University of Kent

School of Sport and Exercise Science

Training, nutrition and exercise immunology: The use of salivary Epstein Barr Virus DNA as a marker of in vivo immunity

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No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other university or institute of learning.

All research within this thesis was conducted according to the guidelines laid down by the Declaration of Helsinki (2008, including 2013 amendments), and all procedures were approved, in advance, by the University of Kent's ethics committee.

Abstract

Aim: The aim of this thesis was to investigate the use of salivary Epstein-Barr virus (EBV) DNA as a marker of in vivo immunity in response to training and nutritional intervention. **Methods:** Initially, an assay for the detection of EBV DNA in saliva was developed which was subsequently used to detect the concentration of EBV DNA in samples collected in subsequent studies. The potential role of EBV as a predictor of URTI in response to endurance exercise are presented, along with the outcome of nutritional interventions, with chapters investigating the effects of supplementation with carbohydrate, and Chlorella pyrenoidosa. Finally, data from all four of these chapters were consolidated, and the role of salivary EBV DNA as a marker of in vivo immunity investigated. **Outcome:** The main finding from this thesis is that salivary EBV DNA does not appear to be a useful marker of in vivo immunity based on the present data. EBV concentration was not a predictor of URTI, nor was there a relationship between EBV concentration and SIgA concentration or secretion rate, or the absolute change or percentage change in EBV from pre-post exercise and the absolute and percentage change in SIgA concentration or secretion rate.

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Abbreviations

A_{260}/A_{280}	Ratio of the UV absorbance at 260 and 280 nm
AGPC	Acid guanidinium thiocyanate-phenol-chloroform
APC	Antigen Presenting Cell
AT	Anaerobic threshold
BSA	Bovine Serum Albumin
CD	Cluster of differentiation (classification determinant)
CHL	Chlorella pyrenoidosa
CHO	Carbohydrate
CHS	Contact hypersensitivity
CP	Critical power
Cq	Quantification value
CWSP	Hot water soluble polysaccharides from Chlorella
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DPCP	Diphenylcyclopropenone
DTH	Delayed type hypersensitivity
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EtBr	Ethidium bromide
GC	Guanine-cytosine
GLR	Granulocyte to lymphocyte ratio
HIIE	High intensity interval exercise
HR	Heart rate
HRP	Horseradish peroxide
IFN	Interferon
IgA	Immunoglobulin A
IGF	Insulin like growth factor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IM	Infectious mononucleosis
IQR	Interquartile range
LPS	Lipopolysaccharide

LT	Lactate threshold
MHC	Major Histocompatibility Complex
$MQdH_2O$	Milli-Q grade water
mRNA	Messenger ribonucleic acid
NBM	Nude Body Mass
NK	Natural killer
OD	Optimal Density
OPD	O-Phenylenediamine
PAR-Q	Physical activity readiness questionnaire
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PLA	Placebo
PMBC	Peripheral blood mononuclear cells
qPCR	Quantitative real-time PCR
RPE	Rating of perceived exertion
RPM	Rotations/Revolutions Per Minute
SCODA	Synchronous coefficient of drag alteration
SD	Standard Deviation
SFT	Skin fold thickness
SIgA	Secretory Immunoglobulin A
SPSS	Statistical Package for the Social Sciences
TAE	Tris-acetate-EDTA
Tc	T-cytoxic
Th	T-helper
T_m	Primer melting temperature
TNF	Tumour Necrosis Factor
TQD	Tandem quadrupole detector
TTE	Time to exhaustion
UPL	Universal probe library
UPLC	Ultra performance liquid chromatography
URI	Upper respiratory infection
URTI(s)	Upper Respiratory Tract Infection(s)
URS	Upper Respiratory Symptoms
URT	Upper respiratory tract
UVB	Ultraviolet B

$\dot{V}O_2$	Rate of oxygen uptake
VT	Ventilatory Threshold
WBC	White blood cells

Chapter 1

Introduction and Literature Review

For many centuries, a relationship between exercise and health has been observed. Research has demonstrated that exercise forms an important component of a healthy lifestyle, including being beneficial to the prevention and/or management of more than twenty chronic diseases or disorders such as heart disease, diabetes and certain types of cancers (Booth et al., 2002) . Observations made in the 1920s on the relationship between exercise and illness reported, that in patients suffering from poliomyelitis, exercise could in fact be detrimental to patients' recovery. Those consigned to bed rest, compared to those who continued to exercise following the onset of symptoms, generally recovered quicker, with the resulting disablement being less severe (Agre et al., 1991). These observations prompted physicians and researchers to explore the relationship between exercise and immune function. The antibiotics and vaccines developed throughout the Second World War, however, halted the need for research in this area as a lot of the common illnesses could be controlled through the use of medications. It was not until the emergence, development and accessibility of new technologies in the early eighties that research looking at the relationship between exercise and disease started to be researched and reported with some regularity. This re-emergence has resulted in exercise immunology becoming a recognised research area in its own right.

The cellular responses to exercise have been well researched over the past decades, but it's these cellular interactions that, cumulatively, result in an *in vivo* immune response. However, the *in vivo* immune responses to exercise have been researched less often and are therefore less well understood. The aim of this thesis is to build upon the existing body of research into the role of training and nutrition on immune function, with a specific focus on the *in vivo* immune outcomes. Within this chapter, the reader will find a review of contemporary literature providing an overview of sport and exercise immunology today, including a variety of studies that have used *in vivo* immune techniques.

1.1 Respiratory Infections in Athletes

1.1.1 Introduction to Respiratory Infections

Respiratory infections caused by the common cold or influenza are the most common illnesses to afflict man (Monto, 2002). They are the leading cause of visits to general practitioners (Graham, 1990), and are one of the main reasons for restricted activity within the adult population (Monto, 2002). The last report published by the U.S. Department of

Health and Human Services which detailed the incidence rate of acute respiratory conditions (including the common cold, other acute upper respiratory infections and influenza) estimated that there were nearly eighty reported cases of acute respiratory conditions per one hundred persons per year in the United States (Adams et al., 1999). Of these, nearly fifty percent could be attributed to common colds and other acute upper respiratory infections. Indeed, on average, an adult living in the developed world can expect to suffer two to five respiratory infections every year (Gwaltney et al., 1966 cited by Heath et al., 1992). In 1996, it was estimated that these symptoms lead to 87.1 days of restricted activity (a day in which symptoms affect normal daily activities) per one hundred persons (Adams et al., 1999). The impact on the average adult can be disruptive, resulting in socioeconomic issues such as missed work days. The impact respiratory infections can have on an athlete, however, may not only be disruptive to work and/or training but can also negatively affect performance (Pyne, et al., 2000) with the potential to limit athletic careers. Understanding the etiology of these infections in athletes, and how best to prevent and manage them, therefore, has resulted in the research field of sport and exercise immunology as we know it today.

1.1.2 The Influence of Exercise Intensity and Training Load on Respiratory Illness

There is a well-established link between exercise and respiratory illness which is based upon anecdotal reports, athlete surveys, epidemiologic data, cross-sectional studies, and studies which have researched the role the immune system plays in response to both acute, and chronic exercise, and the subsequent risk of infection (Nieman, 2000). Populations who take part in regular, moderate activity generally report fewer symptoms associated with upper respiratory tract infections (URTIs) (Matthews et al., 2002; Nieman et al., 2011; Nieman et al., 2005; Nieman et al., 1993), and consider themselves at less risk of developing infections compared with their sedentary counterparts (Shephard et al., 1995). Coaches and athletes have, for many years, however, noticed that during periods of intensified training, athletes are more susceptible to the development of minor infections. Indeed, research has supported these observations and, compared to the general population, athletes training at a high intensity and/or athletes whose training volume is high, are more likely to complain of upper respiratory symptoms (URS) and infections compared to both sedentary and moderately active populations (Bishop, 2006*b*). In addition, increases in training load have been shown to result in an increase in URS (Dias et al., 2011; Klentrou et al., 2002; Schwellnus et al., 2016). The relationship between the incidence of upper

respiratory tract infections (URTIs) and the amount of exercise has been described as a J-Curve (Nieman, 1994*a* & 1994*b*) (Figure 1.1).

