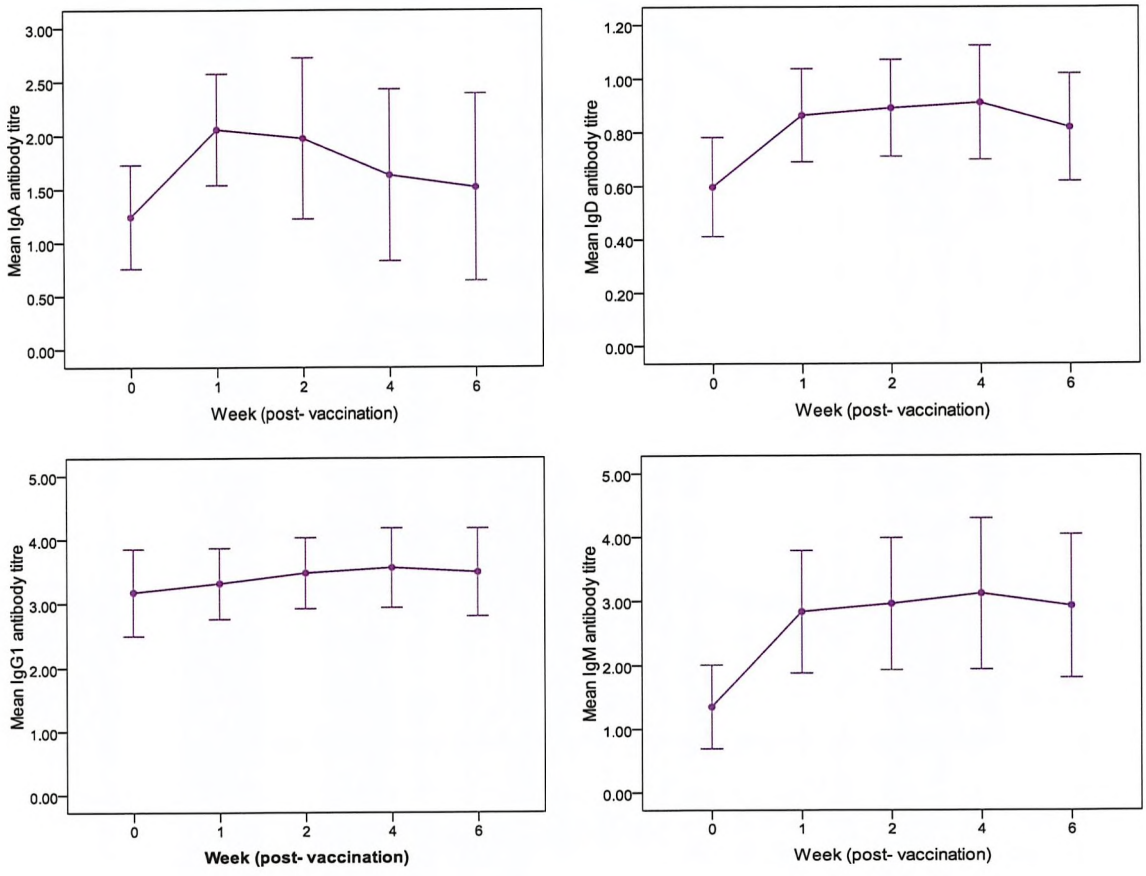


Figure 31 : Vaccine-specific serum antibody concentrations for immunoglobulin classes IgA, IgD, IgG and IgM. Data are mean of all subjects (n = 6); error bars indicate =/- 1 standard deviation

Although there was no significant effect of time on the responses of specific IgA, IgD or IgG1 antibodies (one way ANOVA effect of time: p = 0.276, p = 0.056, p = 0.828, respectively), the response of specific IgM antibodies was significantly affected by time (p = 0.032). The maximal specific IgA response was seen at one week post-vaccination, the near-maximal specific IgM response at one week post-vaccination and the maximal specific IgD and IgG1 responses at four weeks post-vaccination.

3.3.3 TOTAL SERUM AND SALIVARY IMMUNOGLUBULIN CONCENTRATIONS

The total immunoglobulin concentrations in serum (IgA, IgM and IgG) and saliva (sIgA) are shown in Table 10.

Week	Serum IgA (ng/L)	Serum IgM (ng/L)	Serum IgG (mg/L)	Salivary sIgA (µg/L)	Salivary sIgA / [total protein]
0	1082.01 (482.11)	844.34 (658.68)	3.61 (0.53)	530.20 (532.15)	345.06 (88.02)
1	1156.97 (331.69)	948.80 (582.02)	4.52 (1.13)	448.29 (368.31)	427.61 (210.08)
2	1526.64 (298.77)	1119.15 (665.99)	4.16 (0.51)	537.07 (453.95)	473.69 (344.97)
4	1474.78 (509.09)	1154.54 (595.66)	3.77 (1.25)	619.37 (648.03)	818.48 (421.26)
6	1250.99 (755.78)	1040.05 (862.90)	3.97 (1.07)	549.15 (556.03)	811.53 (861.83)

Table 10 : Total serum and salivary immunoglobulin concentrations. Data are mean of all subjects (n = 6); standard deviation indicated in parentheses

Serum immunoglobulins and salivary sIgA concentrations appeared to increase following vaccination. The maximal serum IgA response was seen at 2 weeks post-vaccination, although there was no significant difference between the concentrations seen at the different time points (one-way ANOVA effect of time: $p = 0.52$). The maximal serum IgM response was seen at 2 to 4 weeks post-vaccination, although there was no significant difference between the concentrations seen at the different time points ($p = 0.94$). The maximal serum IgG response was seen at one week post-vaccination, although there was no significant difference between the concentrations seen at the different time points ($p = 0.52$). The maximal salivary sIgA response was seen at four weeks post-vaccination, although there was no significant difference between the concentrations seen at the different time points ($p = 0.99$). When sIgA was adjusted for total protein content of the saliva samples (measured by Bradford assay) the maximal response was still seen at 4 weeks post-vaccination, although again there was no significant difference between the concentrations seen at the different time points ($p = 0.3$).

3.3.4 RESULTS FROM CELL CULTURE WORK

The aim of these experiments (measuring T cell activation, lymphocyte proliferation and lymphocyte cytokine production) was to establish conditions for culturing cells to be used in the main study. It was important to establish conditions for stimulating immune cells with seasonal influenza vaccine in vitro.

3.3.4.1 T CELL ACTIVATION MEASURED BY CD69 EXPRESSION

T cell activation was assessed by CD69 expression on $CD3^+CD4^+$ cells. PBMC cultures were stimulated with the mitogens ConA and PHA and both the percentage of $CD3^+CD4^+$ cells staining positive for CD69 and the mean fluorescence intensity of CD69 on those cells were measured. Figure 32 shows percentage of $CD3^+CD4^+$ cells expressing CD69; both ConA and PHA increased the number of CD69+ cells with ConA inducing a bigger response. The response to both ConA and PHA was enhanced one week post-vaccination.

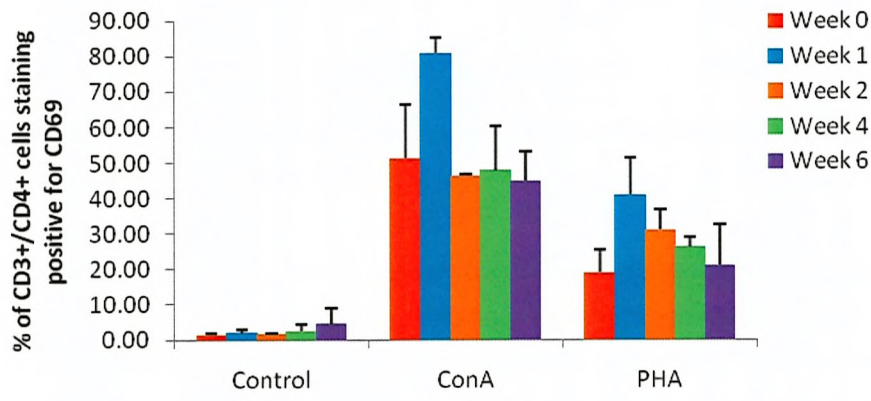


Figure 32 : Percentage of $CD3^+CD4^+$ cells staining positive for CD69 after stimulation with ConA or PHA for 24 hours (control cells were cultured for 24 hours but not stimulated). Different coloured bars represent different weeks post-vaccination (see key). Data are mean + 1 standard deviation (n = 3)

Figure 33 shows the mean fluorescence intensity of CD69 on $CD3^+CD4^+$ cells. Again, ConA was found to induce a larger response than PHA, and the maximal response from both ConA and PHA stimulated cultures was seen at one week post-vaccination.

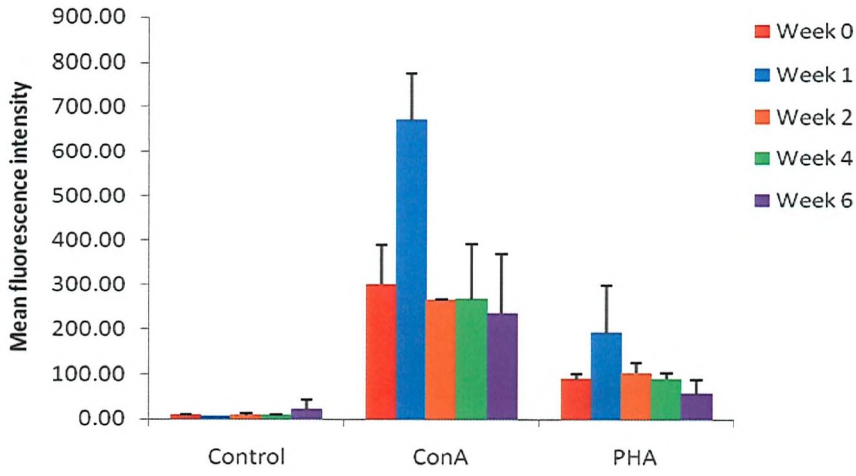


Figure 33 : Mean fluorescence intensity of CD69 staining on CD3+CD4+ cells cultured with ConA or PHA for 24 hours (control cells were cultured for 24 hours but not stimulated). Different coloured bars represent different weeks post-influenza vaccination (see key). Data are mean + 1 standard deviation (n = 3)

PBMC cultures were stimulated with different concentrations of the seasonal influenza vaccine (concentrations are here identified as dilutions of the original vaccine) and the vaccine was used in either an undialysed or dialysed form; the effect of dialysis was examined because it was thought that the vaccine may contain a low molecular weight component (e.g. preservative) that might affect cells in vitro (97). Both dialysed and non-dialysed vaccines induced responses in cell culture experiments. The results from experiments measuring percentage of $CD3^+CD4^+$ cells expressing CD69 and the mean fluorescence intensity of CD69 expression on $CD3^+CD4^+$ cells, at different weeks post-vaccination are shown in Figure 34, Figure 35, Figure 36 and Figure 37. Both dialysed and non-dialysed vaccines induced a response, and the 1/10 dilutions induced the biggest responses. The response to vaccine (either in terms of percentage positive cells or mean fluorescence intensity) was lower than to the mitogens described above.

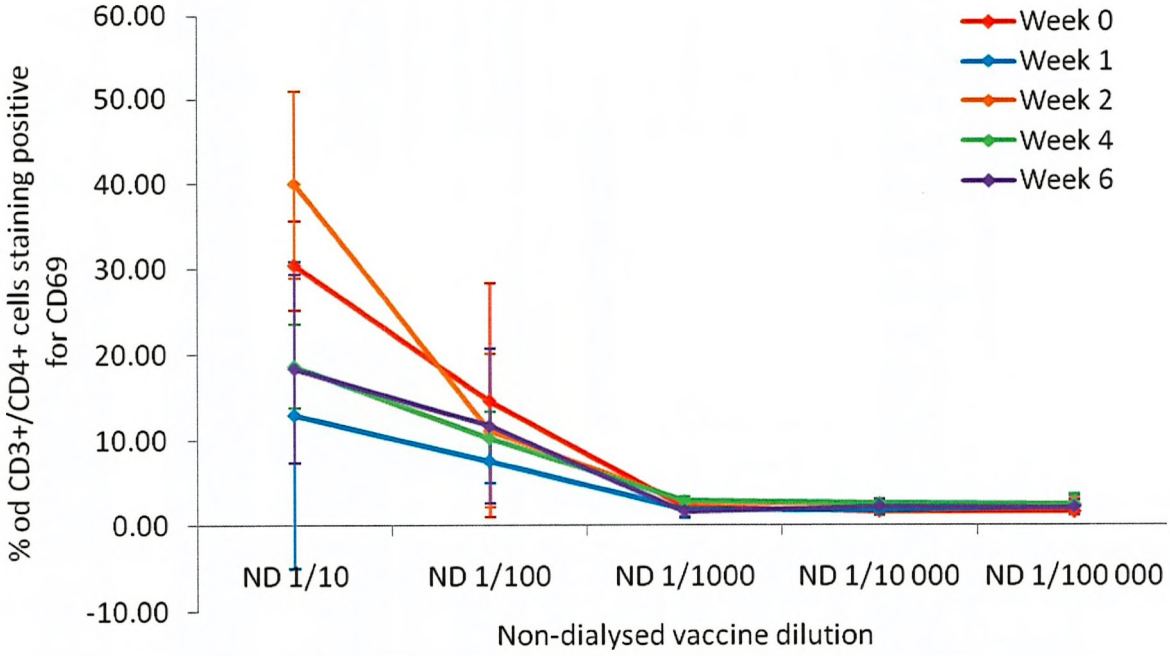


Figure 34 : Percentage of CD3+CD4+ cells staining positive for CD69 after stimulation with non-dialysed (ND) vaccine for 24 hours. Different coloured bars represent different weeks post-vaccination (see key). Data are mean +/- 1 standard deviation (n = 3).

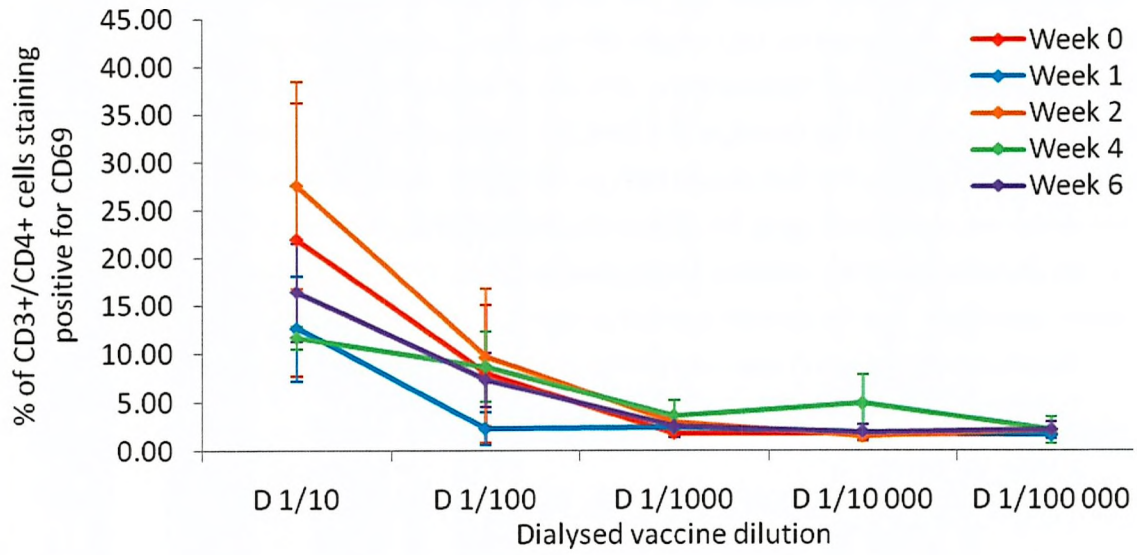


Figure 35 : Percentage of CD3+CD4+ cells staining positive for CD69 after stimulation with dialysed (D) vaccine for 24 hours. Different coloured bars represent different weeks post-vaccination (see key). Data are mean +/- 1 standard deviation (n = 3).

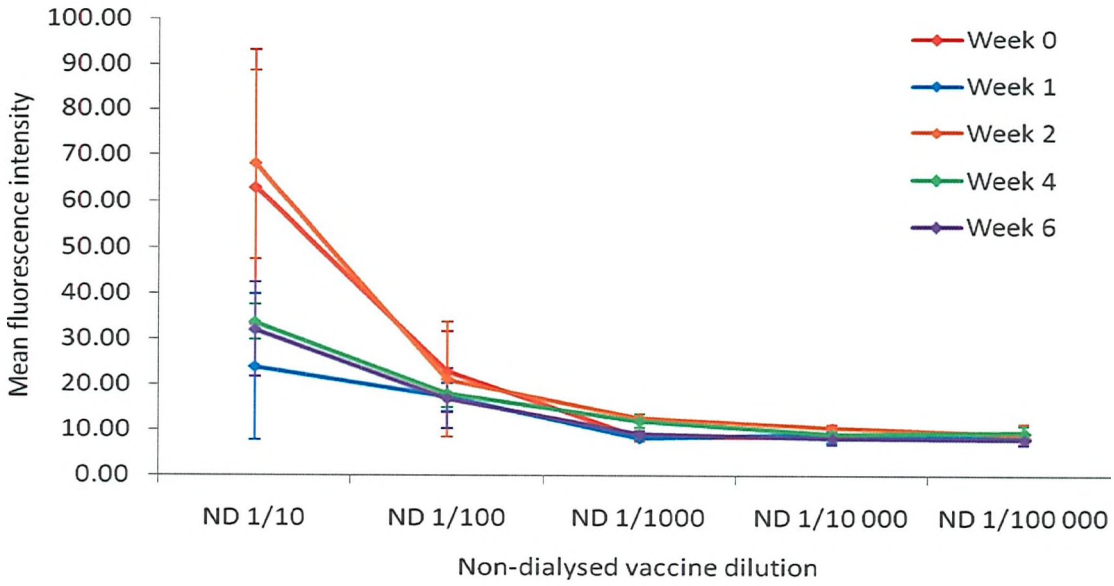


Figure 36 : Mean fluorescence intensity of CD69 staining on CD3+CD4+ cells cultured with non-dialysed (ND) vaccine for 24 hours. Different coloured bars represent different weeks post-vaccination (see key). Data are mean +/- 1 standard deviation (n = 3).

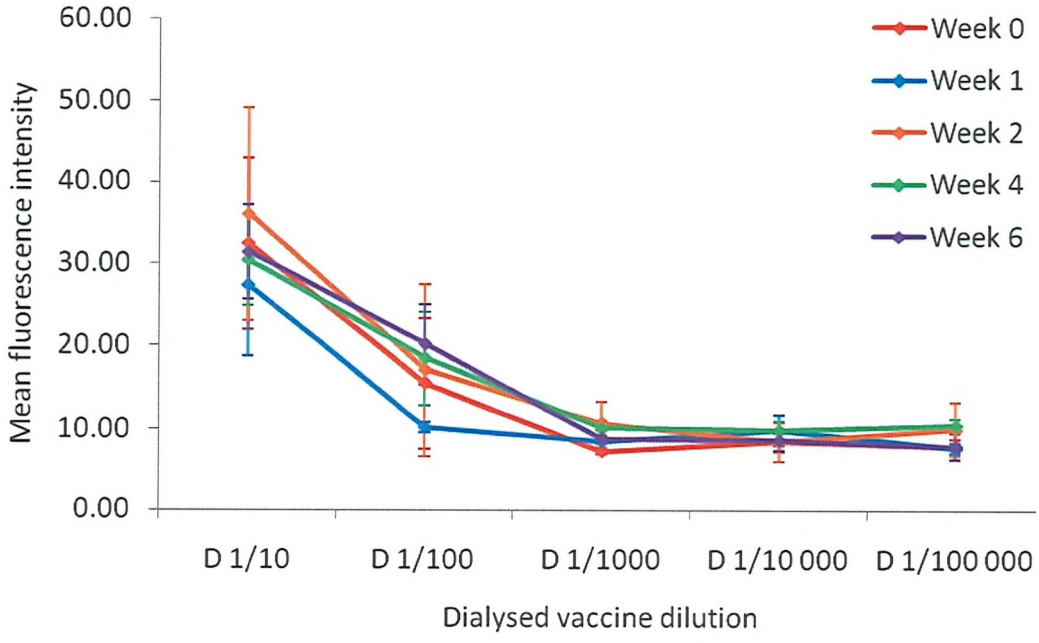


Figure 37 : Mean fluorescence intensity of CD69 staining on CD3+CD4+ cells cultured with dialysed (D) vaccine for 24 hours. Different coloured bars represent different weeks post-vaccination (see key). Data are mean +/- 1 standard deviation (n = 3).

3.3.4.2 DETERMINING THE OPTIMUM CULTURE TIME FOR CYTOKINE PRODUCTION

The concentrations of several cytokines (IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α) in the medium of PBMC cultures were measured by cytometric bead array; PBMCs were cultured for 24, 48 or 72 hours with ConA, PHA or a variety of dilutions of dialysed or non-dialysed vaccine, in order to determine the optimum duration of culture. Concentrations of these cytokines in supernatants from unstimulated cultures and cultures stimulated with ConA and PHA are shown in Table 11, Table 12 and Table 13.

		IL-2		IL-4		IL-6		IL-10		TNF-α		IFN-γ	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Week 0	Control	11.7	17.9	8.7	10.7	4815	4229	48.5	65.4	98.3	112.4	27	40
	ConA	174.9	123.2	39.5	27.9	20898	8824	245.1	157.7	862.4	654.2	5644	6848
	PHA	73.7	39.2	21.7	13.2	5546	5836	101.5	100.5	203.9	69.0	354	357
Week 1	Control	19.4	27.9	6.9	8.6	8652	8215	83.9	70.8	61.1	58.9	51	82
	ConA	368.9	395.5	75.4	78.2	24800	6368	284.6	213.6	610.9	499.6	18388	25441
	PHA	108.1	133.0	24.7	26.8	5896	6943	136.2	141.9	126.2	113.9	633	858
Week 2	Control	11.4	17.5	8.1	11.7	2692	2262	35.1	44.1	49.8	40.9	11	13
	ConA	233.2	280.1	42.5	53.9	19989	17850	291.2	350.9	703.0	715.8	4790	5865
	PHA	49.5	63.3	19.1	25.1	5748	6093	158.5	209.0	83.6	64.2	210	242
Week 4	Control	1.8	0.9	5.9	5.7	5871	5096	54.9	50.9	77.3	105.5	14.8	20
	ConA	230.6	273.9	32.7	45.5	18883	12525	269.1	288.9	354.0	323.2	2637	4054
	PHA	35.5	51.1	13.2	18.2	7999	6794	123.8	131.3	51.2	42.6	79	125
Week 6	Control	15.7	24.9	9.1	13.5	7348	6799	78.2	64.5	48.0	36.5	33	51
	ConA	205.8	280.6	38.4	55.3	20083	10126	258.2	197.4	257.3	200.3	3083	4737
	PHA	23.1	31.5	14.7	20.7	7779	7042	112.7	85.7	58.0	41.1	73	115

Table 11 : Cytokine concentrations in supernatants of unstimulated (control) blood mononuclear cells, and mitogen stimulated blood mononuclear cells, cultured for 24 hours (n=3)

		IL-2		IL-4		IL-6		IL-10		TNF-α		IFN-γ	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Week 0	Control	13.9	19.8	6.6	9.3	3838	3534	17.5	16.9	18.8	14.0	11	8
	ConA	139.6	143.6	52.3	41.9	21227	7413	236.1	140.3	464.4	294.9	38150	52269
	PHA	102.8	51.6	34.1	27.8	5072	5508	105.9	88.2	168.1	47.4	443	460
Week 1	Control	38.9	49.0	11.9	15.0	4227	5474	50.8	64.8	35.9	45.8	259	362
	ConA	94.6	38.8	39.9	19.7	23712	4367	251.9	118.1	417.3	82.7	24026	3158
	PHA	42.5	42.8	22.4	24.3	7656	10097	118.4	138.8	104.3	142.5	560	774
Week 2	Control	5.9	4.4	1.8	0.9	2777	2880	8.7	6.4	8.2	8.1	10	11
	ConA	50.3	22.6	18.8	12.2	15982	518	123.3	22.6	197.8	31.4	3311	2194
	PHA	58.8	70.4	13.4	17.1	2974	2785	39.6	10.4	49.9	48.8	177	149
Week 4	Control	4.2	1.7	1.3	0.0	7416	8258	26.5	20.2	5.8	3.9	8	5
	ConA	119.4	68.6	28.5	17.4	20539	12172	223.6	20.1	426.9	143.3	4336	2583
	PHA	55.5	41.8	11.1	6.5	9608	8750	72.6	15.6	72.1	43.5	161	58
Week 6	Control	4.8	3.5	1.3	0.0	8973	12435	33.1	42.2	10.6	12.2	6	4
	ConA	90.2	55.6	17.4	3.2	20512	12551	190.3	37.6	300.5	133.6	3522	1966
	PHA	39.0	42.3	6.9	4.6	12188	13546	65.8	29.9	35.9	38.2	201	102

Table 12 : Cytokine concentrations in supernatants of unstimulated (control) blood mononuclear cells, and mitogen stimulated blood mononuclear cells, cultured for 48 hours (n = 3)

		IL-2		IL-4		IL-6		IL-10		TNF-α		IFN-γ	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Week 0	Control	20.9	12.2	1.9	0.9	1482	1751	5.8	1.9	3.3	3.3	13	9
	ConA	12.7	3.9	6.6	2.2	10640	2248	181.3	183.7	395.5	435.5	5915	769
	PHA	31.2	7.7	6.4	1.6	5989	1219	50.5	30.7	122.3	142.5	593	118
Week 1	Control	12.9	9.1	1.3	0.0	3135	4066	18.2	10.5	1.4	0.0	17	8
	ConA	20.7	9.3	12.8	8.9	12256	2531	130.7	106.1	676.6	708.5	5779	1456
	PHA	17.1	4.8	5.0	3.1	3986	1052	45.6	33.5	26.6	18.1	139	10
Week 2	Control	14.9	7.3	1.3	0.0	2553.	2149	4.9	0.6	1.4	0.0	31	33
	ConA	22.7	10.3	15.2	5.8	15443	8407	123.9	79.1	276.5	286.6	5037	3152
	PHA	65.6	88.3	11.4	11.5	5312	5620	40.9	41.0	76.8	106.6	445	288
Week 4	Control	16.1	8.7	1.3	0.0	4843	4226	10.3	4.3	1.4	0.0	18	12
	ConA	43.4	34.8	15.7	4.7	21736	13854	137.9	42.1	377.9	310.5	8623	4310
	PHA	53.9	42.5	12.0	8.1	11060	9869	57.1	24.3	75.9	43.2	574	145
Week 6	Control	20.9	6.5	2.7	1.3	9995	12857	22.6	30.3	5.7	7.4	34	27
	ConA	47.9	52.5	12.8	5.1	20969	11587	101.3	69.3	232.0	255.2	5089	3486
	PHA	56.4	41.1	8.0	4.9	13133	15211	39.2	41.5	16.3	13.2	580	347

Table 13 : Cytokine concentrations in supernatants of unstimulated (control) blood mononuclear cells, and mitogen stimulated blood mononuclear cells, cultured for 72 hours (n = 3)

From these results it can be concluded that culturing cells for 24 hours is sufficient to see a good cytokine response, and that culturing cells for longer than this time does not result in a greater response, apart from for IFN-γ which showed a greater concentration after 48 hours of culture. Once again the response to ConA was greater than that to PHA, confirming the findings from the T cell activation experiments. The cytokine response to both non-dialysed and dialysed vaccine was tested, and results from 24 hour cultures shown in Table 14 and Table 15 (values for corresponding unstimulated control cultures are shown in Table 11, Table 12 and Table 13. Again, a response to both non-dialysed and dialysed was observed, and the greatest response was seen with stimulation with a 1/10 dilution of the vaccine. Results from longer duration cultures (48 and 72 hours) with vaccine are not shown. However for vaccine-stimulated cells, 24 hours of culture produced the maximal response for IL-4, IL-10 and TNF-α, 48 hours of culture produced the maximal response for IL-2, and 72 hours of culture produced the maximal response for IFN-γ. All time points produced similar results for IL-6.

Calculation of the ratio of the Th1-type to the Th2-type cytokine response, using the concentrations of IFN-γ and IL-4 revealed that the Th1-type response was favoured particularly following ConA stimulation and that the response to the vaccine becomes more Th1 polarised following vaccination (data not shown).