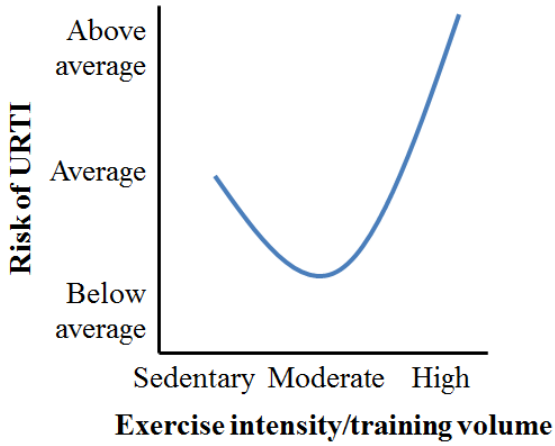


Figure 1.1 The J-Curve which represents the relationship between the risk of developing an URTI and the intensity/volume of training. Adapted from Nieman, 1994a.

Many papers have supported the J-Curve relationship between intensified periods of training and the incidence of URTIs (Cox et al., 2008; Dias et al., 2011; Fahlman, & Engels, 2005; Heath et al., 1991; Klentrou et al., 2002; Matthews et al., 2002; Nieman, et al., 1990; Peters & Bateman, 1983; Spence et al., 2007). Fahlman and Engels (2005) reported on a year's longitudinal study investigating the incidence of URTI and secretory immunoglobulin A (SIgA) responses in an American university football team compared with physically active, non-varsity controls. Symptoms of URTI were recorded retrospectively on a weekly basis for a year, encompassing all seasons and every level of training that occurs throughout the season. They found that there was a significant increase in the number of URTIs reported by football players during intensified periods of training and the competitive season compared to controls. A later study by Spence et al. (2007) compared the incidence of upper respiratory illness in elite triathletes and cyclists, recreational triathletes and cyclists and sedentary populations through both retrospective health questionnaires, and the use of laboratory techniques which identified infection. They reported that, out of all three groups, elite athletes were most likely to suffer upper respiratory illness, with recreational athletes least likely to suffer upper respiratory illness. Most of the illnesses reported by the elite athletes occurred when these athletes were engaged in their heaviest, most intense training periods.

Despite this, a small number of studies have failed to observe Nieman's J-Curve relationship (Ekblom et al., 2006; Neville et al., 2008). This lead to an updated model being proposed in 2006 by Malm (2006) based on an S-shaped relationship whereby the J-shaped relationship remained, but with the proposal that elite athletes can better withstand infection in response to the physical, and psychological demands of elite sport. The authors argued that it is the athlete's ability to withstand infections that allows them to continually maintain their status as an elite athlete by maintaining their training volume and quality.

1.1.3 The Influence of Exercise on Immune Responses

Studies from both human and animals have lead us to the conclusion that physical activity and the immune system are intricately linked. Despite the obvious differences that exist between athletes and non-athletes, what may be arguably more interesting, is the magnitude of change that happens to the immune system following a bout of prolonged exercise. Following intensive or prolonged exercise, researchers have reported decreased levels of immunoglobulins (particularly SIgA); high blood neutrophil counts and low lymphocyte counts caused by an increase in circulating catecholamines, growth hormone and cortisol; decreased delayed type hypersensitivity (DTH) responses; an increase in pro- (e.g. IL-8) and anti-inflammatory cytokines (e.g. IL-6 and IL-10); increases in blood granulocyte and monocyte phagocytosis; decreased oxidative burst by granulocytes; decreased natural killer (NK) cell cytoxic activity; and down regulation of T-cells (Bruunsgaard et al., 1997; Nieman 1997; Nieman 2000; Ostrowski et al., 1999; Pedersen & Bruunsgaard, 1995). All of these responses combined, or in isolation, are believed to lead to an increased risk of succumbing to infections. These alterations in immune function are not permanent, however, but occur only for a few hours or days after each exercise bout (typically 3-72 hours) (Kakanis et al., 2010; Nieman, 2000). These transient alterations in immune function allow viruses and bacteria to gain a foothold, resulting in subclinical, and clinical infection. The period of time in which an athlete is vulnerable. is referred to as the open window (Kakanis et al., 2010; Nieman, 2000).

One of the first studies to provide evidence that URTI risk is associated with reduced SIgA levels, tracked a large group of American collegiate football players over a competitive season. Fahlman & Engels (2005) suggested that if the secretion rate of SIgA dropped below 40 $\mu g.min^{-1}$, athletes were at an increased risk of infection. They further reported

that the risk of illness was not associated with the time of year, and therefore any seasonal variation could be ruled out. Gleeson et al. (1999*b*) also reported an association between SIgA levels and URTI risk in both moderately exercising controls, and elite swimmers. Athletes with an SIgA concentration below 40 $mg.L^{-1}$ were identified as being more at risk of URTI. Further studies have gone on to demonstrate an association between reduced SIgA levels and URTI risk (e.g. Gleeson et al., 1999*a*), but not all studies have established an association. When a group of young, nationally ranked tennis players reported URTIs daily, and provided fortnightly saliva samples for SIgA analysis, the incidence of URTI increased during periods of heavy training, which was not associated with a decrease in SIgA (Novas et al., 2003). The authors attributed the lack of SIgA suppression to the fact that the training sessions monitored in the study were shorter in duration than those in similar studies, and that the data collection period was relatively short. The general consensus is that the monitoring of SIgA is one of the most effective methods of predicting URTI risk within athletes and it continues to be used as a reliable research method and monitoring tool today.

Within a laboratory setting, the severity, intensity, and type of exercise have been shown to be linked to the magnitude and characteristics of the subsequent immune response (Blannin, 2006). A more in depth analysis of these responses can be found below (Section 1.2.5)

1.1.4 The Impact of Respiratory Illness on Competitive Athletes

Intensified periods of training, such as teams' pre-season preparations, can increase the likelihood of athletes developing URTIs (Fahlman & Engels, 2005). A decrease in immunocompetence early on in the season may also lead to the development of URTIs later on in the season (Gleeson et al.1999*a*). Partial humoral immune deficiency may cause athletes to suffer from an increased incidence of URTIs, cause persistent fatigue, and lead to decreases in performance (Reid et al., 2004). The monitoring of immune markers (such as SIgA within athlete populations throughout the season may, therefore, enable the prediction of illness within the same population.

Studies have also shown that the likelihood of an athlete suffering from upper respiratory tract (URT) symptoms or infection post-race are significantly increased if the athlete has suffered with URT symptoms or an infection within the 3 weeks leading up to the race

(Ekblom et al., 2006). The majority of studies have also shown that training mileage can act as a predictor of athletes developing URTI (Heath et al., 1991). Heath et al. (1991) reported an association between running an annual mileage of 486 miles or more and the development of URTIs.

It is generally accepted that if symptoms are above the neck (i.e. confined to the nose, throat, sinuses and ears) and not accompanied by aches, lower respiratory symptoms and/or fever, it is safe for athletes to continue with their training at a moderate intensity without delaying their recovery, exacerbating their symptoms (Weidner & Schurr, 2003; Weidner et al., 1998), or risking the development of any serious health complications associated with viral infections such as myocarditis (Friman & Wesslen, 2000). Despite this, upper respiratory symptoms continue to be one of the main causes of missed, or adjusted training sessions (Engebretsen et al., 2010; Neville et al., 2006) and, although findings have not been significant, differences in race times between athletes suffering from URS in the lead up to competition, and those who don't, have been reported (Pyne et al., 2000). As a result, the interest in the immune system's response to exercise, and limiting the number of URS suffered by athletes has grown exponentially over the past twenty to thirty years (Shephard, 2010).

Approximately 7% of elite athletes experience illness of some description during competition (Engebretsen et al., 2010; Mountjoy et al., 2010; Schwellnus et al., 2016). During the Winter Olympics 2010, 185 illnesses were reported among 2,567 athletes with 54% of these illnesses affecting the upper respiratory system, making URTIs the most frequently diagnosed condition among competitors (Engebretsen et al., 2010). Even during the 1996 Olympic Games which took place in Atlanta during the months of July and August (which generally see a low incidence of URTIs in a normal population sample in the northern hemisphere (Gleeson, 2006*b*), 42.8% (773 out of 1,804) of visits to a physician related to illness (excluding heat-related illnesses), with URTIs being the most common illness requiring attention (9% of physician visits) (Wetterhall et al., 1998). Similar findings have also been reported from international aquatic competitions (Mountjoy et al., 2010), cross-country skiing (Tomasi et al., 1982), endurance yachting events (Neville et al., 2008), and endurance running events (Nieman et al., 1990; Peters & Bateman, 1983).

Not all research is in agreement however, with some authors finding no relationships between workload and the incidence of URTIs (Ekblom et al., 2006; Neville et al., 2008). Some of these differences may be due to varying differences in the definition of a URTI, others may be attributed to study designs, such as the relative exercise intensity. A study by Ekblom et al. (2006) reported non-significant differences in infection rates in marathon runners pre- and post-race, but did find significant findings in the incidence of infections post-race if an infection had been experienced in the three weeks leading up to the race. It is worth noting that this study used the three weeks leading up to the race as its control, and treated this period as *"a normal 3 week training period*". The fourth week (i.e. 21-28 days) preceding a marathon will generally see the greatest weekly mileage in an athlete's marathon training plan before tapering in preparation for the race begins (Noakes, 2002). An increased weekly mileage has, in itself, been shown to be a risk factor in the development of URTIs (Fricker et al., 2005) and the non-significant findings of this study may, therefore, have been influenced by this.

1.2 Immunology

1.2.1 Overview of the Immune System

The immune system is multifactoral with both the innate (natural and nonspecific) and adaptive (acquired and specific) systems working synergistically to defend the host against infection and disease through the detection, isolation, attack, and destruction of invading viruses, bacteria or protozoa (Gleeson, 2006*b* & 2006*c*). The immune system not only protects against infection, but is also constantly monitoring the integrity of host tissues (Delves et al., 2011). Essentially, through elaborate functions, the immune system is designed to recognise and remove foreign substances and organisms from the infected host. The immune system comprises of a variety of cells, tissues, and molecules; each has its own, unique function, but it's when they work together that an effective immune response can be mounted. The immune system has three basic levels of defence: physical barriers; the innate immune system; and the adaptive immune system (Delves et al., 2011). These are discussed below.

1.2.2 Physical barriers

The body is covered with largely impenetrable physical barriers which provide protection against infectious agents trying to gain entry to the host (Delves et al., 2011). Skin provides the most obvious of these barriers, but it is the mucosal secretions which inhabit the respiratory, digestive, and reproductive tracts which trap and work to expel (via coughing and sneezing, and the washing action of tears, saliva, and urine) any infectious agents that enter via these passageways (Delves et al., 2011). More specifically, this response is referred to as *mucosal immunity*.

The mucosal immune system defends against pathogens which enter the host via the gut, mouth, eyes, respiratory system and reproductive tract. Like physical barriers, the mucosal immune system provides the first line of defence against infection, but it combines functions of both the innate and adaptive immune systems to eradicate invading pathogens (Gleeson, 2006*b*). For the mucosal immune system to function effectively, it is reliant upon the production and presence of antibodies which perform immune exclusion; and of immunosuppressive mechanisms involved in the dampening of immune responses to avoid hypersensitivity to exogenous proteins, such as those from food (Delves et al., 2011). The immune system of the gut is arguably the most complex and extensive, with the intestinal mucosa containing approximately 80% of the body's activated B cells. This results in a large production of antibodies, specifically IgA (Gleeson, 2006*b*). B-cells in the upper respiratory tract also secrete IgA into the surrounding saliva, and it is this secretory specific protein which is of particular interest to sport and exercise immunologists.

In addition to SIgA, saliva also contains two additional enzymes, and one additional protein which are of interest to sport and exercise immunologists: amylase, lysozyme, and lactoferrin. Amylase is an enzyme which helps to digest carbohydrates and simple starches but its role within the URT is to help prevent the attachment of bacteria to the epithelial wall (Gleeson, 2006*b*). Lysozyme is a powerful enzyme with the capacity to break down the cell walls of bacteria (Gleeson, 2006*b*). Lactoferrin is a protein also involved in the lysing of bacterial cell walls but, in addition, also has an ability to bind to lipoproteins of cell membranes likely to be targeted by virus particles, thus prohibiting the virus from binding. It also has the capacity to bind to viral particles directly, preventing their attachment to the cell's lipoproteins (Delves et al., 2011).

1.2.3 Innate Immunity

The innate immune system provides the first line of defence against pathogens entering the body. It responds quickly, but lacks specificity (Davison & Simpson, 2011). It is, however, very similar between individuals. Physical barriers, such as the skin and mucous linings of the gut and respiratory system form the body's primary defence mechanisms, restricting the entry of pathogens into the body (Delves et al., 2011). If a pathogen manages to successfully breach a physical barrier and penetrate the body, the innate immune system relies upon soluble factors (which are bactericidal, e.g. the complement system); the mechanism of phagocytosis, undertaken by macrophages and neutrophils; and the lysing of infected host cells by NK cells (Murphy, 2012).

The complement system comprises over 30 plasma proteins which normally circulate in a dormant state (Murphy, 2012). These proteins have a binding capacity, which are almost exclusively designed to target microbial polysaccharides typically found on bacterial membranes (Delves et al., 2011). The by-products produced as a result of complement activation can also act as chemokines (signalling molecules) for phagocytic cells (Gleeson, 2006*b*). This binding action, and the resulting chemotactic factors, greatly increase the chances of invading bacteria being ingested by phagocytes (Delves et al., 2011).

Phagocytes (macrophages, monocytes, dendritic cells, and neutrophils) engulf and ingest invading pathogens (Gleeson, 2006*b*). Once pathogens are ingested by the phagocyte, granules within the phagocyte discharge their contents onto the trapped pathogen. It's the combination of this microbicidal mechanism, and the oxidative burst, which occurs simultaneously to destroy the cell's target (Murphy, 2012).

Neutrophils are the most numerous granulocyte in the immune system and are also one of the most dominant leucocytes in the bloodstream (Gleeson, 2006*b*). They are the first cells recruited, and involved in the fight against infection by the innate immune system (Murphy, 2012). Neutrophils are full of granules that contain digestive enzymes (including elastase). The neutrophil ingests the pathogen, and the granules fuse to, and release their enzymes onto their target. The result of this is called degranulation (Gleeson, 2006*b*) and bacterially stimulated neutrophil elastase can be analysed as a measure of neutrophil function.

If an invading pathogen is not intercepted and eliminated by the innate immune system, then the adaptive immune system steps up its fight against infection.

1.2.4 Adaptive Immunity

Adaptive immunity has two distinctive arms: cell-mediated, and humoral immunity. Cell-mediated responses (type 1) are triggered by intracellular pathogens (such as viruses), and rely upon the differentiation of T-lymphocytes (specifically CD4+ and CD8+ T-lymphocytes) into either T-helper cells, or T-suppressor/cytoxic cells type 1 lymphocytes (Th1/Tc1), characterised by the production of interferon (IFN)-γ and interleukin (IL)-2; whereas humoral responses (type 2) are triggered by extracellular pathogens and rely upon the differentiation of T-lymphocytes (specifically CD4+ and CD8+ T-lymphocytes) into either T-helper cells, or T-suppressor/cytoxic cells type 2 lymphocytes (Th2/Tc2), characterised by the production of IL-4, IL-5, IL-10, and IL-13 (Davison et al., 2014).

T and B cells both start out as circulating immature lymphocytes in the blood stream. These immature lymphocytes then mature in either the thymus, where they become T-lymphocytes, or the bone marrow, where they become B-lymphocytes (Delves et al., 2011). Once matured, naïve T and B cells re-enter the blood stream where they disseminate and circulate throughout the blood, spleen, lymph nodes, and mucosal lymphoid tissue, waiting for adaptive immune responses to be initiated.

When antigen presenting cells (APCs) reach the spleen, lymph nodes or mucosal lymphatic tissue, cytokines are released from the APC which stimulate the naïve T cells. This results in the proliferation and differentiation of these T-cells into their final, functional form – the specific cytokine released from the antigen presenting cells will dictate the final differentiation of the T-cell into either Th1, Th2, Th17, or Treg cells (Delves et al., 2011; Murphy, 2012). The activation of these naïve T-cells is a critical, first stage of an adaptive immune response.