		IL-2		IL-4		IL-6		IL-10		TNF-α		IFN-γ	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Week 0	1/10	64.4	72.1	15.6	15.5	3809	5146	65.3	86.9	75.0	97.8	281.9	385.3
	1/100	43.8	60.1	12.7	16.1	4224	5812	74.1	98.9	113.8	156.4	100.8	131.8
	1/1000	38.9	53.2	17.6	20.8	4740	6563	76.5	104.1	102.8	139.7	57.1	75.7
	1/10 000	19.9	26.3	10.7	13.3	3153	4304	52.3	72.0	62.5	80.9	41.6	53.8
	1/100000	15.9	20.8	15.5	20.1	3532	4850	56.3	77.7	93.9	126.5	3.6	0.0
Week 1	1/10	171.7	215.6	9.6	9.7	5196	6874	90.9	119.7	102.4	135.5	624.4	839.6
	1/100	81.8	107.2	12.9	16.5	3952	5089	67.3	89.4	76.9	101.1	352.1	492.9
	1/1000	27.4	36.8	19.2	22.1	2834	3742	52.9	72.8	47.6	62.6	59.4	79.0
	1/10 000	20.4	23.0	11.5	12.5	5797	7591	81.4	110.8	90.7	117.7	142.8	196.9
	1/100000	22.9	30.6	15.7	17.9	2836	3648	46.1	59.3	53.7	68.4	47.3	61.9
Week 2	1/10	206.8	279.2	12.1	15.2	2710	3646	89.2	124.2	55.1	71.6	491.4	689.9
	1/100	72.7	97.5	12.7	16.1	2323	2954	50.6	63.6	29.5	33.3	68.7	86.7
	1/1000	17.7	23.2	9.6	11.8	3860	5074	79.6	107.4	37.2	42.5	41.6	53.8
	1/10 000	20.7	27.5	16.2	17.3	4893	6759	125.9	173.3	46.6	58.3	65.9	88.1
	1/100000	11.2	14.0	10.4	12.9	1311	1693	30.3	38.7	18.2	19.8	25.2	30.6
Week 4	1/10	91.9	132.8	4.0	4.7	5841	5009	89.4	105.8	38.1	36.9	136.7	203.4
	1/100	48.5	79.1	10.1	13.7	5600	5552	77.1	68.5	26.3	22.4	99.2	159.4
	1/1000	15.6	23.6	8.1	10.6	7318	6246	105.1	143.2	28.8	34.1	32.1	49.4
	1/10 000	10.5	14.5	6.1	6.7	5116	4778	58.1	56.4	27.8	25.8	23.3	34.2
	1/100000	19.6	31.6	8.6	11.5	3379	4563	60.3	61.2	28.7	26.8	28.6	43.4
Week 6	1/10	65.6	108.4	5.5	7.2	7774	6721	86.1	71.1	57.9	47.2	210.9	354.4
	1/100	33.9	54.1	7.3	10.3	7371	6724	92.2	76.0	54.7	44.4	72.6	119.6
	1/1000	19.8	30.6	9.2	11.4	7446	6902	77.7	66.9	45.6	38.3	28.9	43.9
	1/10 000	11.9	18.5	4.5	4.3	7091	6404	65.7	54.1	46.3	42.2	24.7	36.6
	1/100000	7.9	11.4	2.6	2.2	7009	8098	81.2	76.2	24.4	23.1	3.6	0.0

Table 14 : Cytokine concentrations in supernatants of blood mononuclear cells cultured for 24 hours and stimulated with non-dialysed vaccine (n = 3)

		IL-2		IL-4		IL-6		IL-10		TNF-α		IFN-γ	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Week 0	1/10	18.9	30.6	16.1	25.7	22543	9424	319.1	401.9	1725.9	1441.6	925.5	691.5
	1/100	29.3	48.5	13.1	18.9	16422	5035	155.4	194.1	349.6	1121	140.6	219.4
	1/1000	15.9	25.2	7.5	10.7	4818	3876	44.8	57.1	61.8	45.9	35.2	54.9
	1/10 000	14.8	23.5	10.9	16.8	3643	3104	31.1	34.7	33.5	26.7	3.6	0.0
	1/100000	21.5	35.0	8.1	11.7	4166	3568	39.5	48.7	43.7	41.8	17.2	23.7
Week 1	1/10	44.2	72.3	15.6	23.1	24428	8228	502.9	449.5	1793.9	1464.6	3583.8	5284.9
	1/100	31.8	51.5	12.2	18.8	21073	3149	236.4	189.1	378.9	297.9	377.8	620.8
	1/1000	28.7	38.7	12.1	15.2	4336	3714	54.5	69.8	36.8	31.0	67.7	90.7
	1/10 000	7.4	8.6	10.2	12.6	4374	5770	63.9	83.7	47.7	58.4	63.1	84.2
	1/100000	13.6	17.4	7.2	8.4	3448	4588	45.3	62.0	46.5	58.5	25.2	30.6
Week 2	1/10	49.7	64.9	7.1	9.9	22460	8805	446.6	636.7	1692.9	892.9	2109.9	1358.4
	1/100	26.1	42.9	7.2	10.1	16006	2665	176.2	215.8	357.3	38.9	288.7	216.8
	1/1000	9.1	13.5	7.2	9.1	4023	3601	39.3	45.8	33.3	21.5	30.3	23.2
	1/10 000	9.1	13.5	8.9	13.1	2858	2425	41.3	57.9	30.9	23.1	31.2	47.8
	1/100000	7.2	10.2	9.1	13.5	3480	3155	48.3	70.5	37.6	29.7	5.2	2.8
Week 4	1/10	27.3	38.3	6.5	6.3	26678	7958	747.5	690.4	1541.1	911.7	828.8	554.2
	1/100	19.6	30.4	7.1	8.7	13529	12482	192.2	294.0	204.8	178.6	99.9	130.2
	1/1000	7.8	9.7	8.3	12.1	8254	6987	98.2	99.7	35.8	29.7	25.0	33.7
	1/10 000	11.1	14.7	7.6	8.6	7479	6394	100.2	121.9	42.6	40.4	30.8	47.3
	1/100000	9.9	12.8	12.1	16.9	5857	5410	66.9	67.5	30.9	24.4	22.6	33.1
Week 6	1/10	23.8	36.3	2.0	0.9	24951	7475	531.9	545.4	1015.5	161.1	961.4	1426.9
	1/100	12.7	19.3	8.6	12.6	17588	6240	254.7	203.5	312.9	143.3	150.7	238.0
	1/1000	1.3	0.0	6.5	6.9	7154	7543	80.4	68.9	32.5	23.5	14.8	19.5
	1/10 000	15.1	23.9	9.9	14.9	8303	7273	92.3	77.6	46.6	43.0	46.3	74.1
	1/100000	17.2	27.5	6.2	8.4	7092	6734	83.9	69.4	39.6	38.8	16.5	22.3

Table 15 : Cytokine concentrations in supernatants of blood mononuclear cells cultured for 24 hours and stimulated with dialysed vaccine (n = 3)

3.3.4.3 DETERMINING THE OPTIMUM CULTURE TIME FOR LYMPHOCYTE PROLIFERATION EXPERIMENTS

T cell proliferation was measured by ^{3}H-thymidine incorporation and by CFSE staining. Results from ^{3}H-thymidine incorporation experiments are shown, followed by results from CFSE dilution experiments.

3.3.4.3.1 ^{3}H-THYMIDINE INCORPORATION

T cell proliferation was measured by ^{3}H-thymidine incorporation by PBMCs. Results are expressed as the stimulation index (thymidine incorporation in stimulated cultures/thymidine incorporation in control unstimulated cultures). As shown in Figure 38, ConA and PHA induced similar responses, which did not change over time.

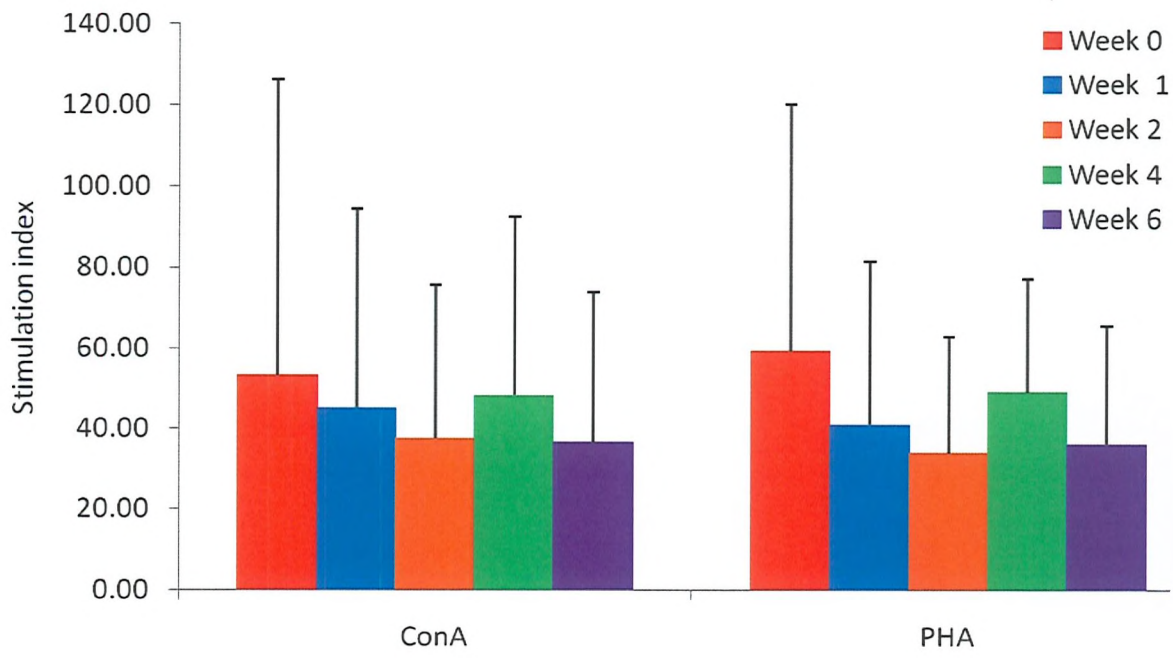


Figure 38 : ^{3}H-thymidine incorporation by mitogen stimulated blood mononuclear cells. Different coloured bars represent different weeks post-vaccination (see key). Data are mean+ 1 standard deviation (n = 3)

In order to determine the optimal vaccine concentration to use to stimulate PBMC cultures, a series of vaccine dilutions was tested, and the vaccine was used in dialysed and non-dialysed states. The results are shown in Figure 39 and Figure 40. The non-dialysed and dialysed vaccine induced a similar proliferative response, with the 1/10 dilution generally inducing the greatest response. The maximal proliferative response was generally seen at week four post-vaccination for the dialysed vaccine stimulated cultures.

For the non-dialysed vaccine stimulated cultures, the pattern is less clear, but for dilutions of 1/10 and 1/100 the greatest response was seen at weeks one and four.

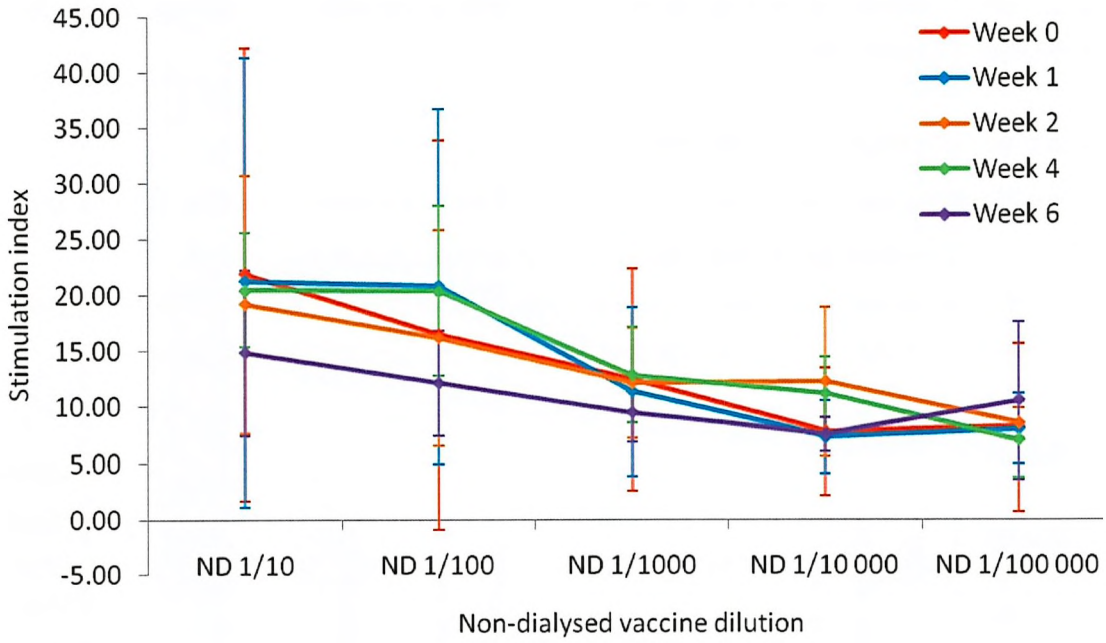


Figure 39 : ^{3}H-thymidine incorporation by non-dialysed (ND) vaccine stimulated blood mononuclear cells (mean of n = 3; error bars indicate +/- 1 standard deviation)

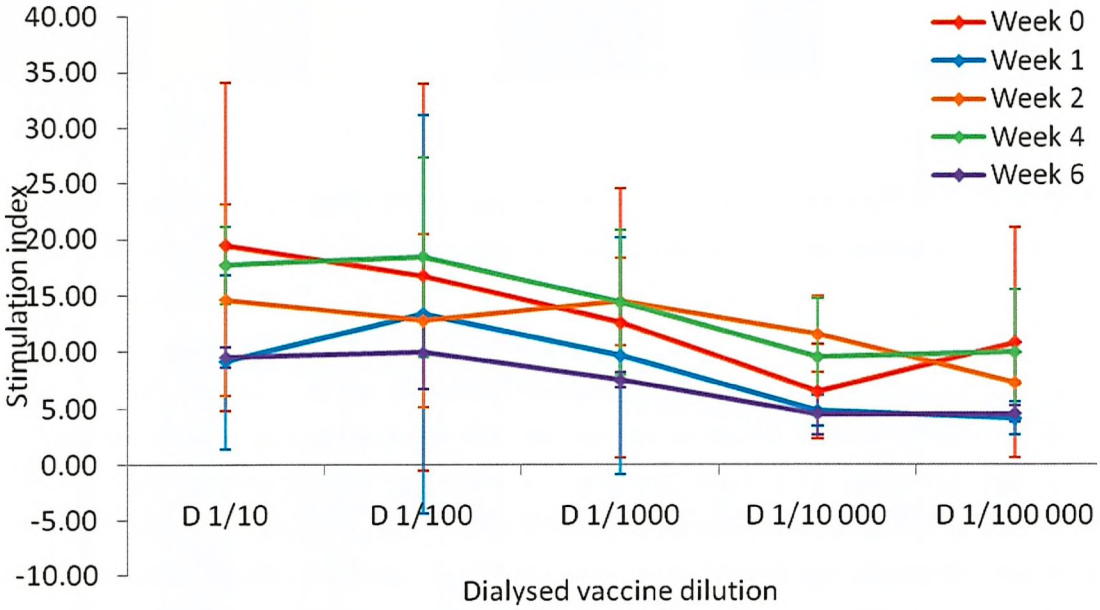


Figure 40 : ^{3}H-thymidine incorporation by dialysed (D) vaccine stimulated blood mononuclear cells (mean of n =3; error bars indicated +/- 1 standard deviation)

3.3.4.3.2 CFSE Dilution

Due to limitations on the cell counts of the samples there were only sufficient cells to do the full CFSE staining experiment for one participant, and sufficient cells from another participant for part of the experiment. Therefore, sample size for the following results is 1 or 2. Results were analysed using Weasel software, and are shown in Figure 41, Figure 42 and Figure 43. ConA and PHA both produced good proliferative responses, and these did not change greatly over time after vaccination. The dialysed and non-dialysed vaccines produced similar responses to one another, and at week two it can be observed that some cells had moved into further stages of proliferation. The non-dialysed vaccine did not produce as big a proliferative response with CFSE staining as was observed when measuring proliferation by ^{3}H-thymidine incorporation, probably because vaccine-stimulated cells were cultured for a shorter time for CFSE staining compared with ^{3}H-thymidine incorporation, and so cells stained with CFSE must be cultured for a longer time period.

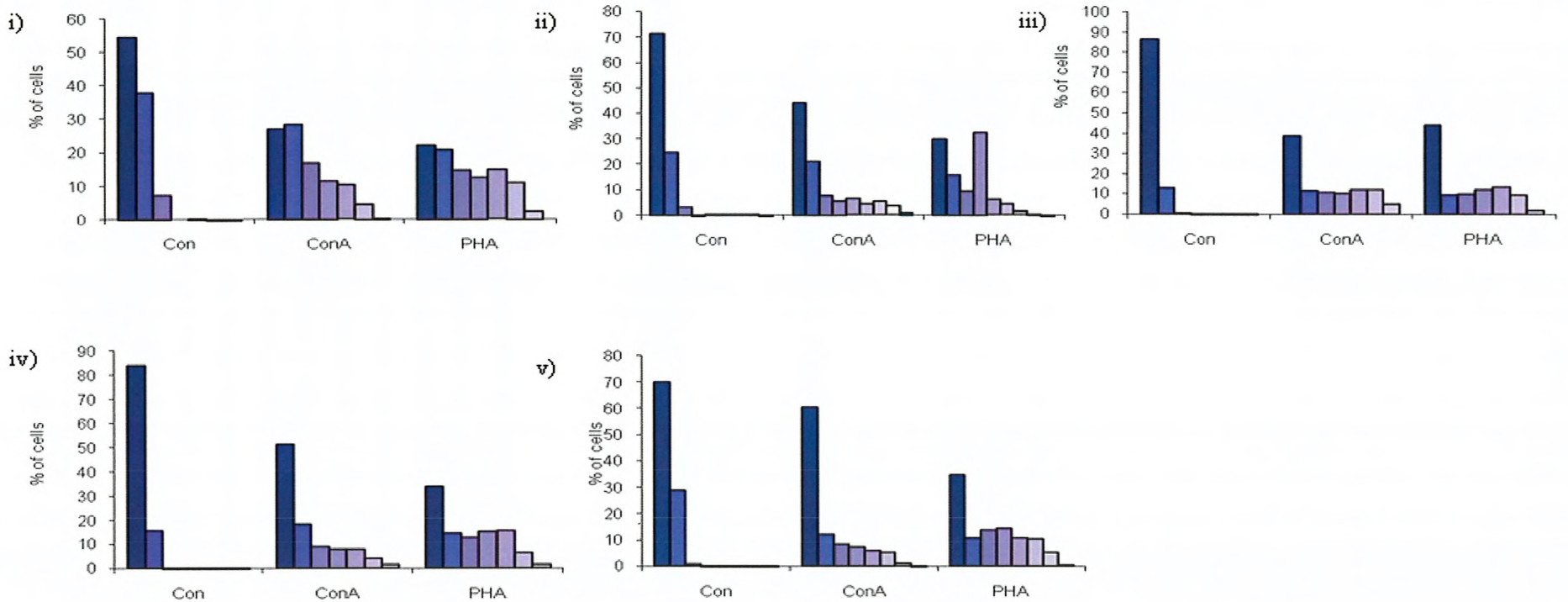


Figure 41 : T cell proliferation measured by CFSE dilution, expressed as the percentage of cells in each division, after stimulation with mitogens (ConA or PHA) for 96 hours. i) week 0, immediately before vaccination; ii) week 1 post-vaccination; iii) week 2 post-vaccination; iv) week 4 post-vaccination; v) week 6 post-vaccination; Legend: darkest purple = division 0 through to lightest purple = division 7. Con indicates unstimulated cells.

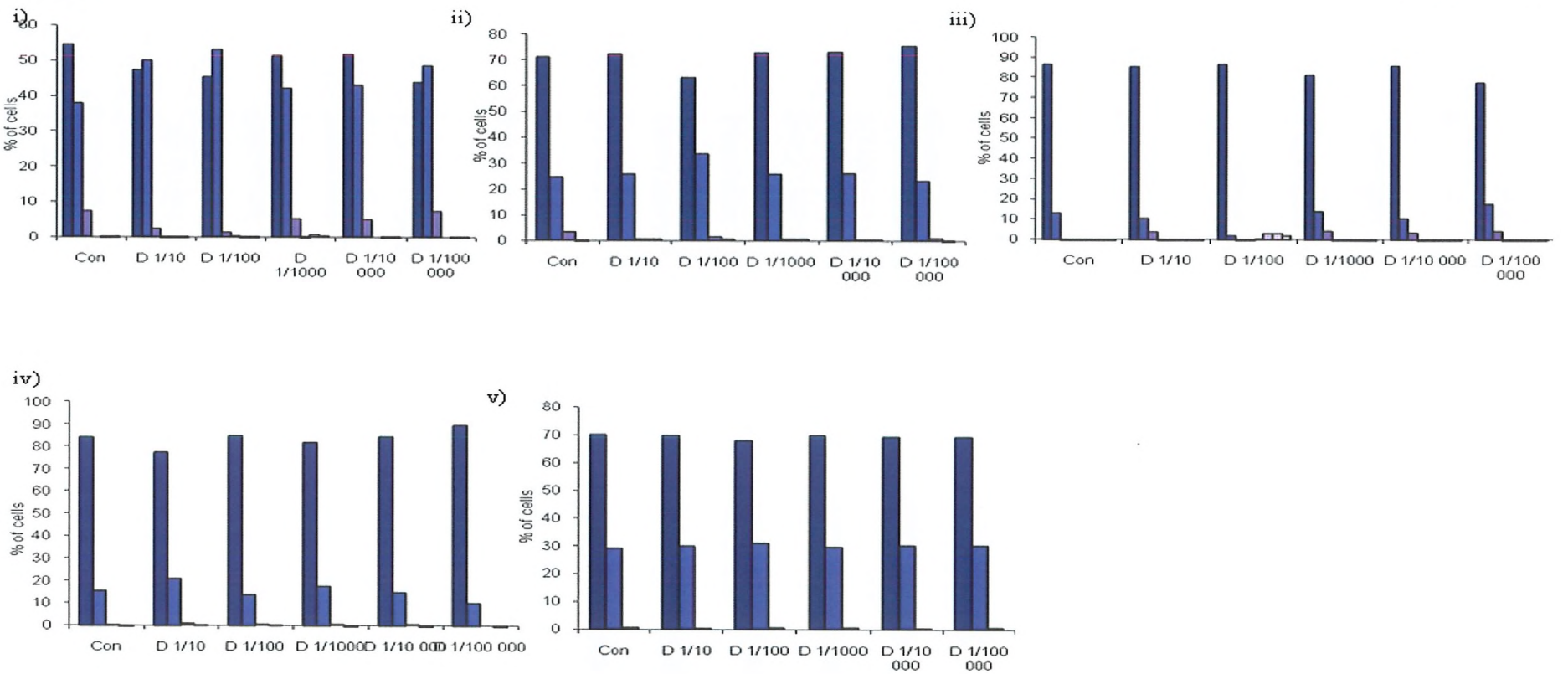


Figure 42 : T cell proliferation measured by CFSE dilution, expressed as the percentage of cells in each division after stimulation with dialysed (D) vaccine for 96 hours. i) week 0, immediately before vaccination; ii) week 1 post-vaccination; iii) week 2 post-vaccination; iv) week 4 post-vaccination; v) week 6 post-vaccination; Legend: darkest purple = division 0 through to lightest purple = division 7. Con indicates unstimulated cells.

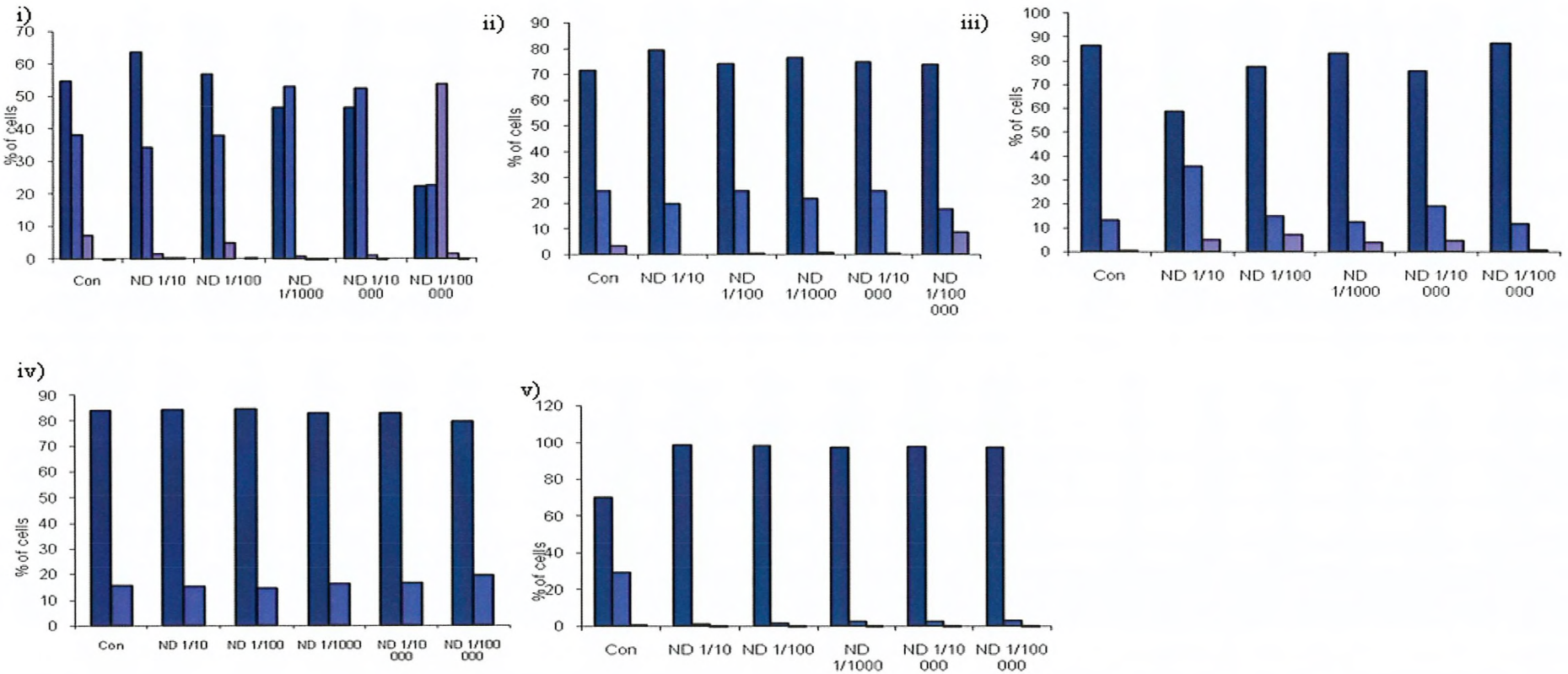


Figure 43 : T cell proliferation measured by CFSE dilution, expressed as the percentage of cells in each division after stimulation with non-dialysed (ND) vaccine for 96 hours. i) week 0, immediately before vaccination; ii) week 1 post-vaccination; iii) week 2 post-vaccination; iv) week 4 post-vaccination; v) week 6 post-vaccination; Legend: darkest purple = division 0 through to lightest purple = division 7. Con indicates unstimulated cells.

3.4 DISCUSSION

3.4.1 ANTIBODY RESPONSES

Vaccination did not cause a statistically significant increase for two out of three strains of vaccine-specific antibodies, although visual inspection of the time course suggests that these did increase. Therefore, considering the small sample size and the large variation in the antibody response to vaccination between individual subjects, it would be anticipated that in a larger study, a statistically significant response for these antibodies would be seen. From the results obtained it is clear that by two weeks post-vaccination, peak serum concentrations of antibodies specific to all three strains of the influenza virus used in the vaccine had been achieved. For the HAH3 and HAB strains, the serum antibody concentrations remained relatively stable until four weeks, while the concentration of anti-HAH1 antibodies had decreased by four weeks. Thus based on these results it seems that two weeks post-vaccination would be the best time to measure this parameter in the main study. However in the main study, which will investigate the effects of a prebiotic supplement (Orafti® Synergy1), on the functioning of the immune system in healthy human adults, the 2008/2009 seasonal influenza vaccine will be used. This will contain different strains of the virus, and it is not certain that subjects will respond in exactly the same way to these strains as subjects did to the strains used in the 2007/2008 vaccine used in the pilot study. Thus, blood samples will be collected at both two and four weeks post-vaccination in the main study in order to ensure that the peak antibody response is not missed. From the results of this pilot study it is clear that four weeks post-vaccination is sufficient to see the peak anti-vaccine antibody response, and so no time points beyond this need to be considered. Vaccine-specific immunoglobulin class-specific antibody concentrations had also all peaked by four weeks post vaccination. The only statistically significant change over time was seen for specific IgM antibodies, although it appears that for the other classes there are also changes, and again the small sample size and variation between subjects must be considered.

Statistically, vaccination did not increase total antibody concentrations. However again, considering the small sample size and the large variation in the total antibody concentration between individual subjects, it would be anticipated that in a larger study, statistically significant differences might be seen. The total serum concentration of IgA peaked at two weeks post-vaccine, while that of serum IgM and of salivary sIgA peaked at four weeks post-vaccination, and so the proposed time points for the main study will be suitable for these measurements. Total serum IgG peaked at one week post-vaccination, but remained elevated at 2 weeks post-vaccination and so the proposed time points for the main study should also be suitable for this measurement

3.4.2 CELL CULTURE EXPERIEMENTS

PBMCs were cultured to measure T cell activation by CD69 expression (percentage positive and mean fluorescence intensity), lymphocyte proliferation, and lymphocyte cytokine production in response to two mitogens (ConA and PHA) and undialysed and dialysed vaccine.

3.4.2.1 STIMULANTS

Comparing responses from stimulation by ConA with PHA, showed that ConA generally produced the greater response, and so will be used as the general stimulant with which to stimulate cells in the main study. Comparing responses from cultures stimulated with dialysed vaccine and those from cultures stimulated with non-dialysed vaccine illustrated that both forms of the vaccine were able to induce responses. To dialyse the vaccine involves a process which could potentially introduce foreign antigens into the vaccine, which could then produce a response in cell cultures that is not related to the vaccine. Therefore, it was considered appropriate to avoid this step if possible. As the non-dialysed vaccine produced good responses, this will be used to stimulate cells in the main study. The 1/10 dilution of the vaccine consistently produced the greatest responses in all experiments, and will be adopted as the concentration of vaccine with which to stimulate cells in the main study.