Antigens can enter the spleen, lymph nodes and mucosal lymphatic tissue without the need of an APC. These free antigens can stimulate the antigen-receptor B-cells but typically help is required from activated T-cells to initiate an optimal antibody response (Gleeson, 2006*b*). Antibodies are proteins which recognise specific antigens and are an essential component of the adaptive immune system (Murphy, 2012). Through the maturation

process of T and B cells, the antibodies they produce become specific against only one antigen which differs between clones of lymphocytes. After primary exposure to a novel antigen, the immune system starts to develop an immunological memory and specific antibodies against this antigen start to be produced by B-cells. Upon secondary exposure to the antigen, the number of circulating antibodies drastically increases, along with the helper and cytoxic effector capacity of the T-cells. This is how immunological memory is formed. Every time the host encounters this antigen again in the future, it will be recognised and an appropriate immune response mounted rapidly (Gleeson, 2006*b*).

1.2.5 The Acute Effects of Sport and Exercise on the Immune System

When a bout of physical activity is undertaken, the immune system mounts a response. The nature of this response, however, varies depending upon the duration and intensity of the exercise undertaken. Exercise results in an increase in oxidative stress, an increase in the release of heat shock proteins; an increase in the release of catecholamines and cortisol; an increase in IGF-1; and an increased metabolism (Walsh et al., 2011*a*). It is thought that these responses to exercise influence cell trafficking, pathogen recognition and effector functions (such as microbial killing, cytokine expression, and antigen processing), although there is very little evidence for these (Walsh et al., 2011*a*).

What has been repeatedly observed in response to an acute bout of exercise, however, is exercise-induced leucocytosis (Blannin, 2006). In the late eighties and early nineties, researchers started reporting changes in circulating leucocyte numbers in response to exercise. One of the earlier studies was undertaken by Gabriel et al. (1992) who investigated leucocyte responses to a 60 second all out maximal sprint on a cycle ergometer. Total leucocytes increased immediately post-exercise, decreased between 15 and 30 minutes post-exercise, increased again, 1-2 hours post-exercise, and had returned to normal within 24 hours. However, not all leucocyte sub populations responded in the same way. They reported increases in NK cell; cytotoxic, not MHC-restricted T-cells; and monocyte numbers circulating in the peripheral blood immediately following exercise. This was followed by the other cells in the subpopulations increasing 15 minutes following exercise. Circulating granulocyte numbers peaked both 15 minutes and 2 hours post-exercise, with the exception of eosinophils which increased 15 minutes post exercise and dropped below baseline 2 hours post-exercise. Most circulating cell numbers had returned to near baseline 24 hours post exercise, with NK cells exhibiting the greatest decrease, not

returning to baseline until 24 hours after exercise. Allsop et al. (1992) presented similar results, reporting increases in peripheral lymphocyte, monocyte and granulocyte numbers within 5 and 10 minutes post-exercise following a 4 minute supramaximal sprint on a cycle ergometer. Indeed, in response to exhaustive submaximal high intensity exercise (80% maximum workload on a cycle ergometer, Field et al., 1991; and a stepwise treadmill protocol to exhaustion, Bieger et al., 1980) a doubling of circulating leucocytes is not an uncommon observation following exercise. In Field et al.'s (1991) study, they observed that, by 1 hour-post exercise, monocytes and neutrophils had returned to baseline levels. Within the same time period however, circulating lymphocyte numbers had actually dropped below baseline levels. A large number of studies observe the biphasic lymphocytosis associated with high intensity exercise which normally occurs immediately, and 2 hours post an intensive exercise bout.

The studies mentioned above outline some of the typical leucocyte responses to short-term, high intensity exercise. But the responses observed when the exercise stimulus is more endurance based are often more pronounced (Chinda et al., 2003; Nieman et al., 1998; Robson et al., 1999; Suzuki et al., 2003). Chinda et al. (2003) investigated the effects of a competitive marathon race on neutrophil function. They reported a threefold increase in total blood leucocytes and neutrophils pre to post race. Interestingly, the increase in neutrophils was not accompanied by an increase in function. The oxidative burst and phagocytic activity of the neutrophils decreased immediately after the marathon, suggesting that the increase in the number of circulating neutrophils may actually be to compensate for the reduced functional capacity. The same year, Suzuki et al. (2003) reported a significant increase in total leucocytes, neutrophils and monocytes in response to a competitive marathon, along with significant decreases in NK cells, and eosinophils within 10 minutes of finishing the race. Robson et al. (1999) compared leucocyte responses to cycling at both 55% $\dot{V}O_2$max for 3 hours (or exhaustion) and 80% of $\dot{V}O_2$max until the work-rate could no longer be maintained. In the 80% $\dot{V}O_2$max condition, subjects fatigued at around 37 minutes. The duration and intensity of this exercise bout was enough to provoke leucocytosis, and bring about a biphasic response. Leucocytosis was much more pronounced in the condition where subjects cycled at 55% $\dot{V}O_2$max, with more than a 3 fold increase in the number of circulating WBCs. In both conditions, leucocyte numbers had returned to baseline within 24 hours. Within sport and exercise, a reduction in monocytes' capability to express TLR-4 has been noted (Oliveira & Gleeson, 2010), alongside a decrease in their antigen presenting ability following acute bouts of exercise.

In addition, chronic periods of intensified training have also resulted in decreases in monocyte numbers, alongside decreases in TNF-α, IL-6, and IL-1β. Nieman et al. (1998) reported that the phagocytic function of monocytes and granulocytes increased in response to 2.5 hours cycling at 75% $\dot{V}O_2$max. Despite these findings, any depression in monocyte function following acute bouts of exercise generally recover to baseline levels after 24 hours, thus attributing to the open-window theory (Shantsila & Lip, 2011).

The abovementioned studies have very much focused on the innate immune system's response to exercise, but acute exercise also impacts on how the adaptive immune system functions, and responds to challenge. Typically, the number of lymphocytes observed in circulation are much higher immediately post exercise, compared to baseline levels. As the immune system starts to recover, the number of circulating lymphocytes drops below baseline, before returning to normal resting levels (Bishop, 2006*a*). The magnitude of change is dependent upon the exercise intensity, and duration. Nielsen et al. (1998) reported a two- to three-fold increase in circulating lymphocytes immediately following 6 minutes of maximal ergometer rowing. A 40% drop in the number of circulating lymphocytes was observed during recovery, before a return to baseline values. Similar responses have been observed following 45 minutes of treadmill running at 80% $\dot{V}O_2$max (Nieman et al., 1994), repeated 1 min treadmill sprints to exhaustion (Gray et al., 1993), 75 minutes cycling at 75% $\dot{V}O_2$max followed by an identical session 3 hours later (Ronsen et al., 2001), and following heavy resistance exercise in women (Miles et al., 2003). However, lymphocytosis has not been observed following 45 minutes of treadmill running at 50% $\dot{V}O_2$max (Nieman et al., 1994); or intermittent exercise of a moderate intensity (Bishop, 2006*a*). The lymphocytosis is very similar to the responses observed in studies above focused on the innate immune system, however these responses influence how the adaptive immune system responds as well. T and B cells are subpopulations of lymphocytes and therefore any decrease in lymphocytes from resting levels (as observed in the recovery phase of studies above) may affect the host's ability to respond in a spontaneous manner to invading pathogens.

T cells respond to acute exercise with a biphasic response similar to that described above (Nielsen et al., 1998; Nieman et al., 1994; Shek et al., 1995;). Shek et al. (1995) reported a 58% increase in circulating T cells following a 2 hour treadmill run at 65% $\dot{V}O_2$max. This was followed by a 42% drop below baseline just 2 hours post-exercise. B cells do not demonstrate such a strong response to prolonged aerobic exercise. Significant changes in

the number of circulating B cells were not noted following 45 minutes of treadmill running at 85%, or 50% $\dot{V}O_2$max (although the magnitude of change was greater following exercise at 85% $\dot{V}O_2$max) (Nieman et al., 1994), or 30 minutes of treadmill running at 65% $\dot{V}O_2$max (Shek et al., 1995). However, greater magnitudes of change in the proliferation of circulating B cells have been reported immediately following high intensity exercise such as 6 minutes maximal rowing (Nielsen et al, 1998) and strenuous resistance exercise (Miles et al., 2003).

Acute exercise typically results in an increase in circulating cytokines, most markedly IL-6. Nehlsen-Cannarella et al. (1997) reported a 753% increase in circulating plasma IL-6 following a 2.5 hour run at ~75-80% $\dot{V}O_2$max. Chan et al. (2004) and Nieman et al. (2003) however, set out to establish what, if any, other cytokines presented a similar response to exercise as IL-6. In skeletal muscle, they reported that IL-1β, IL-6, IL-8, IL-15, TNF-α, IL-12p35 and IFN-γ mRNA are detectable at rest whereas IL-1α, IL-2, IL-4, IL-5, IL-10, and IL-12p40 mRNA remain undetectable. Following exercise, both studies reported that IL-6 and IL-8 increased (Chan et al, 2004; Nieman et al., 2003). Nieman et al. (2003) also reported a post-exercise increase in IL-1β, although this is most likely attributable to the differing protocols (1 hour cycling (Chan et al., 2004) vs. 3 hours running (Nieman et al., 2003). Studies have gone on to demonstrate that the systemic release of IL-6 from the muscle during exercise is mostly responsible for the increase in plasma IL-6, and that the exercise duration is closely intertwined in this relationship (Steensberg et al., 2000).

It is not only haematological markers that are influenced by acute exercise. High intensity exercise typically elicits a decrease in saliva SIgA immediately following exercise, with levels returning to, or near baseline within an hour (Gleeson & Pyne, 2000) although it is not uncommon for studies to report no change, or even increases in SIgA. There is a lot of variation in how SIgA is reported. Secretion rate is generally considered as the most accurate way to express SIgA, as it takes into account both SIgA concentration, and saliva flow rate. Studies that report SIgA using alternative expressions (i.e. concentration only; SIgA in relation to total protein ratio; SIgA in relation to albumin, etc) should, therefore, be interpreted with caution (Blannin et al., 1998). Acute decreases in SIgA have been observed following acute bouts of prolonged exercise. Nieman et al. (2002) reported a 25% decrease in SIgA 1.5 hours following a competitive marathon. Nehlsen-Cannarella et

al. (2000) reported a 20% decrease in SIgA following a 2 hour training session completed by elite female rowers.

There are also problems associated with defining exercise intensity by a percentage of $\dot{V}O_2$max which should be considered when discussing acute exercise responses. The limitations of this methodology should, therefore, be taken into consideration when reviewing sport and exercise immunology research. According to Neiman's (1994*a*) "J" Curve (figure 1.1) it is athletes whose training is at a high intensity or above who are more susceptible to developing URTIs. But what counts as high intensity exercise? Katch et al. (1978) highlighted the fact that, although a correlation exists between percent max heart rate (HR) values and percent $\dot{V}O_2$max, there was a lot of variability as to where athletes' anaerobic thresholds (AT) fell at each intensity. For example, when thirty-one athletes exercised at 80% max HR (62% $\dot{V}O_2$max) seventeen athletes were working at or above their AT (Katch et al., 1978). Coyle et al., (1988) later went on and reported that in a group of well-trained cyclists, the percent $\dot{V}O_2$max at which AT occurred ranged from 59.1% to 86.0% thus confirming the findings previously reported by Katch et al. (1978) that there is a great degree of variability in where the AT occurs even in athletes whose $\dot{V}O_2$max values are the same. Indeed, in terms of endurance performance, Coyle et al. (1988) reported that at 88% $\dot{V}O_2$max, the group with a higher lactate threshold (LT) were exercising 8% above their LT while those with a lower LT were exercising 34% above their LT. At 88% $\dot{V}O_2$max, time to exhaustion (TTE) ranged from 29.1 ± 5.0 min in subjects with a low LT Vs. 60.8 ± 3.1 min in subjects with a high LT. Indeed, the researchers also observed, in some instances, a twofold difference in blood lactate concentrations, glycogen utilisation, and time to fatigue between subjects sharing a similar $\dot{V}O_2$max but different LT. The physiological stress of athletes working above their AT would be very different from those working below their AT and this brings into question the validity of using percent HR or percent $\dot{V}O_2$max values to set intensity. Lansley et al. (2011) reported a method for standardising exercise intensity which took into account both $\dot{V}O_2$max and the gas exchange or first ventilatory threshold (VT1), synonymous with AT, and devised the delta (Δ) concept. Δ is defined as the range between an athlete's VT1 and $\dot{V}O_2$max and, by prescribing work rate as a % Δ as opposed to % $\dot{V}O_2$max, helping to ensure that there is consistency in the prescribed work rate between participants when exercise at a heavy intensity or above is required.

To standardise the work rate further, critical power (CP), which falls above the VT1 but below $\dot{V}O_2$max could also be considered. Moderate exercise is classified as an intensity which falls below the VT1 (Wasserman et al., 1973); heavy exercise is exercise at an intensity above the VT1 but below CP (Poole et al., 1990; Poole et al., 1988); severe exercise is an intensity above CP at which $\dot{V}O_2$max is attained, quickly followed by fatigue (Hill et al., 2002; Poole et al., 1990; and Poole et al., 1988); and extreme exercise is at an intensity greater than severe during which individuals fails to attain $\dot{V}O_2$max before exhaustion (Hill et al., 2002). Thus, in order to ensure athletes are exercising at the same relative intensity, the VT1, $\dot{V}O_2$max and CP should be considered when aiming for work in the heavy domain and, when severe and extreme domains are required, the ability to attain steady state VO_2 should be considered in addition to the aforementioned markers. The majority of laboratory based studies which have investigated the effects of endurance exercise on immune function to date, however, have not adopted this method when setting relative exercise intensity. There may, therefore, be inter-subjects variability with regards to relative exercise intensity within studies depending upon where subjects' VT1 and/or their CP occur and this may be reflected in their findings. Despite this, percent $\dot{V}O_2$max continues to be widely used by researchers, and therefore the immune observations made in response to acute exercise, continue to be expressed in relation to it.

In summary, as a result of an acute bout of exercise, leucocytosis is observed but the severity of the response will depend upon the exercise stimulus undertaken, albeit the intensity, duration, and type of exercise, and the subset of leucocyte under investigation. Typically, strenuous exercise in excess of 1 hour will result in an immediate increase of leucocytes (predominately neutrophils and lymphocytes). The leucocytosis tends to start recovery straight away, with neutrophilia peaking 2-3 hours post exercise. However, in cases where the exercise intensity has been sustained for a longer period, the responses may be more pronounced, and the recovery may take longer. The increased shear stress and release of catecholamines in response to acute exercise contributes to the leucocytosis that is observed in the majority of studies. Neutrophil numbers increase, but their functional capacity tends to decrease. It has been hypothesised that the increase in neutrophils are in response to their decreased capacity. The ability of neutrophils to effectively phagocyse bacteria is very much dependent on the intensity of the exercise session. Respiratory burst rate is increased following moderate intensity exercise, but the respiratory burst rate decreases when the exercise intensity is severe. The acute effects of exercise on T cell function is very much dependent upon the intensity and duration of the

exercise session undertaken, but B cell function is less affected. The functional changes affecting the innate immune system arise as a result of activation of the complement system, and an increase in circulating catecholamines, cortisol, and IL-6. SIgA is the predominate measure of mucosal immune function with a decrease in SIgA typically observed following an intensive bout of exercise.

1.2.6 The Chronic Effects of Sport and Exercise on the Immune System

As discussed above, one off, acute bouts of exercise can cause immune perturbations that are (relatively) short lived. The effects of chronic exercise (discussed below in the context of periods of intensified training and/or overtraining) can influence infection rates and bring about changes in how the immune system functions that are more pronounced than those observed following acute exercise. As part of their training, athletes regularly go through peaks in their training volume, and/or intensity, at various times over the course of their season. These peaks temporarily result in a performance dip which, when following a tapering period, results in a supercompensation and an increase in performance. The effect these peaks in training have on the immune system are also marked, with effects on both innate, and adaptive immunity reported (Gleeson & Robson-Ansley, 2006; Verde et al., 1992).