From results of cell culture experiments it is clear that there are cellular responses to stimulation with the vaccine at week zero. This is an unexpected result, as presumably the immune system had not yet been exposed to the vaccine antigen, and so there should not be any vaccine specific lymphocytes which would respond to the vaccine antigen at this time point. This observation cannot be easily explained, but perhaps is due to a reaction to some other component of the vaccine, such as the chicken egg in which the vaccine is grown.

3.4.2.2 TIMINGS

For the vaccine-stimulated cultures different culture times produced maximal responses for the different cytokines: 24 hours of culture produced the maximal response for IL-4, IL-10 and TNF-α, 48 hours of culture produced the maximal response for IL-2, and 72 hours of culture produced the maximal response for IFN-γ. All time points produced similar results for IL-6. For mitogen stimulated cultures 24 (or in the case of IFN-γ 48) hours produced the maximal response. In the main study only one time point will be used for logistical reasons. Thus, a culture time of 24 hours was chosen, as increased production of all six cytokines was seen by this time point, for both vaccine and mitogen stimulated cultures.

For lymphocyte proliferation measured by CFSE staining, on the small number of samples available, 96 hours of culture with vaccine did not appear to be sufficient. 138-140 hours produced good results regarding ^{3}H-thymidine incorporation. As using a longer culture time will not be detrimental, in the main study cells will be stained with CFSE and then cultured for 168 hours, to ensure that a good proliferative response is observed.

T cell activation and cytokine production tended to be highest soon after vaccination, while lymphocyte proliferation tended to peak a little later. Therefore, the effect the vaccination has upon T cell proliferative responses may take longer to become apparent than the effect the vaccination has upon T cell activation, which seems to peak at two weeks post-vaccination. In the 24 hour cultures, production of all cytokines was (generally) highest early (one or two weeks) after vaccination, which fits well with results from the T cell activation experiments.

3.5 CONCLUSIONS FROM THE PILOT STUDY AND THEIR IMPLICATIONS FOR THE MAIN STUDY

The main decisions made based on the results of this pilot study, and their reasoning, are given in Table 16. In conclusion:

- Vaccine-specific and total antibody measurements have shown that four weeks post-vaccination will be suitable to see the maximum antibody response following influenza vaccination in the main study, and taking a blood sample at two weeks post-vaccination will also be necessary in order that the peak antibody response is not missed.
- ConA will be used as a general stimulant in cell cultures, as this produced a greater response than PHA in all experiments.
- Non-dialysed vaccine at a 1/10 dilution in RPMI will be used to stimulate cell cultures.
- For cytokine production measurements, cells will be cultured for 24 hours before the supernatants are harvested and frozen for later analysis.
- For lymphocyte proliferation measured by CFSE staining, cells will be cultured for 168 hours.

Measurement	Decision	Reason
Serum anti-vaccine antibodies (strain-specific and immunoglobulin class-specific)	Blood samples taken at two and four weeks post-vaccination for measurement	Four weeks is sufficient to see a response, and the measurement should be taken at two weeks to ensure the peak antibody response is not missed, as a different vaccine will be used.
Total serum IgA, IgG and IgM, and salivary sIgA	Blood samples taken at two and four weeks post-vaccination for measurement	Serum IgA peaks at two weeks post-vaccination and serum IgM and salivary sIgA at four weeks, while serum IgG peaks at one week post-vaccination but is still elevated at two weeks. Therefore the time points of 2 and 4 weeks post-vaccination should be suitable.
T cell activation - CD69 expression & percent positive	• Stimulate with ConA, but not PHA • Use non-dialysed vaccine • Use a 1/10 dilution of vaccine in RPMI	ConA produces a greater response. Dialysed and non-dialysed both produce good responses, but using the non-dialysed vaccine will reduce the risk of introducing foreign antigens into the cultures. This produces the biggest response
T cell proliferation - ^{3}H thymidine	• Stimulate with ConA, but not PHA • Use non-dialysed vaccine	Both produce a good response, but for other measures ConA produces a better response. Dialysed and non-dialysed both produce good responses, but using the non-dialysed vaccine will reduce the risk of

		introducing foreign antigens into the cultures.
	• Use a 1/10 dilution of vaccine in RPMI	This produces the biggest response.
T cell proliferation - CFSE staining	• Stimulate with ConA, but not PHA	Both produce a good response, but for other measures ConA produces a better response.
	• Use dialysed vaccine	Dialysed and non-dialysed both produce good responses, but using the non-dialysed vaccine will reduce the risk of introducing foreign antigens into the cultures.
	• Use a 1/10 dilution of vaccine in RPMI	Cannot draw conclusions as incomplete data due to low cell counts, but for other measures, this consistently produces the biggest response.
	• Use 168 hours for culture	Based on the small number of samples available, 96 hours did not appear to be sufficient for measurement of proliferation by CFSE dilution. 138 -140 hours produced good results regarding ^{3}H-thymidine incorporation, using a longer culture time will not be detrimental, therefore will culture for 168 hours.
T cell cytokine production (CBA)	• Stimulate with ConA, but not PHA • Use non-dialysed vaccine	ConA generally produced the greatest response. The dialysed vaccine produced better IL-6, IL-10 and TNF responses, and dialysed and non-dialysed produced equal IFN-γ responses. The non-dialysed vaccine produced a better IL-2 response, while neither produced a good IL-4

	• Use a 1/10 dilution of vaccine in RPMI • Use 24 hours culture time	response. As the non-dialysed vaccine did produce results, the non-dialysed vaccine will be used, to reduce the risk of introducing foreign antigens into the cultures. This produced the biggest response For the non-dialysed vaccine, 24 hours of culture produces the maximal response for IL-4, IL-10 and TNF, 48 hours of culture produced the maximal response for IL-2, and 72 hours of culture produced the maximal response for IFN-γ, while all time points produced similar responses for IL-6. An increase in cytokine production could be seen after 24 hours of culture.

Table 16 : The main decisions made based on the pilot study results

CHAPTER 4

PREBIOTIC INTERVENTION STUDY: STUDY DESIGN, SUBJECT CHARACTERISTICS & IMPACT OF ORAFTI® SYNERGY1 ON FAECAL BIFIDOBACTERIA

4.1 INTRODUCTION

ß2-1 fructans, such as inulin and shorter-chain fructooligosaccharides, have been reported to modulate the intestinal microbiotia (43, 241-245); they act to increase the numbers of bifidobacteria and lactobacilli. It is thought that these types of bacteria influence the host immune system, improving its function. Indeed a small number of studies performed to date indicate that ß2-1 fructans can modulate aspects of immune function (see Chapter 1). This has been reported in children (91, 99, 101, 102), the elderly (43, 92, 94, 96, 97, 103), younger adults (98), and adults with colon cancer (104, 105, 108)), active ulcerative colitis (108) and intensive care patients (109). However, few studies have been performed using the gold standard response to vaccination as a tool to examine immune function (91-93, 96, 97, 101, 103, 106). These studies were performed in infants or children (91, 93, 101, 106), or in elderly adults (92, 96, 97, 103), and there are no studies investigating the potential effects of ß2-1 fructan-type prebiotics on immune function in healthy middle aged adults. The four of the above studies carried out in the elderly all used influenza vaccination as a challenge, but used varying times of ß2-1 fructan supplementation before the vaccine was given, and duration of supplementation after vaccination also differed. Only one of the studies carried out in the elderly used solely ß2-1 fructan supplementation (92), the remaining studies used supplements also containing vitamins, minerals, fats or probiotics. Therefore, it is difficult to be clear about the effect that the ß2-1 fructans may have had. Despite there being eight studies which use the response to vaccination as a way to determine the effect of ß2-1 fructans upon immune function, there are inconsistencies between the studies, thus making the results somewhat difficult to interpret.

The overall aim of the main study described in this thesis is to evaluate the effect of a mixture of inulin and oligofructose (known as Orafti® Synergy1) on immune function, including the response to seasonal influenza vaccination, in healthy middle aged subjects. A pilot study was performed in order to develop a vaccination protocol for use in assessing the immune response (see Chapter 3). This chapter will describe the study design, subject characteristics, compliance to the study, and gastrointestinal sensations experienced by subjects when taking Orafti® Synergy1 or placebo. The effect of Orafti® Synergy1 upon the gut microbiotia will also be described. The remaining chapters of this thesis will describe the effects of Orafti® Synergy1 on immune function prior to (Chapter 5) and following (Chapter 6) influenza vaccination.

4.2 HYPOTHESIS

The hypothesis being tested in the work described in this chapter is that consumption of Orafti® Synergy1 for four weeks will increase the number of bifidobacteria within the gut

microbiotia and that bifidobacteria numbers will be greater than in the control group at four weeks.

4.3 STUDY DESIGN, MATERIALS AND METHODS

4.3.1 STUDY DESIGN

The study was a randomised, double-blind, placebo controlled trial. It was registered as "Prebiotics and Immune Function in Middle Aged Humans" on www.clinicaltrials.gov (identifier: NCT00898599). The study was approved by the Southampton and South West Hampshire Local Research Ethics Committee (09/H0504/2) and clinical governance was provided by the Southampton University Hospitals NHS Trust R&D. All subjects provided written informed consent and the study was performed according to Good Clinical Practice. Orafti® Synergy1 and placebo were provided as powders within sealed paper sachets (4 g/sachet) by Beneo-Orafti, and were identical in packaging (except for the labelling, which was A or B) and in appearance. Orafti® Synergy1 contains inulin and oligofructose in a ratio of one to one. The placebo was maltodextrin. Subjects were asked to consume two sachets per day (one in the morning and one in the evening) by stirring the contents into a glass of water. Subjects were given sufficient sachets for the study at the clinic visits week zero and week four. Randomization to A or B was performed by placing equal numbers of letter "A" and letter "B" into plain envelopes, and picking an envelope at random upon inclusion of a volunteer into the study. Un-blinding was performed upon completion of statistical analysis. Supplementation continued for eight weeks (Figure 44) with blood and saliva samples being taken at weeks zero, four, six and eight and faecal samples at weeks zero and four. The 2008/2009 seasonal influenza vaccination (2008/2009 Imuvac® vaccine; Solvay Healthcare, Southampton, UK) was given at week four. All subject visits were at the Wellcome Trust Clinical Research Facility, Southampton General Hospital. Blood and saliva were used for measures of immune function (Figure 45) to be reported in Chapters 5 and 6. Faeces were used to determine numbers of total and bifidobacteria, reported in this Chapter.

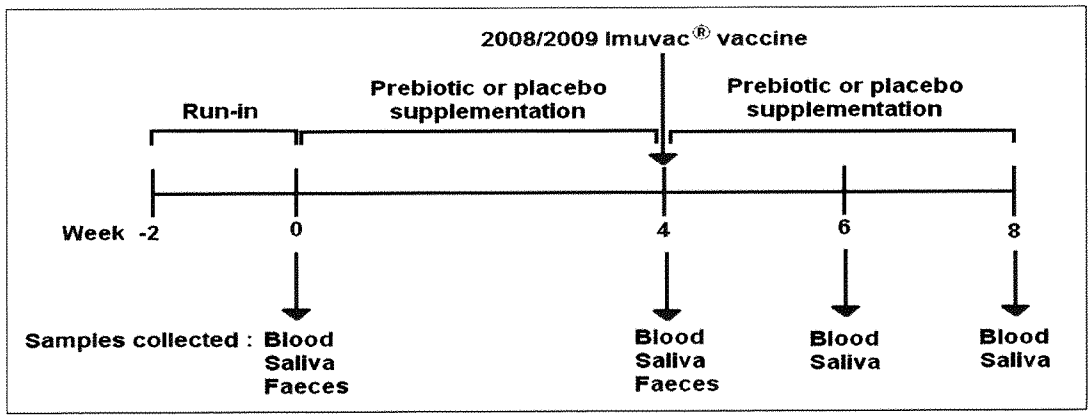


Figure 44 : Prebiotic intervention study design

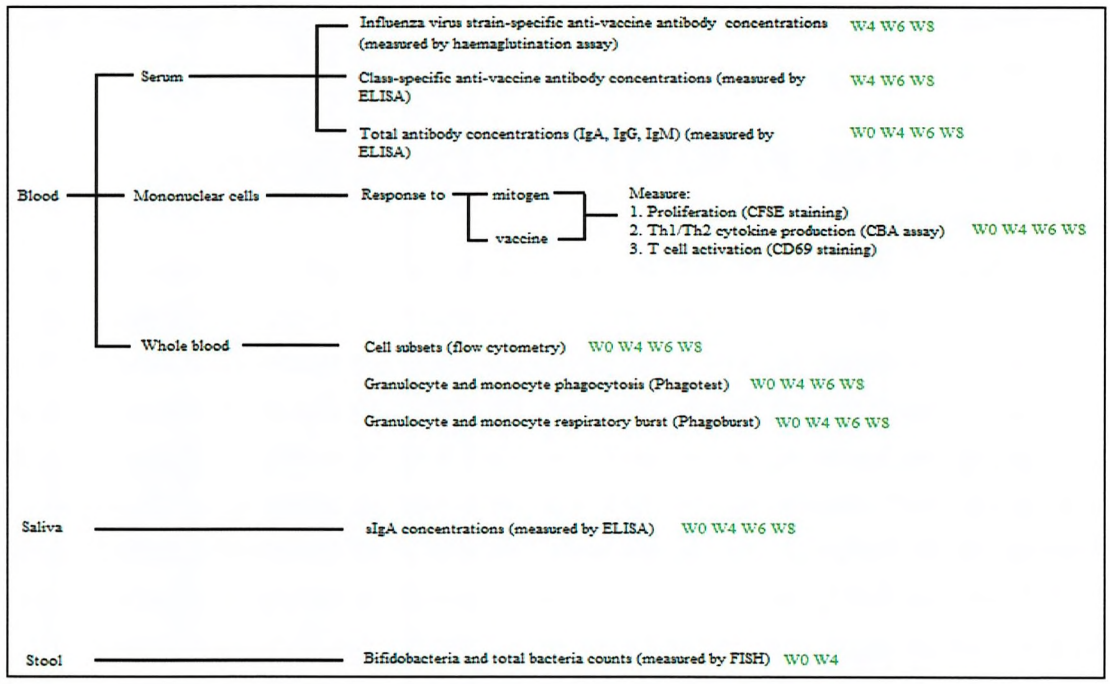


Figure 45 : Sample analysis performed (the green letters indicate at which time points (weeks) the analyses were carried out)

4.3.2 SAMPLE SIZE CALCULATION

The primary outcome for the study was a difference in anti-vaccine antibody response between the two groups (prebiotic versus placebo). The pilot study indicated mean responses to the three vaccine antigens in the 2007/2008 seasonal influenza vaccine with standard deviations of approximately 100% of the mean, using data from the six healthy volunteers. It can be reasonably assumed that the extent of the responses to the 2008/2009 seasonal influenza vaccine would be of the same order as those derived from the pilot study. Possible magnitude of effects of the prebiotic intervention were estimated from those reported previously by others using response to seasonal influenza vaccination as an outcome (92, 96, 97, 103). Thus a 20% higher anti-vaccine antibody response was anticipated in the prebiotic group compared with the control. Using data for mean and standard deviation of responses in the pilot study a sample size of 27 subjects per group would provide 80% power of identifying a 20% difference in mean as being significant at $p < 0.05$. To allow for a drop-out rate of 20%, it was decided to recruit 35 subjects per group (i.e. 70 subjects in total). This number of subjects is consistent with the numbers studied in previous trials of this sort (92, 96, 97, 103). Due to time constraints, a lower number of subjects were recruited (25 in the prebiotic group and 24 in the placebo group). The drop-out rate was also lower than anticipated (12% in the prebiotic group and 14 % in the placebo group). This resulted in 22 subjects completing the study in the prebiotic group, and 21 in the placebo group (see Figure 46).

4.3.3 SUBJECTS

Healthy male and female volunteers, aged between 45 and 65 years, were recruited via poster, word of mouth, e-mail and newspaper/magazine advertisements.

Inclusion criteria were:

1. Aged 45 - 65 years,
2. Body mass index (BMI) between 20 and 32 kg/m^2,
3. Not consuming prebiotic or probiotic supplements, drinks or foods,
4. In general good health,
5. No antibiotic use in the two months prior to entering the study, or during the study,
6. Not vaccinated with the current seasons (2008/2009) influenza vaccination.

Exclusion criteria were:

1. Aged < 45 or > 65 years,
2. BMI < 20 or > 32 kg/m^2,
3. Being diabetic (type 1 or type 2),
4. Displaying manifestations of allergy (asthma, hay-fever or dermatitis) or being treated for these,
5. Being egg allergic,
6. Use of any prescribed medication (unless deemed acceptable by the PI),
7. Suffering from any infectious illness,
8. Chronic gastrointestinal problems (e.g. irritable bowel syndrome, inflammatory bowel disease, cancer),
9. Recent blood donation,
10. Participation in another clinical trial,
11. Use of prebiotic or probiotic supplements, foods or drinks,
12. Consuming vitamin, oil or mineral supplements,
13. Previously vaccinated with the 2008/2009 vaccination or a previous influenza vaccination which contained any of the strains included in the 2008/2009 vaccine.

A total of 49 subjects were recruited, and the flow of subjects through the study is shown in Figure 46.

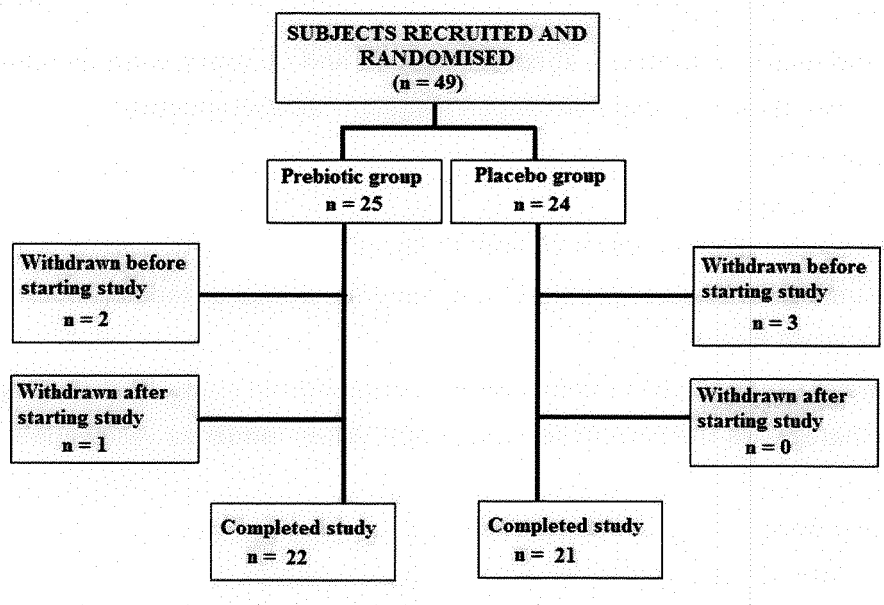


Figure 46 : Flow of subjects through the prebiotic intervention study

Characteristics of the subjects who completed the study are given in Table 17.

Table 17 : Subject characteristics

	Prebiotic group	Placebo group
N	22	21
Age (years); mean (range)*	54 (45 -62)	56 (45 - 63)
Male : female**	3 : 19	8 : 13
BMI; mean (range)***	25.73 (19.40 - 33.30)	24.98 (17.70 - 33.80)

*Not significantly different between groups (analysed by Mann-Whitney test, p = 0.131)

**Not significantly different between groups (analysed by Chi squared test, p = 0.066)

***Not significantly different between groups (analysed by independent T-test, p = 0.533)

Eight subjects (six in the prebiotic group and two in the placebo group) had been vaccinated with an influenza vaccine in previous years (two subjects had received the 2007/2008 influenza vaccine, one in the prebiotic group and one in the placebo group). There was no overlap in the strains used in the 2007/2008 vaccine and those used in the 2008/2009 vaccine. However, it is possible that previous exposure to a similar strain of the virus may affect an individual's response to the vaccine. But it is also possible that subjects who had not been previously vaccinated had been exposed to similar strains of the virus in the past, through natural exposure.

It was considered appropriate to recruit participants with a wide range of BMIs, as overweight and obesity are prevalent in the general population. Therefore inclusion of

overweight individuals as subjects increases the representativeness to the general population of the study population. Individuals who were grossly obese (a BMI > 32 kg/m^2) were excluded.

4.3.4 COMPLIANCE

Compliance was assessed by counting the number of returned sachets that had been used, and subtracting this from the number of sachets which were given out at the beginning of the trial.

4.3.5 SUBJECT SELF-REPORTED GASTROINTESTINAL SENSATIONS

At each clinic visit subjects were asked to report any gastrointestinal sensations or health problems they had encountered since the previous clinic visit.

4.3.6 FLUORESCENCE IN SITU HYBRIDIZATION FOR ENUMERATION OF TOTAL BACTERIA AND BIFIDOBACTERIA IN FAECAL SAMPLES

Storage: Faecal samples were stored at -80°C for approximately ten months following collection.

Fixing: Samples were thawed and diluted 1:10 in phosphate-buffered saline (PBS). Samples were homogenised in a stomacher for 120 seconds, decanted into a 50 ml tube, and vortexed for 30 seconds. Samples were centrifuged at 1500 rpm for 2 minutes, to separate the liquid and solid. Aliquots of the liquid were removed as follows:

- 375 µl for paraformaldehyde fixing (duplicate). 375 µl aliquots were placed into 1125 µl of cold 4% (w/v) paraformaldehyde, vortexed, and incubated at 4°C for 4 hours. Samples were centrifuged at 13000 rpm for 5 minutes, and then washed twice in 1 ml PBS at 13000 rpm for 5 minutes. The washed cells were finally suspended in a mixture of 150 µl of PBS and 150 µl ethanol, and stored at -20°C until fluorescence in situ hybridisation (FISH) analysis.
- 375 µl for ethanol fixing (duplicate). Aliquots were centrifuged at 13000 rpm for 5 minutes. The cell pellet was finally suspended in a mixture of 150 µl of PBS and 150 µl ethanol, and stored at -20°C until FISH analysis.

FISH (Bifidobacteria, stained with Bif164): Ethanol-fixed samples were diluted in a suitable volume of sterile PBS/10 % sodium dodecyl sulphate (SDS) in order to obtain the correct number of cells per microscopic field of view (1:10 for week 0 samples, 1:20 for week 4 samples). 20 µl of each diluted sample was added to a separate well of a six-well slide (Teflon/poly-L-lysine-coated, 6-well, 10-mm diameter well slides; Tekdon Ins., Myakka City, FL, USA). Slides were dried for approximately 15 minutes at 46-50°C in a desktop plate incubator. They were then dehydrated using an alcohol washing series (50, 80 and

96 % [v/v] ethanol) for 3 minutes with each solution. Slides were dried for two minutes at 46-50°C in a desktop plate incubator to evaporate excess ethanol. A synthetic oligonucleotide probe targeting specific regions of the 16S rRNA labelled with the fluorescent dye Cy3 was used for the enumeration of the *Bifidobacterium* genus (Probe: Bif 164, target sequence: CATCCGGCATTACCACCC). 50 µl of the hybridization buffer (5 µl of the probe [50 ng/µl] and 50 µl of the hybridization buffer [1000µl: 799 µl ddH_2O, 20 µl 1 M Tris/HCl (pH 8.0), 180 µl 5 M NaCl and 1 µl SDS] was added to each well and hybridised for 4 hours in a sealed slide tray inside a microarray hybridization incubator. Following hybridisation, slides were washed in 50 ml of washing buffer (50 ml: 40 ml ddH_2O, 1 ml 1 M Tris/HCl (pH 8.0), 9 ml 5 M NaCl, and 20 µl of DAPI [4',6-diamidino-2-phenylindole; 50 ng /µl]) for 15 minutes at 50°C. Slides were dipped into ice-cold distilled water for 2-3 seconds, and dried with compressed air. 5 µl of antifade reagent was added to each well, and a cover-slip applied to the slide. Slides were stored in the dark at 4 °C until analysis. Slides were counted using an epifluorescence Brunel microscope. Filters for the DAPI stain (excitation at 550 nm and emission at 461 nm) and the Cy3 dye (excitation at 550 nm and emission at 564 nm) were used. 15 fields of view were counted for each sample. *Bifidobacteria* counts were determined using the following equation:

$$\text{Bacteria/g of faeces} = DF \times ACC \times 8702.47 \times 50 \times DF_{sample}$$

DF: the dilution factor = 8 (300 [volume of sample]/375 [volume of paraformaldehyde] x 10 [original dilution of stool sample into PBS])
ACC: the average cell count from the 15 fields of view
8702.47: the area of the well/the area of the field of view
50: returns the cell count back to per ml of sample
DF_{sample}: the dilution of the sample used (10 for week 0 samples and 20 for week 4 samples)

FISH (total bacteria, stained with DAPI): Paraformaldehyde-fixed samples were diluted in a suitable volume of sterile PBS/10 % SDS in order to obtain the correct number of cells per microscopic field of view (1:400 or 1:1000). 20 µl of each diluted sample was added to a separate well of a six-well slide (Teflon- and poly-L-lysine-coated, 6-well, 10-mm diameter well slides; Tekdon Inc., Myakka City, FL, USA). Slides were dried for approximately 15 minutes at 46-50°C in a desktop plate incubator. They were then dehydrated using an alcohol washing series (50, 80 and 96 % [v/v] ethanol) for 3 minutes with each solution. Slides were dried for two minutes at 46-50°C in a desktop plate incubator to evaporate excess ethanol. Cells were stained with the nucleic acid stain DAPI. 50 ng/µl DAPI was added to PBS at a dilution of 1:11, and 50 µl of this mixture was applied to each well.

Slides were incubated at room temperature in the dark for 15 minutes. Following incubation, slides were washed in 50 ml of washing buffer (50 ml: 40 ml ddH_2O, 1 ml 1 M Tris/HCl (pH 8.0) and 9 ml 5 M NaCl) for 15 minutes at 50°C. Slides were dipped into ice-cold distilled water for 10 seconds, and dried with compressed air. 5 µl of antifade reagent was added to each well, and a cover-slip applied to the slide. Slides were stored in the dark at 4 °C until analysis. Counting was performed as for Bif164 staining, using the following equation:

$$\text{Bacteria/g faeces} = DF \times ACC \times 5100 \times 50 \times DF_{sample}$$

An example of one field of view from a DAPI stained sample is shown in Figure 47.

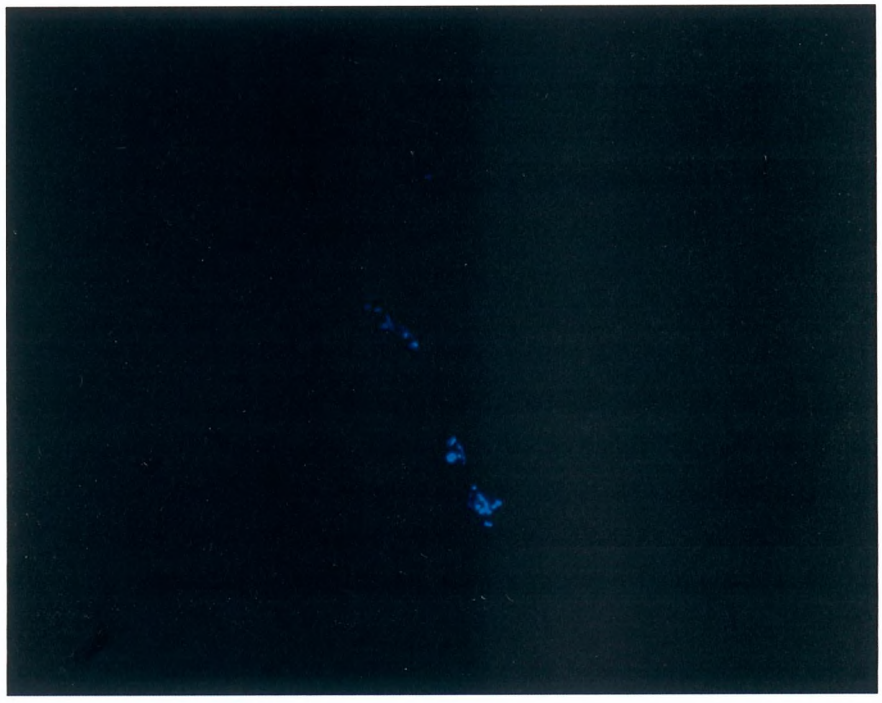


Figure 47 : Example of one field of view from a slide stained with DAPI to enumerate total bacteria (bacteria are shown in bright blue)

4.3.7 STATISTICAL ANALYSIS

Data for bacterial counts were not normally distributed, and therefore were analysed by Mann-Whitney test (comparing groups at each time point, and comparing changes over time between groups), and Wilcoxon Signed Ranks test (comparing changes over time within groups). Correlations were analysed by Spearman's Rank coefficient. Age and compliance were analysed by Mann-Whitney test, BMI by independent T-test, and gender and gastrointestinal sensations by Chi squared test. All analyses were performed using SPSS version 17.0.

4.4 RESULTS

4.4.1 COMPLIANCE

Compliance in both groups was found to be good, and ranged from 74 % to 100 % in the prebiotic group and from 87 % to 101 % in the placebo group. Compliance was significantly higher (Mann-Whitney test, p = 0.05) in the placebo group (median = 100%) compared to the prebiotic group (median = 97.5%).

4.4.2 SUBJECT SELF-REPORTED GASTROINTESTINAL SENSATIONS

The only subject to withdraw after beginning on the study was in the prebiotic group. This subject withdrew due to being unable to tolerate the unpleasant sensations (bloating, nausea, and feeling urgency to empty bowels) experienced upon taking this supplement, in addition to experiencing problems with providing blood samples. Gastrointestinal sensations, as reported among subjects who completed the study are shown in Table 18.

Table 18 : Subject self reported gastrointestinal sensations

	Prebiotic group	Placebo group
Increased bloating	2	1
Decreased bloating	1	0
Increased flatulence	12*	1
Increased regularity	9**	2
Increased constipation	2	2
Decreased constipation	1	0
Loose stools	3	0

Data are number of subjects reporting each sensation

*Significantly higher in the prebiotic group (Chi squared test, p = 0.001)

**Significantly higher in the prebiotic group (Chi squared test, p = 0.034)

From these results it appears that gastrointestinal sensations were experienced by a greater number of subjects in the prebiotic group than in the placebo group.

In addition to the findings presented in Table 18, in the placebo group one subject reported heartburn, one an "irritated bowel" after taking the supplement, and one reported vomiting. One subject in each group reported having an upset stomach. Two subjects in the prebiotic group reported headache and nausea. Six subjects in the prebiotic group and two in the placebo group reported experiencing common cold

symptoms. There were no significant differences between the groups regarding these events.

4.4.3 NUMBERS OF TOTAL BACTERIA AND BIFIDOBACTERIA

Figure 48 shows the numbers of total bacteria and bifidobacteria in the faeces of each group at study entry (week 0) and after 4 weeks of supplementation.

4.4.3.1 COMPARING GROUPS AT EACH TIME POINT

At week 0 there were no differences between the prebiotic group and the placebo group regarding bifidobacteria counts (1.33×10^9 and 1.58×10^9/g faeces), total bacteria counts (1.01×10^{11} and 7.18×10^{10}/g faeces) or bifidobacteria counts expressed as a percentage of total bacteria counts (1.26 % and 1.25 %).

At week 4, bifidobacteria counts were significantly higher ($p = 0.001$) in the prebiotic group (median 2.82×10^9/g faeces) compared to the placebo group (median 1.14×10^9/g faeces). There was no difference in the total bacteria counts between the prebiotic and placebo groups (1.07×10^{11} and 6.81×10^{10}/g faeces), or in bifidobacteria counts when expressed as a percentage of total bacteria counts (2.64 % and 1.62 %).

4.4.3.2 COMPARING WITHIN GROUPS AT THE TWO TIME POINTS

In the prebiotic group, bifidobacteria counts were significantly higher ($p < 0.001$) at week 4 (median 2.82×10^9/g faeces) compared to week 0 (median 1.33×10^9/g faeces). In the placebo group, there was no significant difference in number of bifidobacteria between week 4 (median 1.14×10^9/g faeces) and week 0 (median 1.58×10^9/g faeces).

There was no significant change in either group for total bacteria counts at week 4 compared to week 0 (1.07×10^{11} and 1.01×10^{11}/g faeces in the prebiotic group, and 6.81×10^{10} and 7.18×10^{10}/g faeces in the placebo group).

In the prebiotic group, bifidobacteria expressed as a percentage of total bacteria were significantly higher ($p = 0.001$) at week 4 (median 2.64%) compared to week 0 (median 1.26%). There was no significant difference in the placebo group: week 4 median = 1.62% and week 0 median = 1.25%.

4.4.3.3 COMPARING CHANGES OVER TIME BETWEEN GROUPS

The change in bifidobacteria counts in the prebiotic group between week 0 and week 4 (median increase 1.26×10^9/g faeces) was significantly different ($p = 0.001$) from the change in the placebo group (median increase 3.37×10^7/g faeces). There was no

difference in the change in total bacteria counts between the prebiotic group (median increase = 6.31×10^7/g faeces) and the placebo group (median change = -8.43×10^9/g faeces). There was a significantly greater ($p = 0.002$) change in bifidobacteria counts when expressed as a percentage of total bacteria in the prebiotic group (median increase 0.84 %) compared to the placebo group (median increase = 0.05 %).

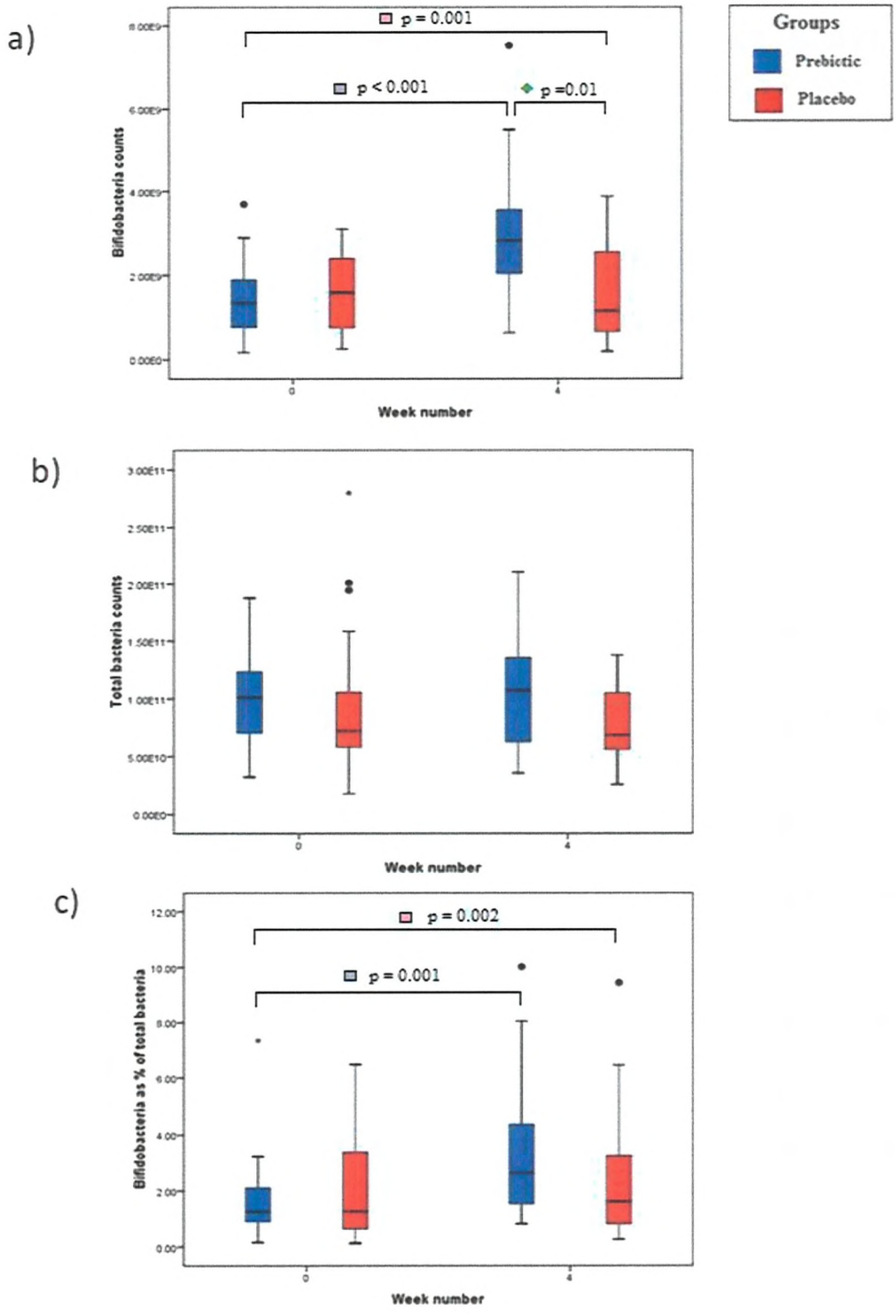


Figure 48 : Faecal bacteria counts before (week 0) and after (week 4) Orafti® Synergy1® or placebo supplementation. a: bifidobacteria counts (/g of faeces), b: total bacteria counts (/g of faeces), c: bifidobacteria expressed as a percentage of total bacteria. Green diamonds: difference between groups at that time point (Mann Whitney test); purple squares: difference between week 0 and week 4 within individual groups (Wilcoxon signed ranks test); pink squares: comparing changes over time between groups (Mann Whitney test). Data are expressed as medians.

4.4.3.4 CORRELATION BETWEEN BIFIDOBACTERIA COUNTS AT WEEK 0 AND THE CHANGE IN BIFIDOBACTERIA IN THE PREBIOTIC GROUP

Baseline counts of bifidobacteria were plotted against the change in bifidobacteria from week 0 to week 4 (Figure 49). No significant correlation was observed (r_s = 0.011).

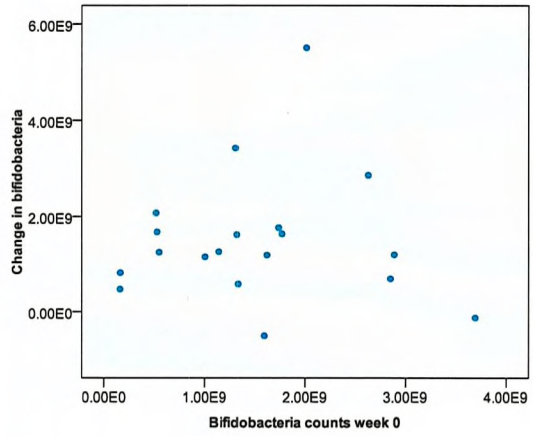


Figure 49 : Correlation of baseline bifidobacteria counts with the change in bifidobacteria counts seen between week 0 and week 4 of Orafti® Synergy1 supplementation

4.5 DISCUSSION

This chapter described the design of the randomised controlled trial of 8 g/day Orafti® Synergy1 in healthy middle aged subjects, the characteristics of the subjects recruited, compliance, gastrointestinal sensations reported by the subjects, and the effect on gut microbiotia with a focus on bifidobacteria. Both males and females were recruited into the study, although more females were recruited, due to a greater number of females volunteering to take part. This may indicate a greater interest in this area of research from the female population. Although there was an imbalance in favour of females, this was not different between the two study groups, and so it is unlikely that this will have had an effect on the results. Fewer subjects than initially planned were recruited to the trial; this was because of the expiry of the 2008/2009 seasonal influenza vaccine which meant it could no longer be used. Compliance, as determined by counting of returned sachets, was very good in both groups. More subjects in the prebiotic group perceived gastrointestinal sensations, particularly increased flatulence and increased regularity of bowel movements. Both of these are suggestive of a change in gut microbiotia. Whilst increased flatulence may be unpleasant, increased regularity of bowel movements may be a desirable outcome both personally and clinically (7, 246). For example, some functional foods already claim "regularity" as a positive aspect. The hypothesis underlying the work presented in this chapter was that consumption of Orafti® Synergy1 for four weeks will increase the number of bifidobacteria within the gut microbiotia and that bifidobacteria numbers will be greater than in the control group at four weeks. Results using FISH clearly

demonstrated an increase in bifidobacteria in the prebiotic group and that bifidobacteria numbers were higher at 4 weeks in the prebiotic than in the control group. Therefore, the results from this set of experiments enable the hypothesis to be accepted, and it can be concluded that the prebiotic supplement has had a bifidogenic effect within the gut. Other studies have also reported the bifidogenic properties of the ß2-1 fructans (247), inulin (241-244), oligofructose (43, 245, 248) and Orafti® Synergy1 (249). There was an approximate doubling of bifidobacteria numbers in the Orafti® Synergy1 group. A similar size of effect has been observed in other studies using fructooligosaccharides (241, 242). Several other studies have reported bigger increases (243-245, 248). Supplementation of 20 g/day of Orafti® Synergy1 to healthy adults for 28 days reported an increase from 7.54 x 10^{10} to 8.19 x 10^{10} bacteria/g faeces (249). The increase in bifidobacteria seen in the current study may be more modest in comparison to that seen in some other studies. Differences in the extent of the bifidogenic effect between studies may be due to the type of fructooligosaccharide used, the dose used, duration of supplementation, the heterogeneity of subjects taking part, as well as the method used to measure the faecal bifidobacteria.

Some studies have shown that the baseline levels of bifidobacteria in the individual can affect the magnitude of the increase seen with prebiotic supplementation, with a higher initial level of bifidobacteria being associated with a smaller increase (242, 243, 245). This observation was not made in the current study, which is in agreement with a study that found no effect of the relationship between baseline levels of bifidobacteria on the bifidogenic effect of inulin (241).

Data presented in this chapter show that supplementation with 8 g/day Orafti® Synergy1 for four weeks has a bifidogenic effect on the gut in healthy middle aged adults. In light of previous studies that have shown that prebiotic supplementation may modulate some aspects of immune function (43, 91, 92, 94, 250, 251), an effect which is presumed to be mediated by a change in gut microbiotia, it may therefore be anticipated that some changes in immune function may be observed in these subjects. Chapters five and six of this thesis will describe the effects that were observed on immune function in these subjects.

CHAPTER 5

PREBIOTIC INTERVENTION STUDY: IMPACT OF ORAFTI® SYNERGY1 UPON IMMUNE FUNCTION IN THE ABSENCE OF IMMUNE CHALLENGE

5.1 INTRODUCTION

Published literature has shown alterations in immune parameters following ß2-1 fructan supplementation in children (91, 99, 101, 102), young adults (98), elderly adults (43, 92, 94, 96, 97, 103), adults with colon cancer (104, 105)), active ulcerative colitis (108) and intensive care patients (109). These studies have used a variety of ß2-1 fructans, often in combination with other nutrients or probiotics, and have used different doses for different lengths of time. Also, different immune outcomes have been measured in the different studies. Therefore, although it appears from looking at the literature as a whole that ß2-1 fructans do appear to alter some aspects of immune function, it is difficult to tell what the effects really are and the extent of their impact ((63), see section 1.4.5.1.2). In order to assess the effect that a nutritional supplement may have upon immune function, it is important to look at a wide a range of markers (10), and in humans these will generally be systemic immune markers (10).

Most of the above studies have been carried out in the young or the elderly, two populations who may be particularly susceptible to modulation of their immune system. Apart from one study in young healthy adults (98), there are no studies looking at the effect of a ß2-1 fructan supplement on immune function in healthy adults who might be expected to have a near optimally functioning immune system. This is an important population to consider because if immune function could be improved before the onset of the immunosenescence that occurs with ageing, then the impact of the age-associated decline in immune function may be delayed.

It is considered that modification of host gut microbiotia is required in order for prebiotics to influence the host immune response. In Chapter 4 it was shown that Orafti® Synergy1 (8 g/day for four weeks) was able to increase faecal bifidobacteria counts in healthy middle aged subjects, thereby confirming its prebiotic effect. Thus, it may be anticipated that some changes in immune function may be observed following supplementation with Orafti® Synergy1. This chapter describes the results of experiments measuring immune outcomes before and after four weeks of 8 g/day Orafti® Synergy1 supplementation and in the absence of an immune challenge.

5.2 HYPOTHESIS

The hypothesis being examined by the experiments described in this chapter is that Orafti® Synergy1 (8 g/day for four weeks) will improve aspects of immune function in healthy middle-aged subjects

5.3 METHODS

5.3.1 STUDY DESIGN AND SUBJECT CHARACTERISTICS

The design of the study is described in Section 4.3 along with inclusion and exclusion criteria, subject characteristics (see Table 17) and the sampling procedure (see Figure 45). Here immune outcomes determined at study entry (week zero) and after four weeks of Orafti® Synergy1 (8 g/day) are described.

5.3.2 MEASUREMENT OF IMMUNE PARAMETERS

The following immune outcomes were measured:

- Immune cell phenotypes in peripheral blood, as described in Section 2.2;
- Neutrophil and monocyte phagocytosis of *E. coli*, as described in Section 2.3.1;
- Neutrophil and monocyte oxidative burst in response to *E. coli* or phorbolmyristoyl acetate (PMA), as described in Section 2.3.2;
- Total serum IgA, IgG and IgM concentrations, as described in Sections 2.8.1 to 2.8.3;
- Total salivary sIgA concentration, as described in Section 2.8.4;
- T cell cytokine production in response to concanavalin A (ConA), as described in Section 2.6;
- T cell proliferation in response to ConA, as described on Section 2.7.2.2;
- T cell activation in response to ConA, as described in Section 2.5.

5.3.3 STATISTICAL ANALYSIS

Where data were not normally distributed, analysis was performed using the Mann-Whitney test (comparing groups at each time point, and comparing changes over time between groups), and Wilcoxon Signed Ranks test (comparing changes over time within groups). Where data were normally distributed, analysis was performed using independent samples T-test (comparing groups at each time point, and comparing changes over time between groups) and dependent samples T-test (comparing changes over time within groups). All analyses were performed using SPSS version 17.0.

5.4 RESULTS

5.4.1 IMMUNE CELL PHENOTYPES

Immune cell phenotypes were determined in whole blood. Data are expressed as percentages of the gated population (e.g. of lymphocytes, of monocytes) and are shown in Table 19. There was no effect of the prebiotic on $CD3^+/CD4^+$, $CD4^+$, $CD3^-/CD19^+$, or $CD14^+$ cells as a percentage of total leukocytes, or on percentage of lymphocytes defined as T_{reg} cells ($CD4^+CD25^+CD127^{lo}$). The percentage of $CD3^+/CD8^+$ cells was higher in the

prebiotic group at week four compared to the placebo group (22.9% and 17.3% respectively, $p = 0.032$) and the percentage of $CD8^+$ cells was higher in the prebiotic group at week four compared to the placebo group (26.7% compared to 21.0%, $p = 0.023$). However, these differences seem to reflect inherent differences between the groups that were present at study entry (Table 19) and are probably not due to the prebiotic. The CD4:CD8 ratio was higher in the placebo group compared to the prebiotic group at week four, which is due to the higher percentage of $CD8^+$ cells seen in the prebiotic group at this time.

The percentage of $CD3^-/CD16^+$ cells decreased in the placebo group (from 10.7% to 9.4%, $p = 0.023$), but this was not observed in the prebiotic group. $CD14^+$ expressed as a percentage of monocytes was increased in the placebo group (63.9% to 77.9%, $p = 0.020$), but this was not observed in the prebiotic group.

When comparing the change between week zero and week four in the prebiotic group, to the change in the placebo group, there were no significant differences. Therefore, it must be concluded that the prebiotic supplement had no effect upon immune cell phenotypes in the blood.

Table 19 : The effect of four weeks supplementation with Orafti® Synergy1 or placebo upon immune cell phenotypes in whole blood

Cell phenotype	Placebo group			Prebiotic group			*p (prebiotic vs. placebo)***		*p (change over time between groups)***
	Week 0	Week 4	*p (week 0 vs. week 4)**	Week 0	Week 4	*p (week 0 vs. week 4)**	*week 0*	*week 4*	
$CD3^+CD4^+$ (% of lymphocytes)	50.9 (8.4)	50.5 (6.9)	*ns*	50.9 (6.8)	50.1 (8.4)	*ns*	*ns*	*ns*	*ns*
$CD4^+$ (% of lymphocytes)	51.2 (8.3)	50.9 (7.1)	*ns*	51.2 (6.9)	50.3 (8.3)	*ns*	*ns*	*ns*	*ns*
$CD3^+CD8^+$ (% of lymphocytes)	17.9 (7.5)	17.3 (7.1)	*ns*	22.8 (9.6)	22.9 (8.9)	*ns*	*ns*	*0.032*	*ns*
$CD8^+$ (% of lymphocytes)	21.3 (8.2)	21.0 (7.4)	*ns*	26.3 (8.8)	26.7 (8.0)	*ns*	*ns*	*0.023*	*ns*
$CD3^-CD16^+$ (% of lymphocytes)	10.7 (4.3)	9.4 (3.7)	*0.023*	9.6 (5.6)	8.2 (5.5)	*ns*	*ns*	*ns*	*ns*
$CD3^-CD19^+$ (% of lymphocytes)	10.4 (3.5)	10.8 (3.7)	*ns*	9.4 (3.7)	10.0 (3.9)	*ns*	*ns*	*ns*	*ns*
$CD14^+$ (% of monocytes)	63.9 (24.8)	77.9 (13.8)	*0.020*	74.7 (15.2)	76.9 (14.3)	*ns*	*ns*	*ns*	*ns*
$CD14^+$ (% of leukocytes)	3.2 (0.9)	3.6 (1.5)	*ns*	3.2 (1.0)	3.2 (0.6)	*ns*	*ns*	*ns*	*ns*
$CD127^{lo}$ (% of $CD4^+CD25^+$ cells)	69.5 (7.4)	73.4 (8.3)	*ns*	73.8 (6.8)	73.1 (8.6)	*ns*	*ns*	*ns*	*ns*
$CD4^+CD25^+CD127^{lo}$(% of leukocytes)	1.1 (0.4)	1.2 (0.6)	*ns*	1.0 (438)	1.2 (0.5)	*ns*	*ns*	*ns*	*ns*
$CD4^+$:$CD8^+$	2.8 (1.2)	2.8 (1.1)	*ns*	2.2 (0.9)	2.1 (0.9)	*ns*	*ns*	*0.045*	*ns*

Data are presented as mean (standard deviation) *Analysed by dependent samples T-test **Analysed by independent samples T-test Note: Some distributions were not normally distributed, and so were also analysed using non-parametric tests (Mann-Whitney or Wilcoxon Signed Ranks) - these gave the same outcomes as the parametric tests indicated above, and so for ease of data presentation, all data are presented as mean (SEM) and analysed with parametric tests (independent or dependent samples T tests).

5.4.2 PHAGOCYTOSIS OF E. COLI BY NEUTROPHILS AND MONOCYTES

Results from experiments measuring neutrophil and monocyte phagocytosis (made using Phagotest kits) in whole blood are shown in Table 20. Data are presented as percentage of cells engaging in phagocytosis (referred to as percent positive) and the extent of phagocytosis of those active cells (mean fluorescence intensity; MFI). No differences were observed between the groups in any of the outcomes measured. However there were differences within the groups, between week zero and week four. The percentage of neutrophils engaging in phagocytosis (percent positive) was decreased from week zero to week four in both groups (from 99.1% to 92.9% in the placebo group, $p < 0.001$, and from 99.1% to 92.2% in the prebiotic group, $p = 0.003$). The same pattern was observed for the percentage of monocytes engaging in phagocytosis (85.6% to 71.9% in the placebo group, $p = 0.013$, and from 86.5% to 67.4% in the prebiotic group, $p < 0.001$). The MFI of neutrophils (the activity of the cells) increased from week zero to week four in both groups (from 210.0 to 334.1 in the placebo group, $p = 0.005$, and from 208.5 to 242.9 in the prebiotic group = 0.031), and the MFI of monocytes was also increased between week zero and week four, although this was only significant in the placebo group (123.0 to 194.9 in the placebo group, $p = 0.001$, and from 117.9 to 137.6 in the prebiotic group, ns). However, when comparing the change between week zero and week four in the prebiotic group, to the change in the placebo group there were no significant differences. Therefore, it must be concluded that the prebiotic supplement had no effect upon blood neutrophil and monocyte phagocytosis.

Table 20 : The effect of four weeks supplementation with Orafti® Synergy1 or placebo upon blood neutrophil and granulocyte phagocytosis of E. coli

	Placebo group			Prebiotic group					
	Week 0	**Week 4**	*p (week 0 vs. week 4)**	**Week 0**	**Week 4**	*p (week 0 vs. week 4)**	*p (prebiotic vs. placebo) week 0***	*p (prebiotic vs. placebo) week 4***	*p (changes over time between groups)***
Neutrophils (% positive)	**99.1 (98.1 - 99.5)**	**92.9 (82.6 - 96.3)**	*<0.001*	**99.1 (94.4 - 99.5)**	**92.2 (82.7 - 95.5)**	*0.003*	*ns*	*ns*	*ns*
Neutrophils (MFI)	**210.0 (183.8 - 246.6)**	**334.1 (228.3 - 408.4)**	*0.005*	**208.505 (174.9 - 242.2)**	**242.9 (191.1 - 353.1)**	*0.031*	*ns*	*ns*	*ns*
Monocytes (% positive)	**85.6 (73.2 - 88.0)**	**71.9 (63.3 - 80.9)**	*0.013*	**86.5 (75.5 - 89.9)**	**67.4 (51.2 - 78.4)**	*<0.001*	*ns*	*ns*	*ns*
Monocytes (MFI)	**123.0 (113.6 - 130.2)**	**194.9 (110.9 - 347.9)**	*0.001*	**117.9 (111.2 - 139.2)**	**137.6 (106.3 - 285.9)**	*ns*	*ns*	*ns*	*ns*

Data are presented as median (25-75 percentiles)

Control samples (incubated on ice) were always set so that ≤ 3 % of the sample had phagocytosed (% positive).

% positive: percentage of neutrophils or monocytes to have engaged in phagocytosis

MFI: mean fluorescence intensity a measure of the activity of the cells (related to the number of ingested bacteria)

*Analysed with Wilcoxon Signed Ranks test **Analysed with Mann Whitney test

Note: Some distributions were normally distributed and were also analysed using parametric (independent t-test or dependent t-test) - these gave the same outcomes as the non-parametric tests, and so for ease of data presentation, all data are presented as median (25^{th} - 75^{th} percentile) and analysed with non-parametric tests.

5.4.3 OXIDATIVE BURST BY NEUTROPHILS AND MONOCYTES

Results from experiments measuring neutrophil and monocyte oxidative burst (made using Phagoburst kits) in whole blood are shown in Table 21. Two different stimulants were used: *E. coli* and the protein kinase C agonist PMA. The response to *E. coli* requires the bacteria to be recognised and taken up by phagocytosis prior to the cell signalling leading to oxidative burst occurring. PMA by-passes these early steps and directly stimulates protein kinase C, initiating the signalling events leading to burst. The main component of the oxidative bust is superoxide anions. Data are presented as percentage of cells engaging in oxidative burst (referred to as percent positive) and the extent of burst of those active cells (MFI). Few changes were observed either within or between groups. However, a larger increase in the MFI of neutrophils incubated with *E. coli* was seen between week zero and week four in the placebo group compared to the prebiotic group (an increase of 29.7 in the placebo group, compared to 7.5 in the prebiotic group, $p = 0.044$). The MFI of neutrophils incubated with *E. coli* was also greater at week four compared with week zero in the placebo group (115.9, compared to 86.1, $p = 0.025$). Also in the placebo group, the MFI of monocytes incubated with *E. coli* was greater at week four compared to week zero (26.9 versus 23.3, $p = 0.050$). There were no other differences observed either within or between groups. When comparing the change between week zero and week four in the prebiotic group to the change in the placebo group, there were no significant differences, other than the greater increase seen in MFI of neutrophils incubated with *E. coli* in the placebo group compared to the prebiotic group. Therefore, it must be concluded that the prebiotic supplement had no effect upon oxidative burst of blood monocytes and little effect on the oxidative burst of blood neutrophils.

Table 21 : The effect of four weeks supplementation with Orafti® Synergy1 or placebo upon blood neutrophil and granulocyte oxidative burst

	Stimulant	**Placebo group**			**Prebiotic group**			*p (prebiotic vs. placebo)***		*p (changes over time between groups)***
		Week 0	**Week 4**	*p (week 0 vs. week 4)**	**Week 0**	**Week 4**	*p (week 0 vs. week 4)**	*week 0*	*week 4*	
Neutrophils (% positive)	*E. coli*	**97.2 (87.3 - 98.9)**	**96.9 (93.6 - 98.6)**	*ns*	**97.9 (95.9 - 98.5)**	**97.1 (89.9 - 98.1)**	*ns*	*ns*	*ns*	*ns*
Neutrophils (MFI)	*E. coli*	**86.1 (75.2 - 122.6)**	**115.9 (100.7 - 134.1)**	*0.025*	**91.6 (74.5 - 157.6)**	**99.1 (73.3 -132.5)**	*ns*	*ns*	*ns*	*0.044*
Monocytes (% positive)	*E. coli*	**79.4 (74.1 - 85.1)**	**76.8 (67.6 - 83.6)**	*ns*	**79.8 (72.9 - 85.8)**	**76.4 (67.2 - 82.6)**	*ns*	*ns*	*ns*	*ns*
Monocytes (MFI)	*E. coli*	**23.3 (19.9 - 30.2)**	**26.9 (22.8 - 30.6)**	*0.050*	**23.9 (20.7 - 28.0)**	**24.3 (22.0 - 29.2)**	*ns*	*ns*	*ns*	*ns*
Neutrophils (% positive)	PMA	**98.9 (98.3 - 99.6)**	**99.1 (98.3 - 99.7)**	*ns*	**99.4 (98.4 - 99.8)**	**99.6 (99.2 - 99.9)**	*ns*	*ns*	*ns*	*ns*
Neutrophils (MFI)	PMA	**280.7 (148.6 - 360.8)**	**303.3 (220.1 - 384.9)**	*ns*	**262.9 (221.7 - 360.7)**	**279.9 (196.8 - 379.1)**	*ns*	*ns*	*ns*	*ns*
Monocytes (% positive)	PMA	**98.9 (93.2 - 99.9)**	**95.3 (91.3 - 99.8)**	*ns*	**98.4 (93.9 - 99.6)**	**97.7 (87.5 - 99.6)**	*ns*	*ns*	*ns*	*ns*
Monocytes (MFI)	PMA	**36.9 (24.8 - 48.4)**	**32.5 (24.9 - 43.1)**	*ns*	**31.1 (26.8 - 41.7)**	**33.2 (27.1 - 49.2)**	*ns*	*ns*	*ns*	*ns*

Data presented as medians (25^{th} - 75^{th} percentile) *Data analysed by Wilcoxon-Signed Ranks test **Data analysed by Mann Whitney test

Control samples (incubated with wash solution) were always set so that ≤ 3 % of the sample had responded (% positive).