Decreased levels of SIgA are the only marker directly linked to an increased risk and/or incidence of URTIs within both athletic, and non-athletic populations (Gleeson et al., 1999*a* & 1999*b*). Gleeson et al. (1999*b*) reported that the lower the concentration of saliva SIgA, the higher the risk of infection. Through regression modelling, they also suggested that pre-season IgA concentrations in swimmers could be used to predict the number of infections reported throughout the season, with a concentration threshold of 40 $mg.L^{-1}$ identified as a useful cut-off point to identify athletes at an increased risk early on in the season. Neville et al., (2008) also collected longitudinal SIgA data from a cohort of elite athletes, this time competitors in the America's Cup yacht racers. Neville et al. (2008), like Gleeson et al. (1999*b*) reported that a drop in SIgA concentration preceded the development of upper respiratory infections (URIs). However, they did not find that a threshold of 40 $mg.L^{-1}$ was a useful predictor, owing to the variability of SIgA concentrations within their population (it should be noted that this within-subject variation is not specific to yacht racers, but elite athletes in general (Francis et al., 2005). Instead, they reported that a drop in SIgA concentration from an athlete's baseline is a more

appropriate and accurate predictor of URI risk. The group reported that a SIgA value less than 40% of their healthy baseline leaves the athlete with a forty-eight percent chance of developing an URI within 3 weeks, and a SIgA value less than 70% of their healthy baseline leaves them with a 28% chance of developing an URI within 3 weeks. It is worth noting, however, that thirty-eight percent of URIs that occurred in the cohort of athletes in the above study were not preceded by a drop in SIgA concentration below baseline, thus highlighting the multifactoral nature of the innate immune system.

Leucocyte counts do not appear to be altered in response to exercising on a regular basis (Gleeson & Bishop, 2005). However, heavy, intense training results in reduced neutrophil function (Hack et al., 1994; Pyne, et al., 1995). Hack et al. (1994) monitored 7 male long-distance runners over the course of a training year. Blood samples were collected in October or November when subjects were engaged in moderate training, and once again in July or August when subjects were engaged in intensified training ahead of competition. The researchers reported no differences in neutrophil phagocytosis at rest during moderate training compared to controls, but there was a significant decrease in ingestion capacity during intensified training (ingestion capacity at rest in controls: 0.21 ± 0.03 particles/cell; moderate training; 0.19 ± 0.07 particles/cell; and intensified training: 0.11 ± 0.02 particles/cell).

T and B cells are sensitive to training load and their functionality may decrease in well-trained athletes when their training load is intensified. It has been observed that an increase in training load results in a decrease in the number of circulating Type 1 T-cells, a reduction in the proliferative responses of T cells, and a reduction in the synthesis of Ig by stimulated B cells (Lancaster, et al., 2004; Verde et al., 1992), however Verde et al. (1992) concluded that these minor, and sometimes transient changes were of limited significance as an indicator of overall immune function, and more of a warning that the training load was becoming excessive.

Numerous studies have investigated the role cytokines play in URS risk. Genotyped athletes with an increased tendency to produce IL-6 have an increased likelihood of URTIs (≥ 3 URS episodes in 12 months), whereas genotyped athletes with a tendency to produce high levels of IL-2 have a decreased likelihood of developing URTIs (Cox et al., 2010). Gleeson et al. (2012) recently reported that increases in antigen-stimulated IL-10 production was a risk factor for the development of URTI in an athletic population. Eighty

athletes provided blood and saliva samples and kept training and illness logs for 4 months. Illness prone athletes in this group (≥ 3 weeks of URTI symptoms) had approximately 2.5 fold higher IL-4 and IL-10 by antigen stimulated whole blood than athletes who did not suffer any URTI symptoms. Illness prone athletes also reported higher training loads, and decreased saliva SIgA secretion rates.

Despite this, it is important not to ignore the influence psychological stress can have on immune function and infection risk. The role of psychological stress may be marked more so during the monitoring of athletes over a prolonged period of time compared to the acute effects of an exercise bout in isolation. Intensified periods of training normally occur in the lead up to competition which may bring anxiety, stress, and/or fatigue to the athlete, all of which can impact immunity (Perna & McDowell, 1995). However, the psychological stress that accompanies many non-athletic life events such as marriage, divorce, monetary issues, and exams, for example, may also play a more substantial role in some athletes risk of infection (Clow & Hucklebridge, 2001; Hardy, 1992).

Functional *in vitro* assays, such as lymphocyte proliferation to an mitogen have shown the cellular responses are sensitive to stress-induced alterations in function (Kiecolt-Glaser et al., 1987*a*; Kiecolt-Glaser et al., 1987*b*; Kiecolt-Glaser et al., 1993; Kiecolt-Glaser et al., 2002). The efficacy of vaccinations have also been shown to be affected by psychological stress (Burns et al., 2003*a*; Burns et al., 2003*b*; Burns et al., 2002; Kiecolt-Glaser et al., 1996). Burns et al. (2003*a*) vaccinated 31 undergraduate students with a trivalent influenza vaccine and measured psychological stressors prior to, and 5 weeks following vaccination. They reported that subjects who lacked full protection from the vaccine 5 months after vaccination (i.e. whose antibody titers for one or more strains of influenza contained in the vaccine) had reported significantly greater exposure to stressful life events and higher perceived stress scores thus indicating that psychological stress may have been detrimental to the long-term maintenance of antibody titters following vaccination. The perceived pressures experienced by adult caregivers has also been shown to affect wound-healing rates (Kiecolt-Glaser et al., 1995).

There are very few studies that demonstrate marked changes in immune function to intensified training that differ from those that occur following acute exercise. However, the risk of infection reported by athletes engaged in intensified training remains higher than those who are sedentary, or moderately active (Nieman, 1994*a*). One mechanism that

has been proposed to cause the reduction of immune function is the cumulative effect of repeated bouts of exercise. Repeated bouts of exercise result in an elevation of stress hormones (most notably glucocorticoids such as cortisol) which dampens the cell-mediated response via the temporary inhibition of Th1 cytokines (Gleeson & Robson-Ansley, 2006). Potentially, training regularly at a high intensity with insufficient recovery does not provide enough time for the immune system to recover (i.e. a prolonged open window), leaving athletes more vulnerable to infection, but more research is required in this area.

1.3 Measurement of Immune Function in Sport and Exercise

As discussed above, the immune system is expansive, recruiting a large variety of cells, tissues (e.g. lymph nodes, Peyer's patches, spleen, and liver), and proteins (Walsh et al., 2011*a*). The way in which the outcome of investigations or interventions are analysed can, therefore, have an impact on the meaningfulness of results. Some studies seek to understand the underlying mechanics of sport and exercise immunology, while others are interested in the end product of their intervention - the host's ability to successfully defend and manage responses. Sport and exercise immunology commonly report leucocyte responses; leucocyte function (i.e. neutrophil function or cytokine production); and salivary Ig concentrations, but there are a variety of methods which can be selected. A selection of these are discussed below.

1.3.1 Subjective Measures

Essentially, the end point for sport and exercise immunology from a practical perspective, is a reduction in the number of URTIs athletes suffer. When undertaking intervention studies, therefore, it can be useful to collect data on URS or illnesses alongside any objective measures collected. Studies have employed various collection methods including self-reporting daily questionnaires (Fricker et al., 2005; Heath et al., 1991; Klentrou et al., 2002; Nieman et al., 2000; Peters et al., 2010; Whitman et al., 2006); daily logs collected by a team physicians or physiologists (Francis et al., 2005); retrospective interviews (Matthews et al., 2002; Peters & Bateman, 1983; Seyfried et al., 1985); retrospective questionnaires (Dias et al., 2011; Ekblom et al., 2006; Fahlman & Engels, 2005; Nieman et al., 1990*a*); self-reporting questionnaires in conjunction with the collection, and subsequent laboratory analysis of nasopharyngeal and throat swabs to confirm the presence of a clinical infection (Spence et al., 2007); and physician examination (Cox et al., 2008;

Pyne et al., 2000; Weidner & Schurr, 2003). Some studies have even gone to the length of deliberately exposing subjects to a low-infectious dose of respiratory illnesses or vaccines and monitoring illness responses (Bruunsgaard et al., 1997; Cohen et al., 1991; Weidner et al., 1998).