% positive: percentage of neutrophils or monocytes to have engaged in oxidative burst MFI: mean fluorescence intensity a measure of the activity of the cells

Note: Some distributions were normally distributed and were also analysed using parametric (independent t-test or dependent t-test) - these gave the same outcomes as the non-parametric tests, and so for ease of data presentation, all data are presented as median (25^{th} - 75^{th} percentiles) and analysed with non-parametric tests.

5.4.4 TOTAL SERUM IMMUNOGLOBULIN AND SALIVARY IgA CONCENTRATIONS

Concentrations of IgA, IgG and IgM were measured in serum and of sIgA in saliva, and results are shown in Table 22. There were no differences between the placebo group and the prebiotic group. However, there were some differences observed within groups.

Total serum IgA decreased from week zero to week four in the prebiotic group (from 3.74 mg/ml to 2.29 mg/ml, $p = 0.013$) and there was also a decrease in the placebo group, but this was not significant.

Total serum IgM decreased from week zero to week four in the placebo group (0.96 ng/ml to 0.90 ng/ml, $p = 0.011$) and also in the prebiotic group (0.98 ng/ml to 0.88 ng/ml, $p = 0.004$).

Total serum IgG increased from week zero to week four in the placebo group (16.34 mg/ml to 27.38 mg/ml, $p = 0.042$), and there was also an increase, but not significant, in the prebiotic group.

There were no differences in salivary sIgA concentrations, either expressed as adjusted for total salivary protein, or unadjusted.

There were no other differences observed either within or between groups. When comparing the change between week zero and week four in the prebiotic group to the change in the placebo group, there were no significant differences. Therefore, it must be concluded that the prebiotic supplement had no effect upon total immunoglobulin concentrations in blood or on salivary sIgA concentration.

Table 22 : The effect of four weeks supplementation with Orafti® Synergy1 or placebo upon total serum immunoglobulin and salivary immunoglobulin A concentrations

	Placebo group			Prebiotic group			*p (prebiotic vs. placebo)***		*p (changes over time between groups)***
	Week 0	**Week 4**	*p (week 0 vs. week 4)**	**Week 0**	**Week 4**	*p (week 0 vs. week 4)**	*week 0*	*week 4*	
Serum IgA (mg/ml)	**2.80 (1.64 - 4.07)**	**2.07 (1.10 - 3.89)**	*ns*	**3.74 (2.82 - 4.34)**	**2.29 (1.53 - 3.92)**	*0.013*	*ns*	*ns*	*ns*
Serum IgM (ng/ml)	**0.96 (0.73 - 1.44)**	**0.90 (0.40 - 1.07)**	*0.011*	**0.98 (0.79 - 1.38)**	**0.88 (0.43 - 1.01)**	*0.004*	*ns*	*ns*	*ns*
Serum IgG (mg/ml)	**16.34 (12.04 - 30.19)**	**27.38 (14.69 - 45.40)**	*0.042*	**20.43 (10.37 - 30.98)**	**28.17 (12.91 - 38.42)**	*ns*	*ns*	*ns*	*ns*
Salivary sIgA (µg/ml)/ total protein (µg/ml)	**191.26 (107.06 - 316.61)**	**192.31 (121.28 - 480.23)**	*ns*	**189.56 (129.93 - 254.74)**	**272.54 (122.14 - 412.49)**	*ns*	*ns*	*ns*	*ns*
Salivary sIgA (not adjusted for protein) (µg/ml)	**257.60 (185.50 - 324.00)**	**233.40 (160.65 - 316.60)**	*ns*	**187.20 (134.63 - 279.78)**	**180.25 (128.65 - 279.95)**	*ns*	*ns*	*ns*	*ns*

Data presented as medians (25^{th} - 75^{th} percentile) *Analysed by Wilcoxon Signed Ranks Test **Analysed by Mann-Whitney Test

Note: Some distributions were normally distributed and were also analysed using parametric (independent t-test or dependent t-test) - these gave the same outcomes as the non-parametric tests, and so for ease of data presentation, all data are presented as median (25^{th} - 75^{th} percentiles) and analysed with non-parametric tests.

5.4.5 LYMPHOCYTE PROLIFERATION MEASURED BY CFSE DILUTION

Lymphocyte proliferation was measured by CFSE dilution, and results are shown in Table 23. Data are presented in two ways. The first is as MFI ([geometric] mean fluorescence intensity); a lower MFI indicates greater proliferation since this is due to dilution of CFSE. The second is as percentage of cells in the M1 and M2 regions of the flow cytometry histogram (see Figure 21); movement of cells from M2 to M1 indicates proliferation. Thus the reduced MFI, the increase in the proportion of cells in the M1 region and the decrease in the proportion of cells in the M2 region are indicative of a proliferative response in the cultures containing ConA. No differences were found either within or between groups regarding the proliferation of lymphocytes. Therefore, it must be concluded that the prebiotic supplement had no effect upon lymphocyte proliferation, measured by CFSE dilution.

Table 23 : The effect of four weeks supplementation of Orafti® Synergy1 or placebo upon lymphocyte proliferation (measured by CFSE dilution)

	Culture condition	Placebo group			Prebiotic group			*p (prebiotic vs. placebo) week 0***	*p (prebiotic vs. placebo) week 4***	*p (changes over time between groups)***
		Week 0	**Week 4**	*p (week 0 vs. week 4)**	**Week 0**	**Week 4**	*p (week 0 vs. week 4)**			
MFI	Control	**753.5 (580.5 - 1086.0)**	**785.0 (633.0 - 1068.0)**	*ns*	**782.5 (662.8 - 1033.0)**	**787.0 (665.8 - 935.8)**	*ns*	*ns*	*ns*	*ns*
% M1	Control	**8.3 (5.5 - 15.7)**	**8.8 (4.9 - 19.0)**	*ns*	**7.5 (4.1 - 15.7)**	**8.6 (5.5 - 14.9)**	*ns*	*ns*	*ns*	*ns*
% M2	Control	**91.5 (83.9 - 94.2)**	**90.9 (80.9 - 94.9)**	*ns*	**92.3 (84.2 - 95.5)**	**90.4 (84.9 - 94.4)**	*ns*	*ns*	*ns*	*ns*
MFI	+ ConA	**160.0 (115.5 - 210.0)**	**167.0 (131.5 - 216.5)**	*ns*	**147.0 (112.2 - 187.8)**	**162.0 (116.0 - 195.5)**	*ns*	*ns*	*ns*	*ns*
% M1	+ ConA	**79.6 (67.7 - 85.9)**	**78.1 (62.9 - 83.3)**	*ns*	**77.3 (71.5 - 88.5)**	**75.5 (67.4 - 86.6)**	*ns*	*ns*	*ns*	*ns*
% M2	+ ConA	**19.8 (13.6 - 32.0)**	**21.4 (16.6 - 36.7)**	*ns*	**22.7 (11.3 - 28.2)**	**24.3 (13.4 - 32.5)**	*ns*	*ns*	*ns*	*ns*

Data presented as medians (25th - 75th percentile) *Data analysed by Wilcoxon-Signed Ranks test **Data analysed by Mann Whitney test

MFI: mean fluorescence intensity (geometric mean); a lower MFI indicates greater proliferation since this is due to dilution of CFSE

% M1 and % M2 refer to the percentage of cells falling into the M1 and M2 regions of the histogram (see Figure 2.6); movement of cells from M2 to M1 indicates proliferation

Note: Some distributions were normally distributed and were also analysed using parametric (independent t-test or dependent t-test) - these gave the same outcomes as the non-parametric tests, and so for ease of data presentation, all data are presented as median (25th - 75th percentiles) and analysed with non-parametric tests.

5.4.6 CYTOKINE PRODUCTION BY LYMPHOCYTES

Cytokine production from blood mononuclear cell cultures was measured by cytometric bead array kits, and results are shown in Table 24. Cells were cultured either unstimulated or they were stimulated with the mitogen ConA which specifically activates T lymphocytes. It is clear that all cytokine concentrations were much higher in the ConA-stimulated cultures than in the unstimulated cultures. The approximate average increases in concentration of cytokines after ConA stimulation were 45-fold for IL-2, 6-fold for IL-4, 5-fold for IL-6, 5-fold for IL-10, 10-fold for TNF-α, and 250-fold for IFN-γ. IFN-γ and IL-2 are the prototypical Th1-type cytokines and the great increase in production of these cytokines suggests that the response of lymphocytes within human PBMC cultures stimulated with ConA is Th1 dominated.

Few differences were observed regarding cytokine concentrations in lymphocyte cultures. No significant differences were noted in IL-2, IL-6, IL-10 and IFN-γ concentrations, either between or within groups.

In control (i.e. unstimulated) cultures, TNF-α concentration was lower at week four compared to week zero in the prebiotic group (23.3 pg/ml compared to 36.7 pg/ml, $p = 0.013$). The change over this time period was also greater in the prebiotic group (a decrease of 13.5 pg/ml) compared to the placebo group where little change was observed (an increase of 1.2 pg/ml; $p = 0.050$).

IL-4 concentration in control cultures was lower in the prebiotic group at week four compared to the placebo group (1.3 pg/ml compared to 1.8 pg/ml, $p = 0.040$). IL-4 concentration in ConA stimulated cultures was lower in the prebiotic group at week zero compared to the placebo group (8.6 pg/ml compared to 11.7 pg/ml, $p = 0.012$).

The Th1/Th2 ratio was also calculated using the concentrations of the prototypical Th1-type (IFN-γ) and Th2-type (IL-4) cytokines and is shown in
Table *25*. No differences between or within groups were observed, apart from that a higher Th1/Th2 ratio was observed in the prebiotic group compared to the placebo group at week zero (144.8 compared to 59.9, $p = 0.044$).

There were no other differences observed either within or between groups. When comparing the change between week zero and week four in the prebiotic group, to the change in the placebo group, there were no significant differences, apart from the greater decrease in TNF-α concentration in control cultures in the prebiotic group compared to the control group. Therefore, it must be concluded that the prebiotic supplement had little effect upon cytokine production by lymphocytes in culture.

Table 24 : The effect of four weeks supplementation of Orafti® Synergy1 or placebo upon cytokine production by lymphocytes

	Culture condition	Placebo group		*p (week 0 vs. week 4)**	Prebiotic group		*p (week 0 vs. week 4)**	*p (prebiotic vs. placebo) week 0***	*p (prebiotic vs. placebo) week 4***	*p (changes over time between groups)***
		Week 0	**Week 4**		**Week 0**	**Week 4**				
IL-2	Control	1.6 (1.3 - 2.1)	1.6 (1.3 - 2.1)	*Ns*	1.8 (1.3 - 2.7)	1.8 (1.3 - 2.2)	*ns*	*ns*	*ns*	*ns*
IL-4	Control	1.5 (1.3 - 2.1)	1.8 (1.3 - 2.5)	*Ns*	1.3 (1.3 - 1.7)	1.3 (1.3 - 1.6)	*ns*	*ns*	*0.040*	*ns*
IL-6	Control	1753.8 (687.4 - 3294.4)	2275.2 (724.2 - 3857.1)	*Ns*	1103.3 (484.3 - 5102.6)	1327.1 (517.9 - 3389.8)	*ns*	*ns*	*ns*	*ns*
IL-10	Control	12.7 (9.4 - 20.7)	18.2 (5.8 - 21.1)	*Ns*	14.8 (8.1 - 29.7)	12.1 (7.9 - 19.0)	*ns*	*ns*	*ns*	*ns*
TNF-α	Control	29.0 (10.1 - 47.6)	30.2 (10.7 - 57.7)	*Ns*	36.7 (4.6 - 50.9)	23.3 (9.4 - 32.9)	*0.013*	*ns*	*ns*	*0.050*
IFN-γ	Control	3.6 (3.6 - 3.9)	3.6 (3.6 - 8.6)	*Ns*	3.6 (3.6 - 4.7)	3.6 (3.6 - 4.4)	*ns*	*ns*	*ns*	*ns*
IL-2	+ ConA	76.3 (52.8 - 152.4)	65.0 (45.2 - 127.4)	*Ns*	79.5 (60.7 - 177.5)	84.0 (58.8 - 154.5)	*ns*	*ns*	*ns*	*ns*
IL-4	+ ConA	11.7 (7.6 - 17.9)	9.8 (6.1 - 15.7)	*Ns*	8.6 (5.0 - 9.9)	8.2 (5.5 - 10.7)	*ns*	*0.012*	*ns*	*ns*
IL-6	+ ConA	9450.6 (4591.4 - 11044.9)	7004.1 (1977.8 - 12521.0)	*Ns*	7764.4 (3891.6 - 13537.4)	7771.4 (6387.1 - 10975.9)	*ns*	*ns*	*ns*	*ns*
IL-10	+ ConA	76.0 (51.0 -116.8)	68.3 (45.1 - 99.8)	*Ns*	72.2 (47.5 - 88.7)	63.5 (55.6- 119.4)	*ns*	*ns*	*ns*	*ns*
TNF-α	+ ConA	382.5 (220.1 - 544.4)	292.3 (152.1 - 477.9)	*Ns*	306.4 (164.6 - 574.3)	360.7 (89.2 - 566.9)	*ns*	*ns*	*ns*	*ns*
IFN-γ	+ ConA	891.9 (387.3 - 1554.7)	712.6 (292.5 - 1479.3)	*Ns*	1235.9 (396.8 - 2313.5)	1209.5 (518.6 - 2069.1)	*ns*	*ns*	*ns*	*ns*

Data presented as medians (25^{th} - 75^{th} percentile) pg/ml *Data analysed by Wilcoxon-Signed Ranks test **Data analysed by Mann Whitney test

Note: Some distributions were normally distributed and were also analysed using parametric (independent t-test or dependent t-test) - these gave the same outcomes as the non-parametric tests, and so for ease of data presentation, all data are presented as median (25^{th} - 75^{th} percentiles) and analysed with non-parametric tests.

Table 25 : The effect of four weeks supplementation of Orafti® Synergy1 or placebo upon ratio of Th1/Th2 (IFN-γ/IL-4) type cytokine production by lymphocytes

	Placebo group			Prebiotic group			*p* (placebo vs. prebiotic week 0)**	*p* (placebo vs. prebiotic week 4)**	*p* (comparing changes between groups)**
	Week 0	**Week 4**	*p* (week 0 vs. week 4)*	**Week 0**	**Week 4**	*p* (week 0 vs. week 4)*			
Control	**2.1 (1.5 - 2.7)**	**2.7 (1.9 - 4.1)**	*ns*	**1.9 (1.4 - 3.0)**	**2.3 (1.2 - 3.4)**	*ns*	*ns*	*ns*	*ns*
+ ConA	**59.9 (19.5 - 148.8)**	**50.4 (26.5 - 223.5)**	*ns*	**144.8 (62.4 - 321.5)**	**138.7 (60.2 - 322.9)**	*ns*	*0.044*	*ns*	*ns*

Data are expressed as medians (25^{th} - 75^{th} percentiles)

*Data analysed by Wilcoxon-Signed Ranks test

**Data analysed by Mann Whitney test

5.4.7 LYMPHOCYTE ACTIVATION

Lymphocyte activation was measured by CD69 expression on $CD3^+CD4^+$ cells, and results are shown in Table 26. Data are presented as the percentage of cells expressing CD69 (percentage positive) and as MFI ([geometric] mean fluorescence intensity), an indication of the extent of expression of CD69 on positive cells. Stimulation of cultures with ConA resulted in an increase in $CD69^+$ cells from about 11% to about 60% and there was about a doubling of MFI on positive cells, indicating a higher level of expression of CD69. No differences were observed between or within groups, except for a decrease in the prebiotic group in the percentage of $CD3^+CD4^+$ cells staining positive for CD69 in ConA-stimulated cultures from week zero to week four (64.8% to 55.9%, $p = 0.031$). There were no other differences observed either within or between groups. When comparing the change between week zero and week four in the prebiotic group, to the change in the placebo group, there were no significant differences. Therefore, it must be concluded that the prebiotic supplement had no effect upon lymphocyte activation, as measured by CD69 expression on $CD3^+CD4^+$ cells.

Table 26 : The effect of four weeks supplementation of Orafti® Synergy1 or placebo upon T cell activation

	Culture condition	Placebo group			Prebiotic group			*p (prebiotic vs. placebo) week 0***	*p (prebiotic vs. placebo) week 4***	*p (changes over time between groups)***
		Week 0	**Week 4**	*p (week 0 vs. week 4)**	**Week 0**	**Week 4**	*p (week 0 vs. week 4)**			
Percent positive	Control	**11.0 (8.8 - 18.3)**	**11.7 (7.9 - 18.3)**	*ns*	**18.7 (10.7 - 24.4)**	**12.3 (8.3 - 20.1)**	*ns*	*ns*	*ns*	*ns*
MFI	Control	**42.4 (35.8 - 47.2)**	**44.3 (36.3 - 53.2)**	*ns*	**41.8 (38.9 - 47.2)**	**42.6 (39.0 - 49.3)**	*ns*	*ns*	*ns*	*ns*
Percent positive	+ ConA	**61.6 (56.0 - 72.6)**	**57.3 (48.8 - 71.2)**	*ns*	**64.8 (56.8 - 69.3)**	**55.9 (52.8 - 65.1)**	*0.031*	*ns*	*ns*	*ns*
MFI	+ ConA	**100.8 (73.6 - 125.1)**	**93.9 (72.7 - 112.0)**	*ns*	**95.8 (76.8 - 112.9)**	**87.4 (74.8 - 113.4)**	*ns*	*ns*	*ns*	*ns*

Data presented as medians (25^{th} - 75^{th} percentile)

Percent positive: Percentage of $CD3^+CD4^+$ cells also expressing CD69

MFI: (geometric) mean fluorescence intensity

*Data analysed by Wilcoxon-Signed Ranks test **Data analysed by Mann Whitney test

Note: Some distributions were normally distributed and were also analysed using parametric (independent t-test or dependent t-test) - these gave the same outcomes as the non-parametric tests, and so for ease of data presentation, all data are presented as median (25^{th} - 75^{th} percentile) and analysed with non-parametric tests.

5.5 DISCUSSION

This chapter described the effect of four weeks supplementation of Orafti® Synergy1 upon various systemic immune outcomes, in the absence of an immune challenge. Few effects were seen, when the change between week zero and week four in the prebiotic group was compared to the change between week zero and week four in the placebo group. Therefore, it must be concluded that the prebiotic supplement had very little, if any, effect upon systemic immune function in healthy middle aged adults, in the absence of an immune challenge.

Little effect of the prebiotic supplement was noted upon the proportions of immune cell phenotypes in whole blood. There was no effect on percentages of $CD3^+/CD4^+$ or $CD4^+$ cells. This is in agreement with two studies which found no effect of ß2-1 fructans on blood $CD4^+$ cell numbers or subsets (98, 103). However, one study has shown an increase in percentage of peripheral blood $CD4^+$ cells in elderly adults after supplementation with 8 g/d of short-chain fructooligosaccharides (sc-FOS) (43), and another study saw a tendency for increased $CD4^+$ cells in elderly adults following supplementation with 1.95 - 3.9 g/d FOS given in a nutritional supplement (94). Possible reasons for these differences are that elderly subjects may be more sensitive to immune-modulating effects of ß2-1 fructans than middle aged subjects as studied here, or that short-chain ß2-1 fructans are more active than longer chain; the mix used in the current study contained 50% inulin which has a high degree of polymerisation.

The percentages of $CD3^+/CD8^+$ and $CD8^+$ cells were higher in the prebiotic group at week four, compared to the placebo group. However, at baseline, values in the prebiotic group were also higher than in the placebo group. Therefore, the difference observed at week four is likely to only reflect baseline differences. An increase in the percentage of peripheral blood $CD8^+$ cells was seen in elderly adults after supplementation with 8 g/d sc-FOS (43). Another study showed a tendency for increased $CD8^+$ cells in elderly adults following supplementation with FOS (94). However, no effect on $CD8^+$ T cell subsets or numbers were reported in other studies using 6 g/d of oligofructose and inulin in the elderly (103), or 9 g/d inulin plus other prebiotics with or without antioxidants in young male adults (98). Again, these findings suggest that differences in study outcomes may relate to the age of subjects studied or the precise composition of the ß2-1 glucans used.

The only effect observed upon the CD4/CD8 ratio was a higher CD4/CD8 ratio at week four in the placebo group compared to the prebiotic group. This general lack of effect on this ratio is in agreement with three other studies (98, 100, 103), although one study

found a tendency (not significant) for an increase in the CD4/CD8 ratio in blood of elderly adults taking a nutrition supplement containing 1.95 - 3. 9 g/d of FOS (94).

There was no effect of Orafti® Synergy1 on percentage of B cells (defined as $CD3^-/CD19^+$). This is in agreement with another study which showed no change in percentage of B cells (defined as $CD20^+$) in the elderly after supplementation with 6 g/d oligofructose and inulin in a ratio of 2:1 (103). These findings differ from other studies which report increases in numbers of $CD19^+$ B cells in elderly adults supplemented with 1.95 - 3.9 g/d FOS in a nutritional supplement (94) and in young male adults supplemented with approximately 9 g/d inulin with other prebiotics with or without antioxidants (98).

There was no effect on $CD14^+$ as a percentage of total leukocytes. However, $CD14^+$ cells as a percentage of monocytes increased from week zero to week four in the placebo group, but not in the prebiotic group. When comparing the change between week zero and week four in the prebiotic group, to the change between week zero and week four in the placebo group, there were no significant differences. No change was seen in the percentage of monocytes defined as $CD14^+$ cells in elderly adults supplemented with a nutritional formula containing ß2-1 fructans (103).

There was no effect on percentage of lymphocytes defined as T_{reg} cells (defined as $CD4^+CD25^+CD127^{lo}$). The only other study that has assessed the effect of ß2-1 fructans on this outcome, found no effect on frequency of $CD4^+CD25^{high}$ T_{reg} cells in cord blood after supplementation of pregnant women with 0.88 g/d inulin plus 8.8 g/d galactooligosaccharides (100).

The percentage of NK cells (defined here as $CD3^-/CD16^+$ cells) decreased between week zero and week four in the placebo group, but not in the prebiotic group. This is in agreement with two other studies which also found no effect of ß2-1 fructans on NK cell numbers (43, 98, 103). However, one study reported an increase in NK cells (defined as $CD16^+CD56^+$ cells) in elderly adults supplemented with 1.95 - 3.9 g/d FOS in a nutritional supplement (94), while another study reported a decrease in $CD3^+NK^+$ cells in young male adults supplemented with approximately 9 g/d of inulin plus other prebiotics, with or without antioxidants (98).

There was little effect of the prebiotic supplement upon neutrophil and monocyte phagocytosis in whole blood. No differences were observed between the groups in any of the outcomes measured. However there were differences within the groups, between

week zero and week four. The percentage of neutrophils and monocytes having undergone phagocytosis was decreased from week zero to week four in both groups. The activity of neutrophils and monocytes was increased from week zero to week four in both groups, but only significantly in the placebo group. This lack of effect of Orafti® Synergy1 upon this outcome is similar to that reported from other studies. No effect of a synbiotic supplement containing Orafti® Synergy1 was found on the percentage of phagocytically active neutrophils or monocytes, or their phagocytic intensity, in patients with cancer, or polypectomised patients (104, 105). No effect on the phagocytosis of *E. coli* by neutrophils was found in healthy males adults after supplementation with inulin (98). However, in elderly adults in a care home, supplementation with 8 g/d sc-FOS decreased monocyte and neutrophil phagocytosis of *E. coli* (43).

Few changes were observed either within or between groups regarding neutrophil and monocyte oxidative burst. However, the MFI of neutrophils and monocytes incubated with *E. coli* was greater at week four compared with week zero in the placebo group. Also, a bigger increase in the MFI of neutrophils incubated with *E. coli* was seen between week zero and week four in the placebo group, compared to the prebiotic group. No other differences between groups were seen. Therefore, there was little effect of Orafti® Synergy1 upon neutrophil and no effect on monocyte oxidative burst. This is similar to the results from a study in cancer and polypectomised patients, where no effect of a synbiotic supplement containing Orafti® Synergy1 was found on the percentage of neutrophils that produced reactive oxygen species and the intensity of this production (104, 105).

Concentrations of IgA, IgG and IgM were measured in serum, and sIgA in saliva. There were no differences between the placebo group and the prebiotic group, but some differences observed within groups. Total serum IgA was decreased from week zero to week four in the prebiotic group, and there was also a decrease in the placebo group, but this was not significant. Total serum IgM was decreased from week zero to week four in both groups. Total serum IgG was increased from week zero to week four in the placebo group, and there was also an increase in the prebiotic group, but this was not significant. There were no differences in salivary sIgA concentrations, either expressed as adjusted for protein, or unadjusted, although sIgA as adjusted for total protein appeared to increase in the prebiotic group between week zero and week four. An increase in sIgA may have been expected following prebiotic supplementation, as this is a measure of mucosal immunity (10). This is more closely related to the site of action of the prebiotic than the systemic immune system is, and so it may be anticipated that an effect would be more likely to be seen.

There is a lack of studies investigating the effect of ß2-1 fructans on total serum and salivary immunolglobulin concentrations before vaccination, and so comparisons with the literature are limited. A study in free living elderly adults found no effect of two weeks supplementation of a mixture of 70 % oligofructose and 30% inulin upon serum IgA, IgG and IgM, or salivary sIgA (92). Two studies have shown that in newborn infants, galactooligosaccharide plus inulin supplementation led to increased faecal sIgA (99, 102), another marker of mucosal immunity.

Three different indicators of lymphocyte responsiveness were measured: activation, proliferation, and cytokine production. No differences were found either within or between groups regarding the proliferation of lymphocytes, as measured by CFSE dilution. No other studies have looked at the effect of ß2-1 fructans on lymphocyte proliferation prior to vaccination. Little effect of the prebiotic supplement was noted upon lymphocyte cytokine production in culture. No effect was seen in this study on lymphocyte production of IL-2. Similarly, there was no effect on IL-2 specific mRNA in PBMCs following FOS supplementation in elderly adults (94). In polypectomized patients, an increase in IL-2 secretion in stimulated PBMCs in the control group was prevented with synbiotic supplementation containing Orafti® Synergy1, but this was not seen in cancer patients (104, 105). In pregnant women supplemented with 0.88 g/d inulin plus 8.18 g/d galactooligosaccharides, there was no effect upon IL-2 production from cord blood cultures (100).

In this study, IL-4 production in control cultures was lower in the prebiotic group at week four compared to the placebo. IL-4 production in ConA stimulated cultures was lower in the prebiotic group at week zero compared to the placebo group. In pregnant women supplemented with 0.88 g/d inulin plus 8.18 g/d galactooligosaccharides, there was no effect upon IL-4 production from cord blood cultures (100).

No effect was seen in this study on PBMC production of IL-6. This is similar to that seen in another study in elderly adults supplemented with ß2-1 fructans, where there was no effect on LPS stimulated mononuclear cell production of IL-6 (103). In pregnant women supplemented with 0.88 g/d inulin plus 8.18 g/d galactooligosaccharides, there was no effect upon IL-6 production from cord blood cultures (100). In patients undergoing elective abdominal surgery (107) or elective colorectal surgery (111) there was no effect of a synbiotic containing OF on serum IL-6 levels. However, other studies showed that IL-6 mRNA expression in PBMCs was decreased after supplementation of elderly subjects with 8 g/d sc-FOS (43) or 1.95 - 3.9 g/d FOS (94). In adult multiple trauma victims in surgical intensive care, supplementation of IN with other fibres and probiotics decreased serum IL-6 levels (109).

No effect was seen in this study on PBMC production of IL-10. This is in agreement with a study which found no effect of synbiotic supplementation containing Orafti® Synergy1 upon IL-10 production from activated PBMCs in cancer or polypectomized patients (104, 105). In pregnant women supplemented with 0.88 g/d inulin plus 8.18 g/d galactooligosaccharides, there was no effect upon IL-10 production from cord blood cultures (100). In patients with active ulcerative colitis, synbiotic supplementation containing Synergy1 had no effect on IL-10 mRNA levels in mucosal tissue (108).

No effect was seen in this study on PBMC production of IFN-γ. IFN-γ production from stimulated PBMCs in colon cancer patients was increased following synbiotic supplementation containing Orafti® Synergy1, but not in polypectomized patients (104, 105). In pregnant women supplemented with 0.88 g/d inulin plus 8.18 g/d galactooligosaccharides, there was no effect upon IFN-γ production from cord blood cultures (100).

In this study, in control cultures, TNF-α production was lower at week four compared to week zero in the prebiotic group, and the decrease over this time period was also greater in the prebiotic group compared to the placebo group, where little change was observed. TNF-α is considered a pro-inflammatory cytokine so a reduction in its production may be of benefit in inflammatory states (252). A decrease was also noted in other studies, where TNF-α specific mRNA was decreased in PBMCs following FOS supplementation in elderly adults (94), and in adult multiple trauma victims in surgical intensive care, supplementation of IN with other fibres and probiotics decreased serum TNF-α levels (109). In patients with active ulcerative colitis, TNF-α mRNA levels in mucosal tissue were decreased following synbiotic supplementation containing Orafti® Synergy1 (108). However, other studies have shown no effect of ß2-1 fructan supplementation upon PBMC production of TNF-α (103-105), or on circulating TNF-α in plasma (94). In pregnant women supplemented with 0.88 g/d inulin plus 8.18 g/d galactooligosaccharides, there was no effect upon TNF-α production from cord blood cultures (100).