Self-reported questionnaires are routinely employed by a large number of studies as a way in which to monitor or record the incidence and severity of URTIs and URS within athletes. Data gathered from validated questionnaires allows for large cohorts of participants to be surveyed, and data collected on immune status and training history. The data collected from self-reported questionnaires is very subjective, however, and may lead to the over reporting of symptoms. In addition, a large percentage of athletes' URS can be attributed to causes such as asthma and/or airway irritation caused by allergens, pollution, or breathing in cold air. The number of URTIs reported by this method may, therefore, be misleading in terms of the incidence of clinical infections caused by pathogens but, with regard to the effect symptoms have on training and competition, the effects of URTI symptoms may cause problems (e.g. disrupted training), regardless of their cause. Retrospective questionnaires appear to be the easiest method of URS data collection, however they are not without their own limitations. Some studies have used one questionnaire to collect URS data for periods up to two months (Nieman et al., 1990). In these cases, the accuracy of the data reported should be questioned. However, when data is collected weekly, as in studies such as those by Dias et al. (2011), Fahlman & Engels (2005), and Gleeson et al. (2011), the risk of inaccuracies in the data is reduced. A further limitation with self-reported questionnaires, which spans both retrospective and daily collection intervals, relates to the subjective nature of the data reported. Often, subjects are asked to rank the severity of their symptoms. The severity of any reported symptom is very often included in the analysis of diaries and will help inform researchers as to whether the symptoms reported should be included as a URTI episode. One subject's interpretation of a severe symptom, however, may be very different to that of a subject in the same study. In addition, there is scope for a symptom to be unrelated to a URTI and actually to be caused by pollutants or allergens. Despite this, the etiology of common colds and/or the presence of a URTIs are rarely clinically confirmed in studies. Studies which have used clinical measures (such as antigen detection, serology and isolation; reverse transcription-PCR; and bacterial cultures and antibody assays) have managed to identify viruses and/or bacteria in approximately seventy percent of cold patients (Makela et al., 1998).

Spence et al. (2007) confirmed, through laboratory diagnosis, that only a small percentage (~30%) of illnesses reported were caused by either viral or bacterial pathogens, with rhinovirus being identified most commonly. This finding was supported by Cox et al. (2008) a year later. In the study by Cox et al. (2008), seventy elite-level athletes complaining of URS were assessed by a qualified physician for the presence of an URTI. In addition to the physician's assessment, athletes had both oropharyngeal throat swabs and blood samples taken for laboratory assessment. The physician deemed eighty-nine percent of cases to be an URTI caused by viral or bacterial infections. Laboratory analyses, however, confirmed that only thirty percent of athletes were suffering with an infection (based on the presence of an identifiable viral or bacterial pathogen). A further twenty-seven percent of athletes' blood work suggested they were suffering with an infection, but no known pathogens could be detected. With hundreds of pathogens known to cause URTIs, it is impossible to screen for every single possible cause. This of course does not account for the pathogens that have not yet been identified.

There will always be problems with self-reported subjective data which is why clinical diagnosis helps objectify the data. However, where a clinical diagnosis cannot be made due to study limitations, the collection of questionnaire data daily should provide a more accurate record of URS symptoms, although the record keeping is more of a burden on study subjects. One of the numerous questionnaires designed, and validated for the collection of this data is the Jackson Score questionnaire (Jackson et al., 1958). However, the way in which results are interpreted can also effect results, and methods for assessing this have also been developed.

The findings discussed in the abovementioned studies demonstrate the need for additional diagnostic techniques to be used in conjunction with self-reported questionnaires when monitoring immune function. One way of doing this, although more costly, both in a monetary and time sense, is through the use of biological and/or biochemical markers, although these techniques are not without their own set of problems.

1.3.2 In Vitro Methods

In vitro refers to experiments performed on samples outside of the host (e.g. in a culture dish or test tube) and can provide valuable insight into functional aspects of the immune system such as neutrophil function and cytokine responses, for example. *In vitro* measures

of immune function, although not as specific or clinically relevant as *in vivo* techniques, can provide valuable mechanistic information on immune function. These techniques are conducted through the use of *in vitro* assays following *in vivo* interventions. The sensitivity of this technique is considered to be greater at detecting differences between groups compared with the abovementioned alternatives and therefore, despite not being as specific as *in vivo* methods, still play an important part in immunological studies.

There are a number of ways in which immune function is measured. In humans, these methods consist of those which consider overall immune function at a whole-body level, to those which asses immune function at a subcellular, mechanistic level. For neutrophil function (i.e. degranulation) and cytokine production, whole blood in typically used. Whole blood, as opposed to purified cell cultures, is more reflective of *in vivo* conditions, but it is worth remembering that these conditions are outside of the host and therefore the humidity, temperature, and concentration of samples during processing and incubation can impact how the cells respond (Lancaster, 2006*b*). Cells are typically incubated in a cell culture for anything from a few minutes to a few days, depending on the nature of the experiment. Any change in the aforementioned conditions may result in an over, or under emphasised response compared to what may be observed *in vivo*. In addition, the responses observed will remain to be dynamic (i.e. the cells still function) and there may be a need therefore for sampling of the culture to happen at multiple time points as opposed to one fixed point in time (Lancaster, 2006*b*).

The results from *in vitro* experiments can provide insightful information on immune responses to an exercise intervention. As discussed above, neutrophils have a strong response to exercise and therefore the measurement of their function is considered to be of value. Stimulatory agents such as lipopolysaccharide (LPS) activate neutrophils, resulting in oxidative burst activity and degranulation which can be measured using flow cytometry, or with a subsequent enzyme-linked immunosorbant assay (ELISA) (Delves et al., 2011). Whole blood samples are typically incubated for an hour at 37°c with a stimulant containing LPS, and gently inverted at regular time points during incubation. Sport and exercise immunology research rarely reports neutrophil function in isolation, however (e.g. Robson et al., 1999), and there is no categorical link between disturbances in neutrophil function within a healthy adult population and the ability to effectively fight infection (Albers et al., 2005).

One popular *in vitro* technique routinely employed is the measure of cytokine production by blood leucocytes (the effects of exercise on cytokine production are discussed in section 1.2.5. Cells (either whole blood, peripheral blood mononuclear cells (PMBC), or purified cell isolations) are most commonly stimulated with an antigenic substances such as a vaccine (Gleeson et al., 2012) which predominately stimulates lymphocytes (Lancaster, 2006*a*), or bacterial LPS (Weinstock et al., 1997) which predominately stimulates monocytes (Lancaster, 2006*a*). Studies employ various incubation times, however, and there is great variation in the array of cytokines analysed, and the downstream analysis techniques employed (Delves et al., 2011). Cytokines belonging to the interleukin class are one of the most important groupings as they provide the communication between leucocytes (Delves et al., 2011). However, other groupings (including TNF which target transformed cells with cytoxicity, or IFN which interfere with viral replication) are also of interest to sport and exercise immunologists. IL-6's production is increased when muscle glycogen is depleted (Lancaster, 2006*a*). It behaves much like a hormone, resulting in the release of glucose from the liver (Febbraio et al., 2004), and fatty acids from adipose tissue (van Hall et al., 2003). It displays one of the strongest responses to prolonged strenuous exercise out of all the cytokines (Nehlsen-Cannarella et al., 1997), which is not surprising, based on its functional role, and is therefore the most researched cytokine within sport and exercise immunology.

As discussed in section 1.2.5, Chan et al. (2004) and Nieman et al. (2003) both reported that IL-10 was undetectable at rest, whereas Gleeson et al. (2012) reported that reduced IL-10 was linked to an increased risk of infection, so how is this possible? Chan et al. (2004) and Nieman et al. (2003) analysed the cytokine mRNA present in muscle biopsy samples, whereas Gleeson et al. (2012) stimulated whole blood samples with a multi-antigen vaccine prior to analysis. The findings of these contrasting studies goes to show that the way in which samples are collected and processed, and the array of cytokines selected are important considerations for immunological research. The majority of studies today will use stimulated blood cultures which will have a downstream analysis by ELISA, or using a biochip, multiplexed array on an semi-automated immunoanalyser.

Neutrophil function alone does not provide a substantial insight into what is happening to the immune system as a whole and, in addition, it is almost impossible to screen for all cytokines during the course of a study owing to the limitations of the downstream analysis techniques (individual manual ELISAs would require a huge amount of cell culture; and

the multiplexed arrays, although requiring less sample than traditional ELISAs, are limited in the number and combination of cytokines that can be multiplexed). *In vitro* methods certainly have their place in sport and exercise immunology, but *in vivo* techniques may provide a better indicator as to how the immune system is functioning as a whole, integrated system.

1.3.3 In Vivo Methods

Because both the innate and adaptive immune systems are so closely intertwined and work so synergistically, individual immune markers may not provide a clear picture of overall immune function or provide indications of athletes' risk of developing infection. Within sport and exercise, a variety of subjective and *in vitro* haematological and salivary analyses are the methods most commonly employed, but this is not necessarily reflective of the measures that are considered to be the best measures of immune function. EBV reactivation, DTH responses, and vaccine responses are a few relatively novel methods which provide an overview of immune function *in vivo* which, when used in conjunction with validated salivary, haematological, and other *in vitro* methods, can provide a better method for understanding both overall immune function and the immunological mechanisms involved.