No differences between or within groups were observed regarding Th1/Th2 type cytokines (IFN-γ/IL4), apart from that a higher Th1/Th2 ratio was observed in the prebiotic group compared to the placebo group at week zero in ConA stimulated cultures. Other studies have not looked at this outcome.

No differences were observed between or within groups regarding $CD3^+CD4^+$ activation (measured by CD69 expression), except for a decrease in the prebiotic group in the percentage of $CD3^+CD4^+$ cells staining positive for CD69 in ConA-stimulated cultures from

week zero to week four. Little effect has been reported from other studies, with no effect of 8 g/d sc-FOS upon total number of activated T cells in the blood in elderly adults (43). Activated T cells, defined as $CD3^+$ $HLA\text{-}DR^+$ cells, were increased after 9 g/d inulin supplementation plus other prebiotics with or without antioxidants in young male adults, but there was no effect on $CD4^+CD25^+$ or $CD4^+CD54^+$ cells in this study (98). In pregnant women supplemented with 0.88 g/d inulin plus 8.18 g/d galactooligosaccharides, there was no effect on CD69 and CD25 expression on $CD4^+$ subsets in un-stimulated cord blood (100).

5.6 CONCLUSIONS

In conclusion, few effects were observed of Orafti® Synergy1 upon systemic immune outcomes, in healthy middle aged adults, in the absence of an immune challenge. A similar lack of effect on some of the outcomes reported here, or related to these outcomes, has been described in previous studies in the literature, although some studies, mainly in elderly subjects, do report some immune effects. Bifidobacteria counts were shown to increase over the four week time period studied here (see Chapter 4), so the lack of effect of Orafti® Synergy1 upon immune function cannot be due to an absence of a prebiotic effect.

Several explanations could be offered for the lack of effect of Orafti® Synergy1 upon systemic immune function. Firstly, in this population of healthy middle aged adults, it could be that a prebiotic supplement will not have any effect upon an immune system that is already functioning optimally; the immune system of elderly subjects may be more sensitive to the effects of prebiotics. Secondly, the dose of prebiotic may be important. It may be that immune effects are seen only with high doses of prebiotic. However, the dose of 8 g/d used in this study is comparable to that used in other studies, which range from 0.88 g/d up to 12 g/d. Thirdly, the type of ß2-1 fructan used may influence whether effects on immune function are seen. As ß2-1 fructans with different chain lengths are fermented in different parts of the intestine, this may influence any effect they may have on immune function. Fourthly, the sample size of the current study, although comparable with several other studies of this type, is fairly small, and therefore, in a bigger study, significant effects may have been observed. Finally, it is also possible that had the change in bifidobacteria been greater, then a greater effect on immune function may have been observed.

In order to further assess the effect of Orafti® Synergy1 upon immune function these subjects were vaccinated with a seasonal influenza vaccination in order to challenge the immune system, and results are presented in Chapter 6.

CHAPTER 6

PREBIOTIC INTERVENTION STUDY: IMPACT OF ORAFTI® SYNERGY1 UPON IMMUNE FUNCTION IN RESPONSE TO AN IMMUNE CHALLENGE (SEASONAL INFLUENZA VACCINATION)

6.1 INTRODUCTION

It is considered that modification of host gut microbiotia is required in order for prebiotics to influence the host immune response. In Chapter 4 it was shown that Orafti® Synergy1 (8 g/day for four weeks) was able to increase faecal bifidobacteria counts in healthy middle aged subjects, thereby confirming its prebiotic effect. Few effects of Orafti® Synergy1 were observed upon systemic immune outcomes in these healthy middle aged adults, in the absence of an immune challenge (see Chapter 5). However, it may be still be anticipated that some changes in immune function will be observed following a controlled immune challenge (e.g. a seasonal influenza vaccination) following supplementation with Orafti® Synergy1.

Response to vaccination has been evaluated as the gold standard method for measuring the functioning of the immune system ((10); see section 1.3.2.1.1). Vaccination is a controlled way to expose the body to a specific amount and type of antigen, so mimicking infection, and the body's response to this antigenic exposure is considered to be the most relevant way in which to assess the functioning of the immune system (in the absence of an infectious challenge). As this response occurs *in vivo*, it is an accurate representation of how the immune system functions in a physiologically natural environment. Several studies have been performed to date using response to vaccination as a tool to examine the effect of ß2-1 fructans upon immune function (91-93, 96, 97, 101, 103, 106). Of these studies, four found an effect of ß2-1 fructans upon the vaccine-specific response (91, 92, 96, 97), whilst four did not (93, 101, 103, 106). These studies were performed in infants or children (91, 93, 101, 106), or in elderly adults (92, 96, 97, 103), and there are no studies investigating the potential effects of ß2-1 fructan-type prebiotics on immune function in healthy middle aged adults. Three out of four studies conducted in elderly adults found an effect of the ß2-1 fructans on response to vaccination, suggesting that in this population ß2-1 fructans may be able to modulate some aspects of immune function. Of the four studies carried out in the elderly, only one used ß2-1 fructans alone (92), the remaining studies used supplements also containing vitamins, minerals, fats or probiotics. Thus there are few studies of ß2-1 fructans alone on the immune response to vaccination in adults and there are no studies in the middle-aged. This chapter describes the results from experiments measuring immune outcomes after four, six and eight weeks of 8 g/day Orafti® Synergy1 supplementation in healthy middle-aged subjects, and following a seasonal influenza vaccination (administered at week four). In this chapter, for clarity and ease of explanation, these time points are labelled 0, 2 and 4 weeks post-vaccination (therefore, week 0 in Chapter 6 is the same time point as week 4 in Chapter 5).

6.2 HYPOTHESIS

The prebiotic effect of Orafti® Synergy1 was confirmed by observing increased bifidobacteria counts in the gut following four weeks supplementation (Chapter 4). Orafti® Synergy1 did not have any major effects on immune function before vaccination (Chapter 5). It is hypothesised that Orafti® Synergy1 will improve the vaccine-specific antibody response following seasonal influenza vaccination and further that Orafti® Synergy1 will enhance responses of immune cells exposed to the vaccine ex vivo. The primary outcome for the study was a difference in anti-vaccine antibody response between the two groups (prebiotic versus placebo).

6.3 METHODS

6.3.1 STUDY DESIGN AND SUBJECT CHARACTERISTICS

The design of the study is described in Section 4.3 along with inclusion and exclusion criteria, subject characteristics (see Table 17) and the sampling procedure (see Figure 29). Here immune outcomes determined at vaccination (week 0) and two and four weeks later (week 2 and week 4) are described. Supplementation of Orafti® Synergy1 (8 g/day) was continued throughout this period. Except for vaccine-specific antibody concentrations (sections 6.4.1 and 6.4.2), results reported for week 0 are the same as the results reported for week 4 in Chapter 5.

6.3.2 MEASUREMENT OF IMMUNE PARAMETERS

The following immune outcomes were measured:

- Immune cell phenotypes in peripheral blood, as described in Section 2.2;
- Neutrophil and monocyte phagocytosis of *E. coli*, as described in Section 2.3.1;
- Neutrophil and monocyte oxidative burst in response to *E. coli* or phorbolmyristoyl acetate (PMA) as described in Section 2.3.2;
- T cell cytokine production in response to concanavalin A (ConA) and vaccine antigen, as described in Section 2.6;
- T cell proliferation in response to ConA and vaccine antigen, as described in Section 2.7.2.2;
- T cell activation in response to ConA and vaccine antigen, as described in Section 2.5;
- Total serum IgA, IgG and IgM concentrations, as described in Sections 2.8.1 to 2.8.3;
- Total salivary sIgA concentration, as described in Section 2.8.4;
- Serum vaccine viral strain-specific antibody concentrations, as described in Section 2.9;

- Serum immunoglobulin class-specific antibodies to the vaccine, as described in Section 2.10.

6.3.2 STATISTICAL ANALYSIS

Analysis was performed by adjusted ANOVA (fixed factors indicated in brackets) in the first instance. If this indicated that there were some relationships in the data, data were analysed further. Where data were not normally distributed, analysis was done by Mann-Whitney test (comparing groups at each time point, and comparing changes over time between groups), and Wilcoxon Signed Ranks test (comparing changes over time within groups). Where data were normally distributed, analysis was done by independent T-test (comparing groups at each time point, and comparing changes over time between groups) and dependent T-test (comparing changes over time within groups). Seroconversion and seroprotection rates were compared using Chi squared test and Fisher's Exact test. All analyses were performed using SPSS version 17.0.

6.4 RESULTS

6.4.1 ANTI-VACCINE ANTIBODY RESPONSE (VACCINE STRAIN-SPECIFIC RESPONSE)

6.4.1.1 ANTIBODY TITRES

Vaccine-specific antibodies were measured in serum, and results shown in Table 27. It is clear that there was a greatly increased anti-vaccine antibody titre (to all three viral strains) post-vaccination. As the data were clearly not normally distributed they were log transformed (Figure 50) and then analysed by adjusted ANOVA (fixed factors: time, group). The antibody response to the HAH3_UR virus strain was enhanced in the prebiotic group (effect of group $p = 0.019$). There was no effect of group on the antibody response to the HAH1_BR or HAB_FL virus strains (effect of group $p = 0.759$ and $p = 0.176$ respectively). The response to the HAH3_UR strain was higher by 1.25 log units at week 2 post-vaccination and by 1.44 log units at week 4 post-vaccination in the prebiotic group compared to the placebo group. There was an increase of 4.54 log units from week 0 to week 2 in the prebiotic group compared to an increase of 3.51 log units in the placebo group, however the difference between groups was not significant ($p = 0.266$). There was an increase of 4.38 log units between week 0 to week 4 in the prebiotic group compared to an increase of 3.16 log units in the placebo group, however the difference between groups was not significant ($p = 0.173$). Therefore, it can be concluded that the prebiotic supplement improves the response to vaccination, although the response to only one of three strains was enhanced.

Table 27 : The effect of supplementation with Orafti® Synergy1 or placebo upon antibody response to a seasonal influenza vaccination (response to the three virus strains in the vaccine: HAH1_BR, HAH3_UR, HAB_FL) at baseline (week 0) and 2 and 4 weeks following vaccination

	Placebo group			*p (w0 vs. w2)**	*p (w0 vs. w4)**	*p (w2 vs. w4)**	Prebiotic group			*p (w0 vs. w2)**	*p (w0 vs. w4)**	*p (w2 vs. w4)**
	Week 0	**Week 2**	**Week 4**				**Week 0**	**Week 2**	**Week 4**			
HAH1_BR	**5.0 (5.0 -5.0)**	**120.0 (40.0 - 560.0)**	**80.0 (37.5 - 260.0)**	*<0.001*	*<0.001*	*ns*	**5.0 (5.0 - 5.0)**	**160.0 (40.0 - 440.0)**	**80.0 (23.8 - 200.0)**	*<0.001*	*<0.001*	*0.001*
HAH3_UR	**5.0 (5.0 - 12.5)**	**320.0 (29.4 - 1160.0)**	**240.0 (11.3 - 960.0)**	*0.001*	*<0.001*	*0.025*	**5.0 (5.0 - 20.0)**	**1600.0 (80.0 - 11840.0)**	**480.0 (102.5 - 20480.0)**	*<0.001*	*<0.001*	*ns*
HAB_FL	**5.0 (5.0 - 5.0)**	**70.0 (27.5 - 160.0)**	**40.0 (8.8 - 180.0)**	*<0.001*	*<0.001*	*ns*	**5.0 (5.0 - 5.0)**	**80.0 (38.8 - 230.0)**	**70.0 (20.0 - 160.0)**	*<0.001*	*<0.001*	*0.027*

Data are presented as medians (25th - 75th percentile)

*Analysed by Wilcoxon Signed Ranks test

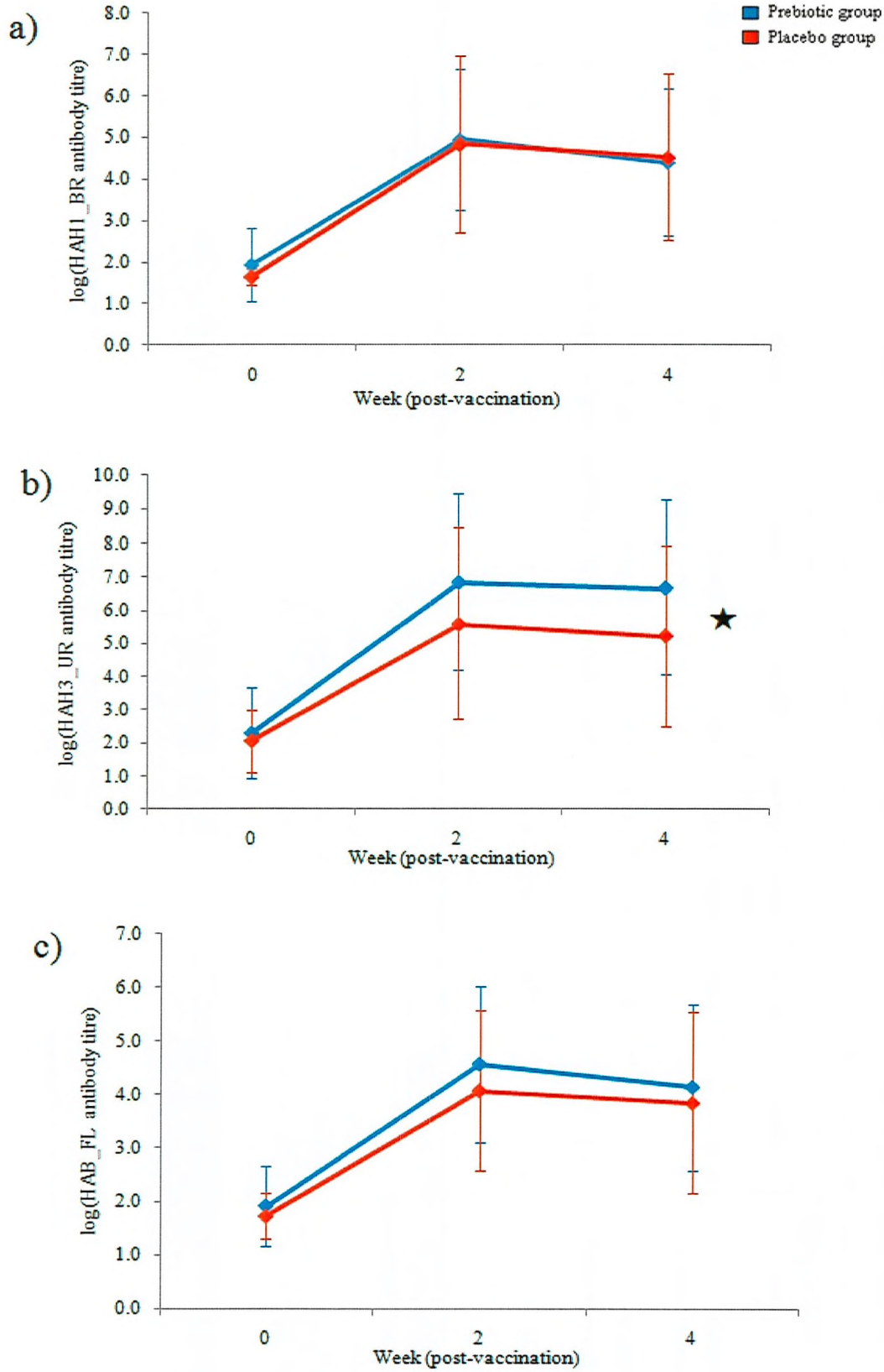


Figure 50 : Vaccine-specific antibody response (to the three virus strains in the vaccine: a = HAH1_BR, b=HAH3_UR, c= HAB_FL) at baseline (week 0) and 2 and 4 weeks post-vaccination. Data are presented as the mean of all subjects (n = 43); error bars indicate +/- 1 standard deviation. ★ significant effect of group (adjusted ANOVA, fixed factors: group, time, p = 0.019)

6.4.1.2 SERCONVERSION

Seroconversion is defined as the percentage of subjects showing at least a four-fold increase in antibody titre (253). Results are shown in Figure 51, and are presented as the percentage of subjects in each group who seroconverted between baseline (week 0) and 2 and 4 weeks post-vaccination. There were no significant differences in seroconversion rates between groups at any time point, for any of the three viral strains. However, Figure 51 b shows that the seroconversion rate to the HAH3_UR strain of the virus is higher in the prebiotic group compared to the placebo group between weeks 0 and 2 (90.5% and 70.0% respectively, $p = 0.130$) and weeks 0 and 4 (85.7 % and 66.7 % respectively, $p = 0.147$).

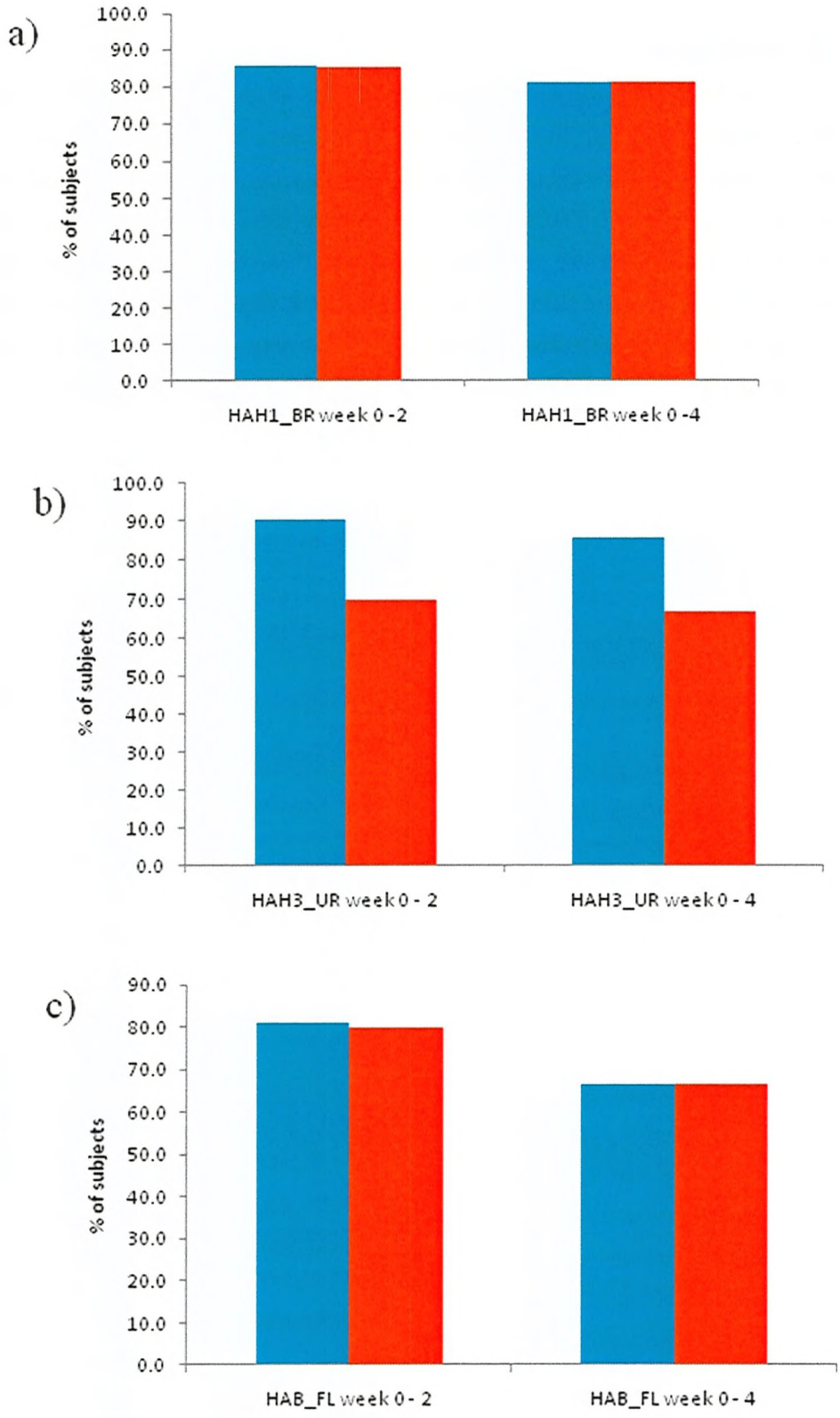


Figure 51 : Percentage of subjects seroconverting (at least a four-fold increase in antibody titre) for each strain of the seasonal influenza vaccine (a= HAH1_BR, b=HAH3_UR, c= HAB_FL) between baseline (week 0) and 2 and 4 weeks post-vaccination. Blue = prebiotic group, red = placebo group. Analysed by Chi squared and Fishers exact tests; no significant differences found.

6.4.1.3 SEROPROTECTION

Seroprotection is defined as an antibody titre of greater than or equal to 40 (253). Results are shown in Figure 52, and are presented as the percentage of subjects in each group who were seroprotected at baseline (week 0) and 2 and 4 weeks post-vaccination. There were no significant differences in seroprotection rates between groups at any time point, for any of the three viral strains. However, Figure 52 b suggests that the post-vaccination seroprotection rate to the HAH3_UR strain of the virus is higher in the prebiotic group compared to the placebo group at week 2 post-vaccination (90.9% and 75.0% respectively $p = 0.229$) and week 4 post-vaccination (90.9% and 71.4% respectively $p = 0.132$).

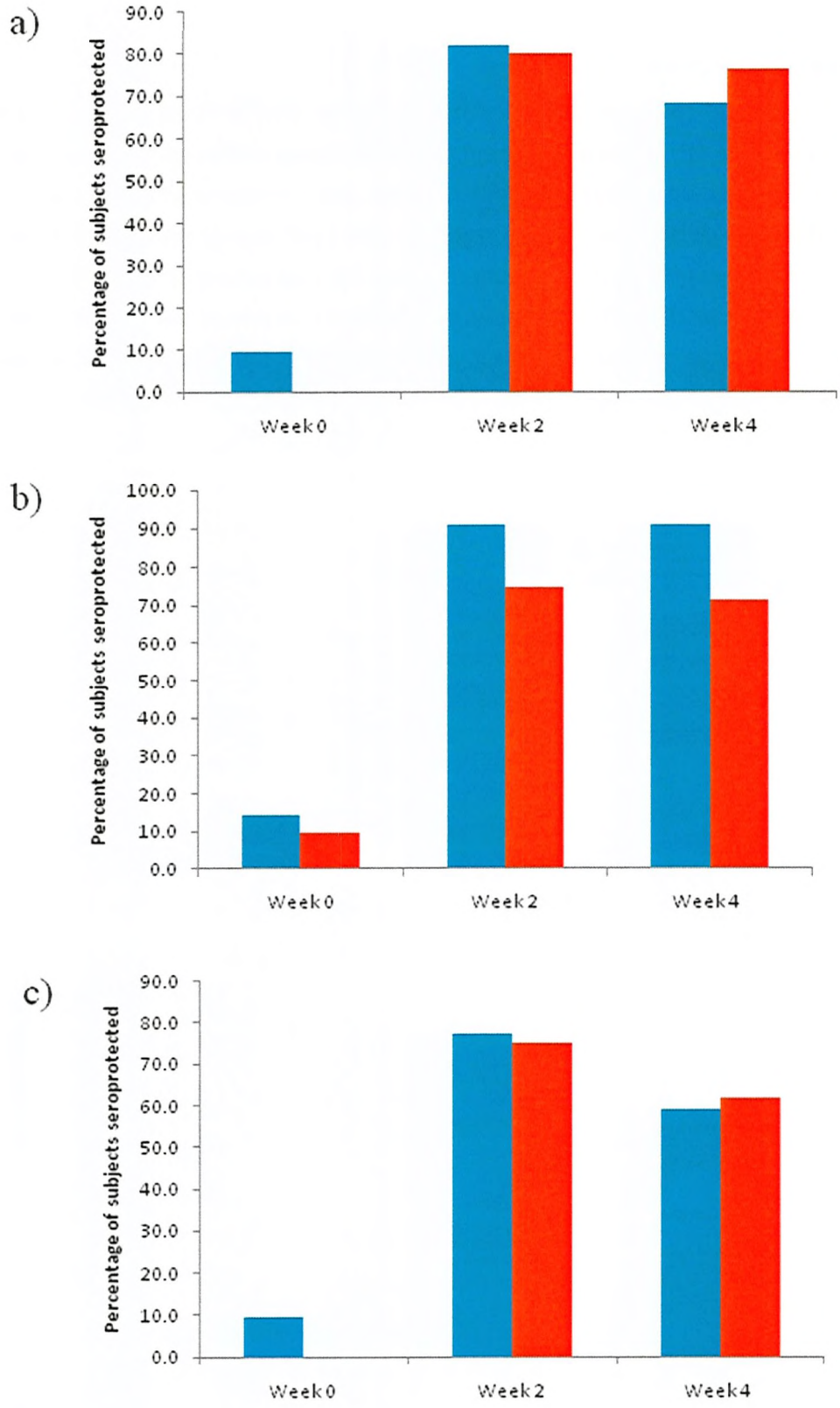


Figure 52 : Percentage of subjects who were seroprotected (antibody titre ≥ 40) for each strain of the seasonal influenza vaccine (a= HAH1_BR, b=HAH3_UR, c= HAB_FL) at baseline (week 0) and 2 and 4 weeks post-vaccination. Blue = prebiotic group, red = placebo group. Analysed by Chi squared test and Fishers Exact Test; no significant differences found.

6.4.2 IMMUNOGLOBULIN CLASS-SPECIFIC ANTIBODIES TO THE VACCINE ANTIGEN

Antibodies to the vaccine antigen, which were immunoglobulin-specific (IgA, IgM, IgG1 and IgD) were measured in serum, and results are shown in Table 28. All vaccine-specific Ig concentrations increased post-vaccination. There was no effect of the prebiotic supplement upon the concentrations of IgA-, IgM- or IgD-specific antibodies to the vaccine antigen. However, the concentration of IgG1-specific antibodies was enhanced in the prebiotic group ($p = 0.019$, adjusted ANOVA, fixed factors: group, time).

The responses of each immunoglobulin class were analysed further, and groups were compared at each time point. No differences were noted for IgA, IgM or IgD. However, at week 2 post-vaccination, IgG1 was higher in the prebiotic group compared to the placebo group (2.25 OD units versus 1.88 OD units, $p = 0.028$, analysed by independent T-test).

The changes over time between groups were analysed. No differences were noted for IgA, IgM or IgD. However, between baseline (week 0) to week 2 post-vaccination, the change in IgG1 was greater in the prebiotic group (increase of 0.99 OD units) compared to the placebo group (increase of 0.70 OD units, $p = 0.036$, analysed by independent T test).

The changes between time points within each group were analysed, and changes between time points were found to be significant for all classes. The only exceptions were between week 2 and week 4 post-vaccination for IgM in both groups, IgG1 in the prebiotic group, and IgD in the placebo group (analysed by dependent T-test).

For vaccine-specific IgA, IgM and IgD no differences between groups were noted, therefore it must be concluded that the prebiotic supplement had no effect on these classes of immunoglobulin. However, there was an enhancement of the IgG1-specific response to the vaccine antigens in the prebiotic group, suggesting that the prebiotic supplement had an effect on this immune outcome.

Table 28 : The effect of supplementation with Orafti® Synergy1 or placebo upon serum immunoglobulin-specific antibodies to vaccine antigen following a seasonal influenza vaccination (administered at week 0)

	Placebo group			Prebiotic group			*p**
	Week 0	**Week 2**	**Week 4**	**Week 0**	**Week 2**	**Week 4**	
IgA	**1.72 (0.88)**	**2.20 (1.00)**	**2.00 (0.97)**	**1.66 (0.62)**	**2.03 (0.79)**	**1.88 (0.82)**	*ns*
IgM	**1.75 (0.67)**	**2.73 (0.73)**	**2.59 (0.70)**	**1.85 (0.62)**	**3.03 (0.63)**	**2.74 (0.51)**	*ns*
IgG1	**1.18 (0.39)**	**1.88 (0.57)**	**2.03 (0.63)**	**1.26 (0.48)**	**2.25 (0.48)**	**2.24 (0.56)**	*0.019*
IgD	**0.47 (0.13)**	**0.58 (0.14)**	**0.60 (0.14)**	**0.51 (0.25)**	**0.58 (0.19)**	**0.60 (0.22)**	*ns*

Data are presented as mean (standard deviation) optical density units

*Analysed by adjusted ANOVA (fixed factors: group, time)

6.4.3 TOTAL SERUM AND SALIVARY IMMUNOGLOBULIN CONCENTRATIONS

Concentrations of IgA, IgG and IgM were measured in serum, and of sIgA in saliva, and results are shown in Table 29.

6.4.3.1 SERUM IMMUNOGLOBULINS

No effect of the prebiotic supplement was seen upon levels of total serum IgA, IgG or IgM (analysed by adjusted ANOVA [fixed factors = time, group]).

Data were analysed further, and no differences were found between the groups at any time point, or when comparing changes between groups. Some changes were observed within groups between some time points. IgM increased between baseline (week 0) and week 2 post-vaccination in the prebiotic group (from 0.8 mg/ml to 0.9 mg/ml, $p = 0.024$), decreased between baseline (week 0) and 4 weeks post-vaccination in the placebo group (from 0.9 mg/ml to 0.7 mg/ml, $p = 0.009$), and decreased between weeks 2 and 4 post-vaccination in both groups (from 0.9 mg/ml to 0.8 mg/ml in the prebiotic group [$p = 0.008$], and from 0.9 mg/ml to 0.7 mg/ml in the placebo group [$p = 0.002$]). IgG decreased between baseline (week 0) and week 2 post-vaccination in both groups (from 36.1 mg/ml to 16.2 mg/ml in the prebiotic group [$p = 0.013$], and from 30.3 mg/ml to 20.0 mg/ml in the placebo group, [$p = 0.013$]) and between baseline (week 0) and week 4 post-vaccination in the placebo group (from 30.3 mg/ml to 18.6 mg/ml, $p = 0.025$).

When comparing the changes between groups, there were no significant differences. Therefore, it must be concluded that the prebiotic supplement had no effect upon total immunoglobulin concentrations in blood.

6.4.3.2 SALIVARY IgA

When salivary sIgA concentrations were not adjusted for total protein concentrations, salivary sIgA was decreased in both groups following the influenza vaccination. This decrease was greater in the prebiotic group compared to the placebo group ($p = 0.007$, analysed by adjusted ANOVA, fixed factors - time, group). When data were analysed further, there was a significant difference between groups at week 2 post-vaccination (162.8 µg/ml in the prebiotic group and 227.2 µg/ml in the placebo group, $p = 0.031$, analysed by independent T-test). sIgA concentrations were also lower at week 2 post-vaccination compared to (baseline week 0) in the prebiotic group (162.8 µg/ml and 205.5 µg/ml, $p = 0.010$, analysed by dependent T-test).

When salivary sIgA concentrations were adjusted for total protein, the group had no effect upon the response (analysed by adjusted ANOVA, fixed factors: group, time; or by parametric tests).

When salivary sIgA concentrations were not adjusted for total protein concentrations, there was a greater decrease in the prebiotic group compared to the placebo group. However this does not take into account the total protein content of the saliva, and so is a less reliable method than adjusting for total protein, and this marker was not significantly different between groups. Therefore, it must be concluded that the prebiotic supplement had little or no effect upon total sIgA concentrations in saliva.

Table 29 : The effect of supplementation with Orafti® Synergy1 or placebo upon total serum immunoglobulin and salivary immunoglobulin A concentrations following a seasonal influenza vaccination (administered at week 0)

	Placebo group			Prebiotic group			*p**
	Week 0	**Week 2**	**Week 4**	**Week 0**	**Week 2**	**Week 4**	
IgA (mg/ml)	**2.5 (1.6)**	**2.8 (1.3)**	**2.5 (1.3)**	**2.6 (1.6)**	**3.4 (0.9)**	**2.6 (1.5)**	*ns*
IgG (mg/ml)	**30.3 (16.6)**	**20.0 (10.0)**	**18.6 (13.4)**	**36.1 (38.4)**	**16.2 (6.7)**	**27.8 (28.3)**	*ns*
IgM (mg/ml)	**0.9 (0.4)**	**0.9 (0.4)**	**0.7 (0.3)**	**0.8 (0.4)**	**0.9 (0.4)**	**0.8 (0.5)**	*ns*
sIgA/total protein (µg/mg)	**261.1 (192.1)**	**348.9 (436.0)**	**355.7 (256.6)**	**340.5 (391.4)**	**344.0 (280.5)**	**343.1 (343.0)**	*ns*
sIgA (µg/ml)	**233.8 (100.9)**	**227.2 (105.5)**	**221.4 (100.4)**	**205.5 (94.1)**	**162.8 (81.1)**	**176.5 (87.6)**	*0.007*

Data are presented as mean (standard deviation)

*Analysed by adjusted ANOVA (fixed factors: group, time)

6.4.4 IMMUNE CELL PHENOTYPES

Immune cell phenotypes were determined in whole blood. Data are expressed as percentages of the gated population (e.g. of lymphocytes, of monocytes) and are shown in Table 30. There was no effect of the prebiotic on $CD3^+/CD4^+$, $CD4^+$, $CD3^-CD16^+$, $CD3^-/CD19^+$, or $CD14^+$ cells as a percentage of monocytes or total leukocytes, or on percentage of lymphocytes defined as T_{reg} cells ($CD4^+CD25^+CD127^{lo}$, or $CD127^{lo}$ as a percentage of $CD4^+CD25^+$ cells) (analysed by adjusted ANOVA, fixed factors: group, time). The percentages of $CD3^+CD8^+$ and $CD8^+$ cells were higher in the prebiotic group (analysed by adjusted ANOVA, fixed factors: group, time, $p < 0.001$ for both outcomes). However, these differences seem to reflect inherent differences between the groups that were present at baseline (week 0) and are probably not due to the prebiotic. The CD4:CD8 ratio was higher in the placebo group compared to the prebiotic group (analysed by adjusted ANOVA, fixed factors: group, time, $p = 0.001$), which is due to the higher percentage of $CD8^+$ cells seen in the prebiotic group at this time.

Data were analysed further, and the percentage of $CD3^+/CD8^+$ cells was found to be higher in the prebiotic group compared to the placebo group at week 0 baseline (22.9 % versus 17.3 %, $p = 0.032$), week 2 post-vaccination (22.7 % versus 17.2 %, $p = 0.029$), and week 4 post-vaccination (23.2 % versus 17.8 %, $p = 0.042$, analysed by independent T-test). The percentage of $CD8^+$ cells was higher in the prebiotic group at week 0 baseline (26.7 % versus 21.0 %, $p = 0.023$), week 2 (26.7 % versus 20.9 %, $p = 0.021$) and week 4 (27.1 % versus 21.4 %, $p = 0.026$, analysed by independent T-test). The change in $CD4^+CD25^+CD127^{lo}$ cells as a percentage of total lymphocytes between weeks 2 to 4 post-vaccination was greater in the placebo group compared to the prebiotic group (an increase of 0.16 % in the placebo group compared to a decrease of 0.11 % in the prebiotic group, $p = 0.021$, analysed by independent T-test).

The percentage of $CD3^-CD19^+$ cells decreased in both groups between baseline (week 0) and week 4 post-vaccination (10.0 % to 9.1 % in the prebiotic group [$p = 0.001$] and 10.8 % to 9.9 % in the placebo group [$p = 0.006$], analysed by independent T-test). $CD14^+$ cells as a percentage of monocytes increased in the placebo group between baseline (week 0) and week 4 post-vaccination (77.9 % to 84.3 %, $p = 0.049$, analysed by independent T-test). The percentage of $CD3^-CD19^+$ cells decreased in the placebo group between weeks 2 and 4 post-vaccination (10.6 % to 9.9 %, $p = 0.027$).

When comparing the changes between groups, the only significant differences found were a greater proportion of $CD8^+$ cells in the prebiotic group, and also a greater increase in $CD4^+CD25^+CD127^{lo}$ cells as a percentage of total lymphocytes between weeks 2 to 4 post-vaccination in the placebo group compared to the prebiotic group. Therefore, it must be concluded that the prebiotic supplement had little effect upon immune cell phenotypes in the blood following a seasonal influenza vaccination.

Table 30 : The effect of supplementation with Orafti® Synergy1 or placebo upon immune cell phenotypes in whole blood following a seasonal influenza vaccination (administered at week 0)

	Placebo group			Prebiotic group			*p* *
Cell phenotype	**Week 0**	**Week 2**	**Week 4**	**Week 0**	**Week 2**	**Week 4**	
$CD3^+CD4^+$ (% of lymphocytes)	**50.5 (6.9)**	**51.8 (7.5)**	**51.7 (5.9)**	**50.1 (8.4)**	**50.7 (8.4)**	**51.2 (7.6)**	*ns*
$CD4^+$ (% of lymphocytes)	**50.9 (7.1)**	**52.2 (7.6)**	**51.8 (5.9)**	**50.3 (8.3)**	**50.9 (8.3)**	**51.4 (7.6)**	*ns*
$CD3^+CD8^+$ (% of lymphocytes)	**17.3 (7.1)**	**17.2 (6.6)**	**17.8 (6.9)**	**22.9 (8.9)**	**22.7 (9.0)**	**23.2 (9.4)**	*<0.001*
$CD8^+$ (% of lymphocytes)	**21.0 (7.4)**	**20.9 (7.3)**	**21.4 (7.2)**	**26.7 (8.0)**	**26.7 (8.1)**	**27.1 (8.5)**	*<0.001*
$CD3^-CD16^+$ (% of lymphocytes)	**9.4 (3.7)**	**10.1 (4.4)**	**9.8 (5.1)**	**8.2 (5.5)**	**8.1 (5.9)**	**8.5 (5.9)**	*ns*
$CD3^-CD19^+$ (% of lymphocytes)	**10.8 (3.7)**	**10.6 (3.4)**	**9.9 (3.3)**	**10.0 (3.9)**	**9.8 (3.8)**	**9.1 (3.8)**	*ns*
$CD14^+$ (% of monocytes)	**77.9 (13.8)**	**78.3 (16.9)**	**84.3 (9.8)**	**76.9 (14.3)**	**81.7 (11.9)**	**81.9 (11.9)**	*ns*
$CD14^+$ (% of leukocytes)	**3.6 (1.5)**	**3.6 (1.0)**	**3.8 (1.2)**	**3.2 (0.6)**	**3.5 (1.1)**	**3.4 (0.9)**	*ns*
$CD127^{lo}$ (% of $CD4^+CD25^+$ cells)	**73.4 (8.3)**	**71.9 (11.4)**	**68.4 (12.3)**	**73.1 (8.6)**	**71.7 (8.1)**	**71.5 (9.4)**	*ns*
$CD4^+CD25^+CD127^{lo}$ (% of total leukocytes)	**1.2 (0.6)**	**1.1 (0.4)**	**1.3 (0.5)**	**1.2 (0.5)**	**1.2 (0.5)**	**1.1 (0.4)**	*ns*
CD4:CD8	**2.8 (1.1)**	**2.8 (1.3)**	**2.7 (1.0)**	**2.1 (0.9)**	**2.2 (0.9)**	**2.2 (0.9)**	*0.001*

Data presented as means (standard deviation)

*Analysed by adjusted ANOVA (fixed factors: group, time), the effect of group on the outcome

6.4.5 PHAGOCYTOSIS OF E. COLI BY NEUTROPHILS AND MONOCYTES

Results from experiments measuring neutrophil and monocyte phagocytosis (performed using Phagotest kits) in whole blood are shown in Table 31. Data are presented as percentage of cells engaging in phagocytosis (referred to as percent positive) and the extent of phagocytosis of those active cells (mean fluorescence intensity; MFI). No differences were observed between groups (adjusted ANOVA [fixed factors: group, time], or independent T-tests). However, there were differences within the groups between some time points (analysed by dependent T-tests). The percentage of neutrophils engaging in phagocytosis (percent positive) was decreased between baseline (week 0) and week 2 post-vaccination in the placebo group (89.6 % to 81.9 %, $p = 0.029$). The percentage of monocytes engaging in phagocytosis was also decreased between baseline (week 0) and week 2 post-vaccination in the placebo group (71.9 % to 62.7 %, $p = 0.013$), but was increased between weeks 2 and 4 post-vaccination in both groups (60.8 % to 69.7 % in the prebiotic group [$p = 0.030$], and 62.7 % to 70.4 % in the placebo group [$p = 0.011$]). In the placebo group, the MFI of neutrophils (the activity of the cells) decreased between baseline (week 0) and week 4 post-vaccination (330.8 to 259.2, $p = 0.005$), and the same pattern was observed for the MFI of monocytes between these times in this group (232.9 to 146.4, $p = 0.009$).

When comparing the changes between groups there were no significant differences. Therefore, it must be concluded that the prebiotic supplement had no effect upon blood neutrophil and monocyte phagocytosis.

Table 31 : The effect of supplementation with Orafti® Synergy1 or placebo upon blood neutrophil and granulocyte phagocytosis of E. coli following a seasonal influenza vaccination (administered at week 0)

	Placebo group			Prebiotic group			*p* *
	Week 0	**Week 2**	**Week 4**	**Week 0**	**Week 2**	**Week 4**	
Neutrophils (% positive)	**89.6 (8.9)**	**81.9 (14.2)**	**84.7 (14.0)**	**86.4 (14.9)**	**83.0 (16.7)**	**87.1 (15.0)**	*ns*
Neutrophils (MFI)	**330.8 (136.2)**	**311.9 (182.4)**	**259.2 (71.0)**	**274.1 (114.7)**	**288.8 (121.8)**	**241.1 (68.5)**	*ns*
Monocytes (% positive)	**71.9 (9.2)**	**62.7 (13.2)**	**70.4 (10.3)**	**65.5 (14.3)**	**60.8 (13.1)**	**69.7 (14.6)**	*ns*
Monocytes (MFI)	**232.9 (126.6)**	**205.9 (159.1)**	**146.4 (71.5)**	**212.9 (159.3)**	**227.6 (180.0)**	**169.9 (68.8)**	*ns*

Data are presented as means (standard deviation)

*Analysed by adjusted ANOVA (fixed factors: group, time), the effect of group on the outcome

6.4.6 OXIDATIVE BURST BY NEUTROPHILS AND MONOCYTES

Results from experiments measuring neutrophil and monocyte oxidative burst (performed using Phagoburst kits) in whole blood are shown in Table 32. Two different stimulants were used: *E. coli* and the protein kinase C agonist PMA. The response to *E. coli* requires the bacteria to be recognised and taken up by phagocytosis prior to the cell signalling leading to oxidative burst occurring. PMA by-passes these early steps and directly stimulates protein kinase C, initiating the signalling events leading to burst. The main component of the oxidative bust is superoxide anions. Data are presented as percentage of cells engaging in oxidative burst (referred to as percent positive) and the extent of burst of those active cells (MFI). No differences were observed between groups (analysed with adjusted ANOVA or by independent T-tests). However, there were differences within the groups between some time points (analysed by dependent T-tests).

For neutrophils and monocytes incubated with *E. coli*, some differences within groups between time points were observed. The percentage of neutrophils engaging in phagocytosis (percent positive) was decreased between baseline (week 0) and week 2 post-vaccination in both groups (from 93.7 % to 88.5 % in the prebiotic group [$p = 0.036$], and from 93.4 % to 86.7 % in the placebo group [$p = 0.034$]). The MFI of neutrophils also decreased from baseline (week 0) to week 2 post-vaccination in the placebo group (121.4 compared to 97.8, $p = 0.022$). The MFI of neutrophils decreased from baseline (week 0) to week 4 post-vaccination in both groups (107.6 to 88.8 in the prebiotic group [$p = 0.027$], and 121.4 to 98.3 in the placebo group [$p = 0.007$]). The MFI of monocytes decreased between baseline (week 0) to week 2 (post-vaccination) in both groups (from 25.6 to 22.6 in the prebiotic group [$p = 0.001$], and from 27.0 to 23.5 in the placebo group [$p = 0.007$]). The MFI of monocytes decreased between baseline (week 0) to week 4 post-vaccination in both groups (from 25.6 to 21.3 in the prebiotic group [$p < 0.001$], and from 27.0 to 23.7 in the placebo group [$p = 0.003$]).

For neutrophils and monocytes incubated with PMA, some differences within groups between time points were observed. The percentage of neutrophils engaging in phagocytosis (percent positive) was decreased between baseline (week 0) and week 2 post-vaccination in the placebo group (from 98.9 % to 96.9 %, $p = 0.025$). The percentage of neutrophils engaging in phagocytosis was decreased between baseline (week 0) to week 4 post-vaccination in the prebiotic group (from 99.3 % to 96.4 %, $p = 0.044$). The MFI of neutrophils also decreased from baseline (week 0) to week 2 post-vaccination in both groups (from 298.9 to 239.7 in the prebiotic group [$p= 0.004$], and from 314.6 to 264.4 in the placebo group [$p= 0.005$]). The MFI of neutrophils was decreased from baseline (week 0) to week four post-vaccination in both groups (from 298.9 to 215.8 in

the prebiotic group [$p < 0.000$], and from 314.6 to 262.1 in the placebo group [$p = 0.004$]). The MFI of monocytes was decreased from baseline (week 0) to week 4 post-vaccination in both groups (from 36.5 to 30.7 in the prebiotic group [$p = 0.028$], and from 39.6 to 30.2 in the placebo group [$p = 0.017$]).

When comparing the changes between groups there were no significant differences. Therefore, it must be concluded that the prebiotic supplement had no effect upon oxidative burst of blood neutrophils or monocytes.

Table 32 : The effect of supplementation with Orafti® Synergy1 or placebo upon blood neutrophil and granulocyte oxidative burst following a seasonal influenza vaccination (administered at week 0)

		Placebo group			Prebiotic group			*P**
	Stimulant	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4	
Neutrophils (% positive)	*E. coli*	93.4 (8.7)	86.7 (13.3)	89.8 (7.5)	93.7 (6.4)	88.5 (9.9)	89.9 (11.8)	*ns*
Neutrophils (MFI)	*E. coli*	121.4 (32.7)	97.8 (32.6)	98.3 (25.9)	107.6 (45.8)	101.7 (57.9)	88.8 (33.1)	*ns*
Monocytes (% positive)	*E. coli*	75.8 (11.8)	72.9 (14.1)	72.8 (13.4)	74.7 (13.2)	67.6 (14.6)	73.3 (12.3)	*ns*
Monocytes (MFI)	*E. coli*	27.0 (4.4)	23.5 (5.4)	23.7 (4.3)	25.6 (4.9)	22.6 (5.3)	21.3 (3.9)	*ns*
Neutrophils (% positive)	PMA	98.9 (1.1)	96.9 (4.5)	98.1 (1.9)	99.3 (0.9)	98.8 (1.7)	96.4 (6.9)	*ns*
Neutrophils (MFI)	PMA	314.6 (106.0)	264.4 (98.4)	262.1 (87.2)	298.9 (123.6)	239.7 (98.3)	215.8 (115.9)	*ns*
Monocytes (% positive)	PMA	94.9 (5.2)	92.3 (9.9)	94.8 (4.7)	93.3 (10.1)	90.8 (13.4)	91.8 (12.6)	*ns*
Monocytes (MFI)	PMA	39.6 (23.1)	35.1 (14.4)	30.2 (14.7)	36.5 (12.2)	30.9 (9.7)	30.7 (8.9)	*ns*

Data presented as means (standard deviation) *Analysed by adjusted ANOVA (fixed factors: group, time), the effect of group on the outcome

6.4.7 LYMPHOCYTE PROLIFERATION MEASURED BY CFSE DILUTION

Lymphocyte proliferation was measured by CFSE dilution, and results are shown in Table 33. Data are presented in two ways. The first is as MFI ([geometric] mean fluorescence intensity); a lower MFI indicates greater proliferation since this is due to dilution of CFSE. The second is as percentage of cells in the M1 and M2 regions of the flow cytometry histogram (see Figure 21); movement of cells from M2 to M1 indicates proliferation. Thus a reduced MFI, an increase in the proportion of cells in the M1 region and a decrease in the proportion of cells in the M2 region are indicative of a proliferative response.

Stimulating cells with ConA induced an approximate 3.5-fold decrease in MFI, indicating that cells stimulated with this mitogen had undergone proliferation. The proportion of cells in M1 increased from approximately 15 % in unstimulated cultures, to approximately 70 % in cultures stimulated with ConA. Correspondingly, the proportion of cells in M2 decreased from approximately 85 % in unstimulated cultures, to approximately 29 % in cultures stimulated with ConA. In cultures stimulated with vaccine antigen, no differences from the unstimulated cultures and no changes over time were noted in MFI or the proportion of cells in M1 or M2, indicating a poor response to stimulation.

There was no effect of the prebiotic supplement upon lymphocyte proliferation in unstimulated cultures, or cultures stimulated with ConA or vaccine antigen (analysed by adjusted ANOVA, fixed factors: group and time). Therefore, it must be concluded that the prebiotic supplement had no effect upon lymphocyte proliferation, measured by CFSE dilution.

Table 33 : The effect of supplementation of Orafti® Synergy1 or placebo upon lymphocyte proliferation (measured by CFSE dilution) following a seasonal influenza vaccination (administered at week 0)

	Stimulant	**Placebo group**			**Prebiotic group**			*p**
		Week 0	Week 2	Week 4	Week 0	Week 2	Week 4	
Geometric mean	None	736.8 (316.5)	803.2 (233.2)	661.3 (444.1)	773.1 (292.9)	778.2 (299.9)	815.2 (335.6)	*ns*
	ConA	285.4 (326.9)	168.3 (84.9)	258.3 (345.1)	220.2 (163.5)	214.8 (243.1)	208.1 (158.5)	*ns*
	Vaccine	829.3 (327.9)	743.8 (223.8)	803.6 (510.9)	860.9 (257.4)	821.9 (304.5)	850.7 (405.7)	*ns*
% proliferated (M1)	None	17.8 (21.9)	11.6 (5.5)	15.7 (17.9)	16.5 (19.6)	13.1 (14.1)	13.7 (16.3)	*ns*
	ConA	66.4 (24.3)	76.6 (15.4)	64.9 (26.6)	69.2 (23.9)	73.2 (20.5)	69.4 (25.1)	*ns*
	Vaccine	10.7 (6.9)	14.8 (8.3)	8.8 (5.7)	9.9 (4.9)	11.3 (7.9)	13.6 (13.1)	*ns*
% not proliferated	None	81.9 (21.9)	88.1 (5.6)	84.1 (17.9)	83.2 (19.6)	86.6 (14.3)	86.2 (16.3)	*ns*
(M2)	ConA	33.2 (24.1)	23.1 (15.1)	34.5 (26.2)	30.5 (23.9)	26.4 (20.3)	26.7 (19.6)	*ns*
	Vaccine	89.0 (6.9)	84.9 (8.2)	90.8 (5.7)	89.8 (4.7)	88.4 (7.7)	86.1 (13.2)	*ns*

Data are presented as means (standard deviation)

*Analysed by adjusted ANOVA (fixed factors: group & time), the effect of group on the outcome

6.4.8 CYTOKINE PRODUCTION BY LYMPHOCYTES

Cytokine production from blood mononuclear cell cultures was measured by cytometric bead array kits, and results are shown in Table 34. Cells were cultured either unstimulated or they were stimulated with the mitogen ConA which specifically activates T lymphocytes, or with vaccine antigen.

It is clear that all cytokine concentrations were much higher in the ConA-stimulated cultures than in the unstimulated cultures. The approximate average increases in concentration of cytokines after ConA stimulation were 45-fold for IL-2, 5-fold for IL-4, 2-fold for IL-6, 4-fold for IL-10, 9-fold for TNF-α, and 260-fold for IFN-γ. IFN-γ and IL-2 are the prototypical Th1-type cytokines and the great increase in production of these cytokines suggests that the response of lymphocytes within human PBMC cultures stimulated with ConA is Th1 dominated.

The approximate average increases in concentrations of cytokines after vaccine antigen stimulation were 13-fold for IL-2, 30-fold for IFN-γ, no change for IL-4, IL-6, or IL-10, and around a 30 % increase in TNF-α. This suggests that the response of lymphocytes within human PBMC cultures stimulated with influenza vaccine antigen is also Th1 dominated, but is smaller than the response induced by ConA. The effect of vaccine was evident at week 0 (i.e. prior to vaccination) which was not an expected finding.

When time and group were taken into account, the prebiotic supplement had no effect on IL-2 or IL-10 concentrations. IL-4 was lower in the prebiotic group in all culture conditions ($p = 0.004$ for control cultures, $p = 0.032$ for ConA stimulated cultures, and $p = 0.006$ for vaccine stimulated cultures, analysed by adjusted ANOVA, fixed factors: group and time). Significant differences were seen between groups in ConA stimulated cultures for IL-6 (higher in the prebiotic group, $p = 0.031$), IFN-γ (higher in the prebiotic group, $p = 0.007$) and TNF-α (higher in the prebiotic group, $p = 0.023$). However these differences were present prior to vaccination and so do not appear to represent an altered response to the vaccination.

The Th1/Th2 ratio was also calculated using the concentrations of the prototypical Th1-type (IFN-γ) and Th2-type (IL-4) cytokines and is shown in Table 35. The prebiotic group had a significantly higher Th1/Th2 ratio in ConA stimulated cultures ($p = 0.004$, analysed by adjusted ANOVA, fixed factors: group and time). However again, this was seen prior to vaccination and so does not appear to represent an altered response to the vaccination.

Therefore, it appears that Orafti® Synergy1 may be able to alter the patterns of production of certain cytokines produced by cultured PBMCs (IL-4, IL-6, TNF-α, IFN-γ), but not of others (IL-2, IL-10), but this effect is not related to the vaccination response.

Table 34 : The effect of supplementation of Orafti® Synergy1 or placebo upon cytokine production by lymphocytes, following a seasonal influenza vaccination (administered at week 0)

Cytokine	Stimulant	Placebo group			Prebiotic group			*P**
		Week 0	Week 2	Week 4	Week 0	Week 2	Week 4	
IL-2	None	2.1 (1.4)	1.9 (0.9)	1.7 (0.7)	1.9 (0.6)	2.1 (1.9)	1.9 (1.1)	*ns*
	ConA	97.2 (81.1)	85.7 (81.2)	63.4 (48.7)	109.8 (68.5)	73.7 (55.3)	86.2 (66.1)	*ns*
	Vaccine	21.2 (23.0)	25.7 (22.9)	22.9 (29.8)	24.8 (19.4)	24.9 (16.4)	32.5 (22.1)	*ns*
IL-4	None	2.1 (1.0)	2.0 (0.9)	1.9 (1.0)	1.6 (0.5)	1.6 (0.4)	1.6 (0.7)	*0.004*
	ConA	12.2 (9.1)	11.9 (9.7)	10.1 (7.9)	10.8 (15.0)	6.3 (3.8)	6.5 (3.1)	*0.032*
	Vaccine	2.0 (1.2)	2.0 (0.8)	2.1 (0.9)	1.7 (0.5)	1.6 (0.7)	1.6 (0.4)	*0.006*
IL-6	None	3036 (3337)	2112 (2275)	2323 (3058)	2639 (3145)	3856 (3415)	3108 (2937)	*ns*
	ConA	7235 (5105)	4913 (4058)	4588 (6110)	8310 (3876)	6003 (4371)	8944 (5095)	*0.031*
	Vaccine	3611 (3648.0)	2273 (2086)	1967 (3200)	2834 (2928.8)	3241 (3132)	2914 (2601)	*ns*
IL-10	None	16.2 (10.1)	12.9 (9.4)	23.9 (46.9)	18.3 (18.5)	24.7 (23.2)	24.9 (37.3)	*ns*
	ConA	76.2 (42.7)	74.2 (66.2)	75.1 (91.8)	92.7 (75.4)	83.1 (119.8)	69.1 (36.1)	*ns*
	Vaccine	17.9 (14.0)	17.4 (13.1)	26.2 (62.5)	17.4 (12.8)	19.5 (15.6)	18.5 (15.8)	*ns*
TNF-α	None	37.3 (31.0)	42.9 (76.1)	41.7 (43.3)	22.7 (16.8)	60.7 (63.9)	64.9 (75.3)	*ns*
	ConA	333.9 (232.6)	270.3 (229.6)	271.5 (261.7)	434.4 (449.1)	388.9 (414.0)	486.5 (439.5)	*0.023*
	Vaccine	47.8 (44.5)	57.9 (73.3)	56.8 (61.7)	40.7 (48.6)	65.8 (81.5)	75.9 (93.2)	*ns*
IFN-γ	None	6.4 (4.4)	4.4 (2.3)	4.1 (1.3)	4.6 (2.3)	11.1 (23.6)	3.7 (0.6)	*ns*
	ConA	1056.3 (1028.9)	662.9 (732.4)	723.7 (985.1)	2080.9 (3126.5)	1603.5 (2886.7)	1754.3 (2006.1)	*0.007*
	Vaccine	190.3 (317.8)	101.6 (141.7)	159.8 (328.9)	114.4 (212.0)	100.1 (105.8)	193.5 (311.5)	*ns*

Data are presented as means (standard deviation) pg/ml *Analysed by adjusted ANOVA (fixed factors: group & time), the effect of group on the outcome

Table 35 : The effect of supplementation of Orafti® Synergy1 or placebo upon ratio of Th1/Th2 (IFN-γ/IL-4) type cytokine production by lymphocytes following a seasonal influenza vaccination (administered at week 0)

Stimulant	Placebo group			Prebiotic group			*p**
	Week 0	**Week 2**	**Week 4**	**Week 0**	**Week 2**	**Week 4**	
None	**3.1 (1.7)**	**2.5 (1.2)**	**2.4 (0.6)**	**3.1 (1.4)**	**6.8 (12.8)**	**2.5 (0.5)**	*ns*
ConA	**151.3 (222.4)**	**102.5 (177.3)**	**83.4 (97.7)**	**248.4 (282.4)**	**181.5 (211.8)**	**297.3 (369.6)**	*0.004*
Vaccine	**108.0 (207.3)**	**51.6 (70.4)**	**66.8 (132.9)**	**78.3 (155.3)**	**62.3 (63.2)**	**130.4 (239.0)**	*ns*

Data are presented as mean (standard deviation)

*Analysed by adjusted ANOVA (fixed factors: group & time)

6.4.9 LYMPHOCYTE ACTIVATION

Lymphocyte activation was measured by CD69 expression on $CD3^+CD4^+$ cells, and results are shown in Table 36. Data are presented as the percentage of cells expressing CD69 (percentage positive) and as MFI ([geometric] mean fluorescence intensity), an indication of the extent of expression of CD69 on positive cells.

Stimulation of cultures with ConA resulted in an increase in $CD69^+$ cells from about 14% to about 55% and there was about a doubling of MFI on positive cells, indicating a higher level of expression of CD69.

Stimulation of cultures with vaccine antigen resulted in an increase in $CD69^+$ cells from about 14% to about 30% and there was roughly a 30 % increase of MFI on positive cells, indicating a higher level of expression of CD69. The effect of vaccine was evident at week 0 (i.e. prior to vaccination) which was not an expected finding.

There was no effect of the prebiotic supplement upon CD69 T cell activation in control or ConA stimulated cultures (analysed by adjusted ANOVA, fixed factors: group and time). However, in vaccine stimulated cultures, there was a greater proportion of $CD69^+$ cells and a higher MFI on positive cells in the prebiotic group ($p = 0.015$ and $p = 0.070$ respectively, analysed by adjusted ANOVA, fixed factors: group and time). However, this does not seem to be due to an improved response to the vaccine in the prebiotic group. Before vaccination, values in both groups were similar. In the prebiotic group, proportion of $CD69^+$ cells and MFI on positive cells did not change much after exposure to vaccine, and the between group differences seem to be caused by the fall in both of these measures in the placebo group following vaccination, which is an unexpected finding (MFI on positive cells also decreased slightly in the prebiotic group following vaccination). Therefore, it must be concluded that the prebiotic supplement had no effect upon lymphocyte activation, as measured by CD69 expression on $CD3^+CD4^+$ cells.

Table 36 : The effect of supplementation of Orafti® Synergy1 or placebo upon T cell activation following a seasonal influenza vaccination (administered at week 0)

	Stimulant	Placebo group			Prebiotic group			*p**
		Week 0	Week 2	Week 4	Week 0	Week 2	Week 4	
Percent positive	None	14.9 (12.6)	12.5 (9.6)	11.5 (8.1)	13.5 (6.2)	15.1 (6.4)	14.9 (9.5)	*ns*
	ConA	57.4 (16.1)	55.9 (11.9)	53.3 (16.1)	55.7 (12.6)	52.5 (13.1)	50.6 (14.1)	*ns*
	Vaccine	29.8 (16.1)	24.9 (13.8)	24.2 (13.6)	31.6 (10.3)	32.9 (11.3)	32.2 (12.6)	*0.015*
Geometric mean	None	45.4 (11.7)	40.1 (10.7)	38.7 (11.5)	45.5 (13.8)	43.3 (10.0)	39.6 (9.8)	*ns*
	ConA	101.0 (48.4)	91.9 (33.0)	88.5 (31.7)	92.3 (27.5)	88.4 (25.6)	82.9 (26.5)	*ns*
	Vaccine	55.5 (16.0)	49.0 (15.2)	49.1 (17.5)	58.9 (16.2)	55.0 (13.0)	55.2 (15.1)	*0.070*

Data are presented as means (standard deviation)

*Analysed by adjusted ANOVA (fixed factors = group & time), the effect of group on outcome

6.5 DISCUSSION

This chapter described the effect of supplementation of Orafti® Synergy1 (8 g/day) upon various systemic immune outcomes, at 2 and 4 weeks following a seasonal influenza vaccination (administered at week 0). Few differences were seen between groups in most immune outcomes measured. However, two important and novel observations were made. First, the prebiotic supplement enhanced the antibody response to the HAH3_UR strain of the vaccine, and secondly the IgG1-specific antibody response to the vaccine was also enhanced in the prebiotic group. Therefore, although it must be concluded that the prebiotic supplement did not alter many aspects of systemic immune function in healthy middle aged adults following an immune challenge, it was able to enhance some aspects of the antibody response to vaccination. Response to vaccination is considered to be the most valid marker of immune function (10). Results from this study are discussed in the context of the eight studies in the literature that have looked at the response to vaccination as a way to assess the effect of a ß2-1 fructan supplement upon immune function.

Although the antibody response to the HAH3_UR strain of the seasonal influenza vaccine was enhanced following prebiotic supplementation, the responses to the HAH1_BR and HAB_FL strains were not affected. Although there were no significant differences in seroconversion or seroprotection rates between groups at any time point for any of the three viral strains, both seroconversion and seroprotection to the HAH3_UR strain of the vaccine were higher in the prebiotic group. It is possible that the high rates of seroconversion and seroprotection seen in the placebo group could explain why no significant differences were seen between the placebo and Orafti® Synergy1 groups. For the HAH3_UR strain of the vaccine where seroconversion and seroprotection rates in the placebo group were lower, some trends for an improvement with Orafti® Synergy1 were seen. These observations, particularly the significantly enhanced response to the HAH3_UR strain mean that the principal hypothesis of the study ("that Orafti® Synergy1 will improve the vaccine-specific antibody response following seasonal influenza vaccination") can be accepted. However, it is not clear why the response to only one of the three strains was improved.

Several studies have looked at the effect of a ß2-1 fructan supplement upon the antibody response following vaccination and some studies have reported some improvement. In elderly adults supplemented with a formula in which FOS contributed 4.95 % of the energy, and immunised against influenza, the response to the H1N1 strain of the vaccine was improved at six weeks post-vaccination (96). However, in common with the current study, there was no effect of FOS on the response to the other two strains of the vaccine

(H3N2 or B strains), or on any antibody response at 24 weeks post-vaccination (96). A study using the same supplement in frail adults immunized with influenza found an increase in the number of subjects with antibody titres of greater than 100 for the H1N1 component, and similar trends for the percentage of subjects with four-fold increases against the B strain or with H3N2 antibody titres of 40 or more. However, there was no effect on geometric mean antibody titres (97). In elderly adults supplemented with 6 g/d of a mixture of 70 % oligofructose and 30 % inulin, there was an increase in the antibody response to Influenza B virus and *Streptococcus pneumonia* at six weeks post-vaccination in both control and prebiotic groups, but no change in the serum antibody titres against influenza A virus at this time (92).

Other studies have found no effect of ß2-1 fructans upon the antibody response to vaccination. In Peruvian infants, supplemented with approximately 0.55 g/d of oligofructose and vaccinated against Influenza B virus, there was no effect on the post-vaccination antibody response measured one month post-vaccination (93). In elderly adults supplemented with 6 g/d of oligofructose and inulin in a two to one ratio in a nutritional supplement and vaccinated against influenza and pneumococcus, there was no effect on the magnitude of the rise in influenza or pnuemococcus antibodies at two months post-vaccination (103). In children supplemented with a synbiotic containing inulin and oligofructose, there was no effect on serum vaccine-specific (tetanus or pneumococcus) antibody levels (106).

The current study is quite unique in measuring vaccine-specific immunoglobulins in serum. These were elevated post-vaccination in both control and prebiotic groups. There was no effect of prebiotic on vaccine-specific IgA, IgM or IgD. However, there was an enhancement of the IgG1-specific response to the vaccine antigens in the prebiotic group. This is in agreement with findings from a study in healthy infants, immunised against measles, and supplemented with a mixture of 70 % oligofructose and 30 % inulin at 0.2 g/kg body weight/d, where an increase in post-vaccination IgG measles antibody levels in blood at six weeks post-vaccination was seen (91). However, in infants at risk from atopy and vaccinated with typical childhood vaccinations at three months of age, and given a supplement of GOS and FOS in a nine to one ratio, there was no effect on the DTP (diphtheria, tetanus, polio)-specific IgE, IgG1, IgG2 or IgG3 levels at three months post-vaccination (101).

The prebiotic supplement had no effect upon total immunoglobulin (IgA, IgG or IgM) concentrations in blood and these were not affected by the vaccination. Other studies have also reported a lack of effect of ß2-1 fructans upon total blood immunoglobulins. In elderly adults supplemented with 6 g/d of a mixture of 70 % oligofructose and 30 %

inulin, there was no effect on serum IgA, IgG or IgM 6 weeks post-vaccination (92). In children supplemented with a synbiotic containing inulin and oligofructose, there was no effect on serum IgG, IgA, IgM or IgE levels following tetanus or pneumococcal vaccination (106). In infants at risk from atopy and given a supplement of GOS and FOS in a nine to one ratio, there was a decrease in total plasma IgE, IgG1, IgG2 and IgG3 levels three months post-vaccination, but no effect on total plasma IgG4 (101).

Salivary sIgA is a marker of mucosal immunity (10), although it probably is more representative of the upper respiratory tract mucosal immune system than the low gastrointestinal immune system. When salivary sIgA concentrations were not adjusted for total protein concentrations, there was a greater decrease in the prebiotic group compared to the placebo group. However this does not take into account the total protein content of the saliva, and so is a less reliable method than when sIgA concentrations are adjusted for total protein, and this was not significantly different between groups. Therefore, the prebiotic supplement had no effect upon total sIgA concentrations in saliva. In agreement with this finding, a study in elderly adults supplemented with 6 g/d of a mixture of 70 % oligofructose and 30 % inulin, found no effect on salivary sIgA six weeks post-vaccination (92). It would be useful in future studies to measure faecal sIgA as a marker of gut mucosal immunity.

The enhancement in antibodies to the HAH3_UR strain and in vaccine-specific IgG1 suggests that Orafti® Synergy1 does impact on the host immune system. This may be the result of the change in faecal (and so gut) microbiotia reported in Chapter 4. It is important to identify which aspect of the immune response is affected by Orafti® Synergy1. Possibilities include an alteration in the types of immune cells present and/or an alteration in the function of one of more key cell types. Among the latter, candidates are: antigen presenting cells and the processes of antigen uptake, processing and presentation; T cells and the processes of activation, proliferation and cytokine production; B cells and the process of antibody production. The current study focussed on identifying whether Orafti® Synergy1 affected the profile of immune cells (in the bloodstream) and the functional responses of T lymphocytes; activation, proliferation and cytokine production were all examined. These processes were induced (ex vivo) using the general T cell stimulant ConA and the influenza vaccine itself. ConA induced strong activation, proliferation and cytokine responses. In addition some aspects of the innate immune response (phagocytosis and respiratory burst), not previously examined in the context of prebiotic supplementation and vaccination responses were examined.

There was no effect of the prebiotic upon immune cell phenotypes in the blood following a seasonal influenza vaccination, apart from an increase in $CD3^+/CD8^+$ and $CD8^+$ cells, and

a decrease in the CD4/CD8 ratio, but this was likely due to differences already present at week four. Only one other study has looked at the effect of a ß2-1 fructan supplement upon immune cell phenotypes following vaccination (97). This study was in elderly adults, supplemented with a nutritional formula to which FOS contributed 4.95 % of the energy. There was an increase in B cells, a decrease in cytotoxic memory T cells, non-significant increases in NK T cells and naïve Th cells, and no effect on $CD4^+$ or $CD8^+$ T cells six weeks post-vaccination (97). It is concluded that, in the current study, the improved response to vaccination seen with Orafti® Synergy1 does not involve an alteration in immune cell types present. However, these were examined in the bloodstream and it is still possible that immune cells types present in gut lymphoid tissue were affected by the prebiotic.

The prebiotic supplement had no effect upon blood neutrophil and monocyte phagocytosis or upon oxidative burst of blood neutrophils or monocytes. No other studies have looked at the effect of a ß2-1 fructan supplement upon neutrophil and monocyte phagocytosis or oxidative burst, following vaccination.

The prebiotic had no significant effect on lymphocyte activation, measured by enhanced CD69 expression on Th lymphocytes (defined as $CD3^+CD4^+$). In frail elderly adults supplemented with FOS as part of a nutritional formula, there was an increase in influenza activated lymphocytes (CD69 and CD25) at six weeks post-vaccination, but no effect on PHA-stimulated cells (97).

There was no effect of the prebiotic supplement upon lymphocyte proliferation in unstimulated cultures, or cultures stimulated with ConA or vaccine antigen, measured by CFSE dilution, although there was a poor response to the latter. This poor response may be attributed to a low number of vaccine-specific T cells in the cultures or to the inability of the method to detect a (small) vaccine-induced increase in lymphocyte proliferation because of its insensitivity. Two other studies have looked at the effect of a ß2-1 fructan supplement upon lymphocyte proliferation, following vaccination. However these studies did not use the CFSE dilution method to follow the proliferative response, instead they used ^{3}H-thymidine incorporation (92, 96). One study in free-living elderly subjects supplemented with 6 g/d of a mixture of 70 % oligofructose and 30 % inulin did not find any effect of the supplement upon influenza antigen- or phytohaemmaglutinin (PHA)-stimulated lymphocyte proliferation 6 weeks post-vaccination (92). A further study in elderly adults supplemented with a formula in which FOS made up 4.95 % of the energy, reported an increase in lymphocyte proliferation to influenza vaccine components at six weeks but not at 24 weeks post-vaccination (96).

It appears that supplementation with Orafti® Synergy1 is able to alter the patterns of certain cytokines produced by cultured blood mononuclear cells (IL-4, IL-6, TNF, IFN-γ), but not others (IL-2 and IL-10). In the prebiotic group IL-4 was lower than in the control group in all cultures conditions, and IL-6, TNF-α and IFN-γ higher than in the control group in ConA stimulated cultures; however this effect was present prior to vaccination and so does not appear to represent an altered response to the vaccination. Several other studies have looked at the effect of ß2-1 fructans upon lymphocyte cytokine production following vaccination, and report varying results. A study in elderly adults supplemented with 6 g/d of a mixture of 70 % oligofructose and 30 % inulin found no effect on IL-4 and IFN-γ production by cultured blood mononuclear cells six weeks post-vaccination (92). In elderly adults supplemented with 6 g/d of oligofructose and inulin in a two to one ratio in a nutritional supplement, the decrease in IL-2 production from PHA-stimulated mononuclear cells seen in the control group was prevented in the prebiotic group at two months post-vaccination. There was no effect on IFN-γ production by influenza antigen-stimulated blood mononuclear cells (103). In frail elderly adults supplemented with FOS as part of a nutritional formula, there was a decrease in IL-6 and a trend for a decrease in IL-10 production by PHA-stimulated mononuclear cells six weeks post-vaccination. However there was no effect upon IL-2, IL-4, IFN-γ or TNF-α production by PHA-stimulated mononuclear cells, or upon IL-2, IL-4, IL-6, IL-10, IFN-γ or TNF-α production by influenza antigen stimulated mononuclear cells at this time point (97).

The lack of effect of Orafti® Synergy1 on lymphocyte activation, lymphocyte proliferation and lymphocyte cytokine production post-vaccination suggests that the enhanced antibody response may not involve an improvement in T cell function. However there are two caveats to this. First, the response to ConA is a general T cell response and has no antigen specificity and so it may not really be informative about how T cells respond to vaccine. Secondly, the investigation of ex vivo T cell responses to vaccine proved to not be very useful. T cell proliferation measured by CFSE dilution was not detectable in response to the vaccine. As stated above, this may be attributed to a low number of vaccine-specific T cells in the cultures, or to the inability of the method to detect a (small) vaccine-induced increase in lymphocyte proliferation because of its insensitivity. In contrast, a marked T cell activation (CD69 expression) and Th1-type cytokine response was seen in cultures with vaccine. This suggests a T cell response to vaccine. However this response was present in cultures from cells taken prior to vaccination (i.e. at baseline - week 0). This was unexpected and means that the T cell responses seen post-vaccination do not necessarily reflect the vaccination process. Rather they suggest that T cells from these middle aged subjects are able to mount a response upon exposure to a seasonal influenza vaccine to which the T cell donor has not been exposed. The T cell responses may be due to some inherent property of the vaccine itself or may be due to a

characteristic of the subjects. For example, the vaccine may contain a component which can stimulate T cells; an examination of the manufacturer's description of the components of the vaccine did not reveal any potential candidates although the vaccine dose contain a small amount of ovalbumin, because the influenza virus is grown in hens eggs. A small proportion (eight) of the subjects had previously received a seasonal influenza vaccination. However, it seems highly likely that all, or most, of them would have been exposed to many different influenza viruses. They would have retained immunological memory of such exposures, although the virus-specific antibody concentrations would have been low. A small number of virus antigen-specific memory T cells could have been responsible for the T cell responses seen upon exposure of PBMC cultures to the vaccine. One way to examine this further would be to expose umbilical cord blood T cells to the vaccine. These cells would be immunologically naïve and so would not mount any vaccine (or influenza virus) antigen-specific response. Thus, a lack of response in cord blood cell cultures would indicate that in the study subjects there were virus antigen specific memory T cells in the circulation. In contrast, a response in cord blood cell cultures would indicate that the vaccine contains some sort of general T cell stimulant; cord blood T cells response to such stimulants because the response does not require a prior antigen exposure.

Despite these caveats, the findings presented here suggest that the enhanced antibody response to the vaccine seen with Orafti® Synergy1 probably does not involve an improved T cell function. Thus an effect on antigen presenting cells or on B cells is possible. Future studies might examine the functional activities of these cell types.

6.6 CONCLUSIONS

In conclusion, Orafti® Synergy1 (8 g/day for 4 weeks prior to vaccination and continuation post-vaccination) enhanced the antibody response to one of the three strains of the seasonal influenza vaccine and improved the anti-vaccine IgG1 response. There was also a tendency for Orafti® Synergy1 to enhance seroprotection and seroconversion rates to the same strain of the vaccine to which the antibody response was enhanced. Orafti® Synergy1 did not affect the blood immune cell profile, a marker of mucosal immunity (salivary sIgA), innate immune responses, or T cell responses to general T cell stimulant, and ex vivo T cell responses to vaccine were not useful. The enhanced antibody response with Orafti® Synergy1 may not involve an effect on T cells. It is also possible that had the change in bifidobacteria been greater, then a greater effect on immune function may have been observed.

CHAPTER 7

FINAL DISCUSSION

Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth, and/or activity, of one or a limited number of beneficial bacteria in the colon and thus improve host health" (24). Prebiotics include the ß2-1 fructans, molecules which contain fructosyl-fructose linkages. A summary of the existing literature revealed that ß2-1 fructans are able to modulate some aspects of immune function, to improve the host's ability to respond successfully to certain intestinal infections, and to modify some inflammatory conditions (63). The existing literature describing probiotics on immune, infectious and inflammatory outcomes in humans was also summarised, as this is related to the mechanism through which prebiotics are believed to exert their beneficial effects. Results from this summary were mixed, and there appear to be large species and strain differences in the effects seen (179). There are large differences between the studies using both prebiotics and probiotics, regarding the supplement given, the dose, the duration of supplementation, heterogeneity of the subjects, sample size, differences in the outcomes measured, and technical differences in how the measurements were made. Therefore it is difficult to draw sound conclusions about the effects that prebiotics and probiotics exert on immune function in humans.

Studies of prebiotics and immune function in middle-aged adults are underrepresented in the existing literature. Although several studies in older adults (43, 92, 94, 96, 97, 103) and one study in younger adults (98) have reported mixed effects of prebiotics on the adaptive immune system, there are no studies in healthy middle-aged adults. Therefore, a study was performed to investigate the effect of supplementing healthy human middle-aged adults with 8 g/day for eight weeks of Orafti® Synergy1 on the functioning of the immune system.

Response to vaccination is regarded as the gold standard method to measure the functioning of the immune system (10), but there are no standard procedures to follow for this method. Therefore, a pilot study was carried out in order to develop a standardised protocol for use in a prebiotic intervention study measuring immune function. Volunteers were vaccinated with the 2007/2008 seasonal influenza vaccine and provided blood and saliva samples for analysis at one, two, four and six weeks post-vaccination. From the subsequent analysis, it was identified that vaccine-specific and total antibody concentrations were maximal by four weeks post-vaccination and that cell culture conditions could be optimised for measuring various immune outcomes.

The prebiotic intervention study itself was a randomised controlled trial in healthy middle-aged adults who were supplemented with 8 g/day of Orafti®Synergy1 for eight weeks. Four weeks into the trial they received the 2008/2009 seasonal influenza vaccine. Fewer

subjects than initially planned were recruited to the trial; this was because of the expiry of the 2008/2009 seasonal influenza vaccine which meant it could no longer be used. Compliance, as determined by counting of returned sachets, was very good in both groups. More subjects in the prebiotic group perceived gastrointestinal sensations, particularly increased flatulence and increased regularity of bowel movements. Both of these are suggestive of a change in gut microbiotia. Results using FISH clearly demonstrated an increase in bifidobacteria in faeces of subjects in the prebiotic group (an approximate doubling) and that bifidobacteria numbers were higher at week four in the prebiotic than in the control group. Therefore, the prebiotic supplement had a bifidogenic effect within the gut, which is in agreement with other studies that have also reported the bifidogenic properties of ß2-1 fructans (43, 241-245, 247, 248), although the increase in this study may be considered to be quite modest. This could be related to the group that was studied, who were healthy middle aged adults. Although diet wasn't assessed, it is possible that this group of people consumed a fairly healthy diet, and had a "healthy" gut microflora to begin with, making it difficult to modify this for the better. In the light of previous studies that have shown that prebiotic supplementation may modulate some aspects of immune function (43, 91, 92, 94, 96-99, 101-105, 108, 109, 250, 251), an effect which is presumed to be mediated by a change in gut microbiotia, it was anticipated that some changes in immune function would be observed in these subjects.

A range of systemic immune outcomes were measured in the subjects at before and after four weeks of supplementation with Orafti® Synergy1 (in the absence of vaccination). These included immune cell phenotypes, neutrophil and monocyte phagocytosis and oxidative burst, total serum IgA, IgG and IgM concentrations, salivary sIgA concentration and lymphocyte responsiveness (activation, proliferation, and cytokine production). In conclusion, few effects of Orafti® Synergy1 upon systemic immune outcomes in the absence of an immune challenge were observed. A similar lack of effect on some of the outcomes reported here, or related to these outcomes, has been described in previous studies in the literature looking at immune function in the absence of a vaccination challenge. However, some studies in children (99, 102), young adults (98), the elderly (43, 94, 103), adults with colon cancer (104, 105), adults with active ulcerative colitis (108) and adults in intensive care (109) do report some effects on immune function.

The same systemic immune outcomes were then measured in the subjects prior to and then two and four weeks following vaccination. The main outcome was the effect that supplementation with Orafti® Synergy1 had upon response to the seasonal influenza vaccination, both upon the antibody response to vaccination and upon ex vivo T cell responses to vaccine components. Few differences were seen between groups in most immune outcomes measured. However, two important and novel observations were made.

First, the prebiotic supplement significantly enhanced the antibody response to the HAH3_UR strain of the vaccine; additionally, the seroconversion and seroprotection rates to this strain of the virus were enhanced in the prebiotic group, although not significantly. Secondly, the IgG1-specific antibody response to the vaccine was enhanced in the prebiotic group. It appeared that this particular antibody response occurred more quickly in the prebiotic group; by week 4 post-vaccination, the serum concentrations of IgG1-specific antibodies to the vaccine had reached a plateau in the prebiotic group, but the concentration was still increasing in the placebo group. Therefore, although it must be concluded that the prebiotic supplement did not alter many aspects of systemic immune function in healthy middle aged adults following an immune challenge, it was able to enhance some aspects of the antibody response to vaccination, which is considered to be the most valid marker of immune function (10). These observations, particularly the significantly enhanced response to the HAH3_UR strain, mean that the principal hypothesis of the study ("that Orafti® Synergy1 will improve the vaccine-specific antibody response following seasonal influenza vaccination") can be accepted. However, it is not clear why the response to only one of the three strains was improved, although other studies have reported varying effects of β2-1 fructan supplementation upon the different viral strains in the influenza vaccination in elderly subjects (96, 97).

Therefore, this study is a valuable addition to the existing literature. It has investigated the use of a ß2-1 fructan supplement in a population which has not been studied before, and has measured a range of immune markers, among them the gold standard method - the response to vaccination.

Limitations and future work

It is acknowledged that the study reported in this thesis has limitations. Firstly, the sample size of this study was smaller than initially planned (see section 4.3.2), although still comparable with several other studies of this type (92, 96, 103). Immune function varies widely between individuals, as demonstrated by the large standard deviations reported for some of the outcomes measured. Some differences between the groups were observed, confirming that the sample used was large enough to detect differences between the groups for these outcomes. However, it may be that for some other outcomes measured, the sample size was not large enough to detect differences between the groups. Therefore, it is possible that in a larger study more differences may have been detected between groups. This study is therefore a valuable addition to the literature, and to people designing other studies of this type, as a guide when deciding how large future studies need to be.

Orafti® Synergy1 was shown to alter the faecal microbiotia, and it may therefore be expected that changes in immune function may be most likely to be detected in the gut associated lymphoid tissue. However, no markers of immune function in the gut were measured in the current study, due to logistical and ethical constraints on sample collection. However in future studies it would be useful to look at faecal markers of immunity. It was expected in the current study that an alteration in salivary sIgA (as a marker of mucosal immunity) would be observed upon supplementation with Orafti® Synergy1, but this was not found. However, as salivary sIgA is more representative of the upper respiratory tract immune system it would be more suitable to look at faecal sIgA, as a marker of the lower gastrointestinal immune system, as this is closer to the site of action of prebiotics. Faecal sIgA has been shown to increase in newborn infants supplemented with a combination of GOS and FOS (99, 102). It may also be useful to look at immune cell types at this site, to determine if any changes are observed. It is also possible that had the change in bifidobacteria been greater, then a greater effect on immune function may have been observed.

The enhancement in antibodies to the HAH3_UR strain and of vaccine-specific IgG1 shows that Orafti® Synergy 1 does impact on the host immune system. This may be the result of the change in faecal (and so gut) microbiotia reported in Chapter 4. It is important to identify which aspect of the immune response is affected by Orafti® Synergy 1. The current study focussed on identifying whether Orafti® Synergy1 could affect the profile of immune cells in the bloodstream, and the functional responses of T cells (activation, proliferation, cytokine production) induced ex vivo using the general stimulant ConA and the influenza vaccine. ConA induced strong activation, proliferation and cytokine responses. There was a lack of effect of Orafti® Synergy 1 on lymphocyte activation, lymphocyte proliferation and lymphocyte cytokine production post-vaccination, suggesting that the enhanced antibody response may not involve an improvement in T cell function. However, it is possible that effects may be seen on antigen presenting cells and the process of antigen uptake, processing and presentation, or on B cells and the process of antibody production. Future studies should examine the functional activities of these cell types.

The response of T cells to the vaccine *ex vivo* was investigated by culturing blood mononuclear cells with the vaccine antigen, with the aim of stimulating vaccine-specific T cells and assessing the function of these cells. However, this method proved not to be very useful as a response to the vaccine was observed in cells before vaccination, suggesting that T cell responses seen to the vaccine post-vaccination do not necessarily reflect the vaccination process, but that T cells from these subjects are able to mount a response upon exposure to a seasonal influenza vaccine to which the subject has not been exposed. Therefore, the information gained from these experiments may not

provide a good insight into the effect that Orafti®Synegy1 had upon the response of T cells to vaccination. This area clearly needs more development before the results gained can be interpreted in a useful way. The T cell responses may be due to some inherent property of the vaccine itself (a component which can stimulate T cells) or may be due to a characteristic of the subjects (such as exposure to many different influenza viruses, resulting in immunological memory of such exposures). Further work could investigate the effect of exposing umbilical cord blood T cells (immunologically naïve) to the vaccine. A response seen in cord blood cells would indicate that the vaccine contains some sort of general T cell stimulant that is stimulating the cells. In this case, purifying the vaccine before use in cell culture work may improve the responses seen. However, a lack of response in cord blood cell cultures would indicate that in the study subjects there were virus antigen specific memory T cells in the circulation, in which case it would be difficult to modify the method used.

The current study investigated the effects of a prebiotic supplement upon immune function in healthy middle-aged human adults. Little effect was seen on the functioning of the immune system in these subjects, and several explanations for this lack of effect are given above. However, it may be that in this population, immune function is near optimal, and cannot be enhanced. This idea is supported by a study in healthy male adults, which reported only a small effect of inulin supplementation upon immune function (98). Studies in children (91, 99, 101, 102) and the elderly (43, 92, 94, 96, 97, 103) have shown that immune function can be altered in these populations, whose immune system is more susceptible to modification. Therefore it may be interesting to investigate the effect of prebiotics in these populations further and maybe to look at the effects that prebiotics may have in slightly older (for example, school age) children, as the majority of studies have been carried out in infants. Also it would be interesting to look at other groups of people who may have suppressed immune systems, such as athletes undergoing intensive training, or people under stress. Studies of probiotic supplementation in such groups have found little effect in students taking exams (254) and small effects on immune markers or infectious outcomes in distance runners and recreational athletes (255-258), but as prebiotics have not been investigated in these populations yet, it may be interesting to see if they might have an effect. This could identify new target groups for prebiotics.

Final conclusions

This thesis has described a study that investigated the effect of Orafti® Synergy1 on the functioning of the immune system in healthy middle-aged adults, an age group that is underrepresented in the literature. A pilot study was conducted initially in order to

develop a vaccination protocol, as response to vaccination has been identified as the most meaningful measure of the immune response. This protocol was then used in an intervention study to investigate the effect of Orafti®Synergy1 (8 g/day for eight weeks) on immune responses in healthy middle-aged adults, who were vaccinated with the 2008/2009 seasonal influenza vaccination after four weeks. Supplementation with Orafti® Synergy1 (8 g/day) for four weeks was shown to have a bifidogenic effect. No effect of the supplement was seen on immune function measured in the absence of vaccination. Furthermore, few differences were seen between groups in most immune outcomes measured post-vaccination. However, two important and novel observations were made: the prebiotic supplement enhanced the antibody response to the HAH3_UR strain of the vaccine, and enhanced the IgG1-specific antibody response to the vaccine. Therefore, it can be concluded that Orafti®Synergy1 is able to enhance some aspects of immune function in healthy middle-aged adults. This is a valuable addition to the existing literature on ß2-1 fructans and immune function, but it is accepted that several improvements could be made, which are suggested as future work in this area.

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