EBV is a member of the herpes simplex family of viruses and is associated most commonly with infectious mononucleosis (IM) (glandular-fever), among other diseases. It is estimated that between eighty and ninety-five percent of the adult population are EBV seropositive, with athletes demonstrating a similar (Pottgiesser et al., 2006), if not slightly increased rate of infection (Pottgiesser et al., 2012) compared to the general population. Many individuals who are seropositive come into contact with the virus at a very young age with many remaining asymptomatic throughout their adult lives. Once infected the carrier remains infected for the rest of their life. The virus primarily infects B lymphocytes and, following primary exposure, a latent infection is established in memory B cells which circulate in a benign state, hidden from the immune system as the main cytoxic T lymphocyte targets are not expressed (Chen et al., 1995; Qu & Rowe, 1992; Tierney et al., 1994). Occasionally it can infect other types of cell, including epithelial cells. The virus can reactivate and replicate in memory B cells. This results in an expression of viral DNA and infectious virus into saliva via the epithelial cells of the parotid glands located either side of the oral cavity. B-cells infected with EBV are generally contained by T-cells

(Clancy et al., 2006), but periods of physical (Gleeson et al., 2002; Pottgiesser et al., 2012; Yamauchi et al., 2011) and/or psychological (Glaser et al., 1999) stress have been shown to result in the viral shedding of EBV into saliva. Clancy et al. (2006) provided the first evidence that this reactivation may be associated with a T cell defect in athletes. The study set out to establish if there was an immune defect in fatigued athletes and, if present, whether or not this deficiency could be corrected with supplementation of a probiotic (*L acidophilus*). Blood and saliva samples were collected from fatigued and non-fatigued subjects pre and post supplementation. T cell function was determined via the secretion of IFN-γ from blood CD4+ T cells, and EBV reactivation in the fatigued subjects was assessed by polymerase chain reaction (PCR) on saliva samples. The secretion of IFN-γ from CD4+ T cells was significantly lower pre-supplementation in the fatigued group compared to the healthy controls. Following 4 weeks' of supplementation with *L acidophilus*, there was a significant increase in the secretion of IFN-γ in the fatigued group which brought concentrations back within a similar range to that of the non-fatigued controls. Interestingly, EBV DNA was detected in 25% of saliva samples collected from fatigued athletes pre supplementation, compared to only 4% post supplementation. Owing to the fact that the containment of EBV in its benign form is understood to be T cell mediated (Young et al., 2007), the decrease of EBV DNA detected in saliva samples combined with the increase in IFN-γ points toward a relationship between T cell function and EBV expression in athletes.

Reactivation of EBV does not necessarily mean the carrier will display symptoms typically associated with EBV (e.g. IM), however. Indeed many of those in whom viral-shedding occurs remain asymptomatic (Crucian et al., 2009) and some athletes carrying EBV may in fact suffer fewer URTI episodes compared to their seronegative counterparts (He et al., 2013*b*). EBV's reactivation can, however, provide information on the carrier's immune status. The reactivation of EBV suggests that, for whatever reason, immune function may have been depressed and, it has been postulated in some cases, that it may contribute to some, but not all cases of URS (Walsh et al., 2011*a*).

Gleeson et al. (2002) monitored the relationship between URS and EBV reactivation within a group of 14 elite swimmers engaged in a 30 day, intensified training period.. They reported that athletes who were seropositive for EBV were significantly more likely to suffer with URS during intensive training. The detection of EBV DNA in saliva was also linked to the onset of URS. It is not yet fully understood, however, whether the

shedding of the latent virus contributes to the development of URS, or if it is an unrelated result of the immune system being stressed. A relationship between EBV infection (or rather reactivation/shedding) and the development of URS has been observed in some studies. The detection of viral DNA in saliva, therefore, is considered to be a good measure of overall, *in vivo* immune function in subjects who are seropositive to EBV.

An alternative *in vivo* method which assesses integrated responses of immune function is the monitoring of DTH responses. DTH works though the intracutaneous application of an antigen, or allergen which then provokes a T cell-mediated immune response at a local, dermal level. Along similar lines is contact hypersensitivity, which uses a stimulus applied at a cutaneous level provoking a hypersensitivity response. DTH responses (generally skin fold thickness and/or erythema readings) are measured 24-48 hours after application which reflects the integrated outcome of a cell-mediated immune response.

Upon initial sensitisation, a hapten from the allergen binds to a carrier protein and is absorbed by a macrophage. The macrophage processes the hapten, and produces a class II MHC which then attract naïve T-cells. These T-cells bind with the class II MHC, activates, and the number of T-cells (Th1 Cells) then increase over the next few weeks. Upon secondary and subsequent exposure, the allergen is recognised by the body, and thus Th1 cells are activated which then release cytokines and recruit macrophages into action. It is the activation of these macrophages which result in the inflammation observed at the site of subsequent exposure and allow for the intensity of the immune response to be measured (Delves et al., 2011).

A relationship between DTH and URI was reported by Zaman et al. (1997). Zaman et al. (1997) studied 512 rural Bangladeshi children aged 0-59 months for one year and recorded instances of URI and DTH responses to the CMI Multitest® at regular intervals throughout the study. They reported a relationship between DTH responses and URI with children displaying anergic responses to the CMI Multitest® being 20% more likely to suffer with an URI compared with those whose responses suggested that they were immunocompetent.

Despite being considered to be a good measure of *in vivo* cell-mediated immune response, the application of DTH has been thwarted, somewhat, as a result of the CMI Multitest® no longer being available commercially. In addition to this, the antigens used by the CMI Multitest® are no longer novel and this means that some people may have been exposed to

these antigens in the past in various doses, and at various points in time making variation in responses more likely between individuals. This means that when monitoring the immune system's response to the CMI Multitest®, standardisation is difficult and more variation is likely to be observed between individuals. Researchers have, however, started to explore the use of alternative methods and techniques. Sleijffers et al. (2001), for example, measured contact hypersensitivity responses to diphenylcyclopropenone (DPCP) following cutaneous exposure and recorded primary allergic reactions 14 days after primary exposure, and elicitation responses 48 and 96 hours after secondary exposure which occurred 4 weeks after primary exposure. The purpose of their study was to assess the effect of ultraviolet B (UVB) radiation on immune responses following vaccination with hepatitis B, and subjects were assigned to either an experimental UVB exposure, or a control group. Although no changes were reported in humoral or cellular immune responses, contact hypersensitivity responses were suppressed in the experimental group, thus demonstrating that *in vivo* studies that measure immune function at a whole organism level can detect immunosupression where cellular measures may not. Only a handful of studies to date have used contact hypersensitivity (CHS) and DPCP within an exercise setting.

Harper-Smith et al. (2011) recently used contact sensitisation and DTH responses to DPCP within an exercise setting. 32 males were assigned to either an experimental group, or a control group. The experimental group exercised for 2 hours at 60% of their $\dot{V}O_2$peak. The control group remained seated in the laboratory for 2 hours. Primary exposure to DPCP occurred 20 minutes after the end of the trial and, as in Sleijffers et al.'s study (2001), primary allergic reactions were measured 14 days after primary exposure and participants were exposed to a secondary dose at 5 different concentrations 4 weeks post-initial exposure. Skin fold thickness (SFT) and erythema readings were taken at each site and significant differences with reported between groups, with the exercise group displaying a lesser immune response. This suggests that the exercise imposed on the experimental group provoked a depression in immune function post-exercise, thus diminishing the immune system's capability to tackle a novel antigen or allergen (in this case, the allergen DPCP) and develop immune memory. The diminished responses noted 4 weeks later upon secondary exposure demonstrate that the cell-mediated immune responses in the experimental group were less than that of the control group. Harper Smith et al. (2011) went on to further back up these findings by undertaking a pilot study following on from the original trial in which 13 males from the original study were

systematically exposed to DPCP until they demonstrated a plateau response to recall challenges (typically achieved after the third recall challenge) (Friedmann, 2006; Friedmann, 2007; Friedmann et al., 1983; Friedmann & Pickard, 2010). This pilot study followed the same protocol as the first study, but used a within group crossover design with 4 weeks between experimental conditions. Both SFT and erythema readings were, once again, significantly lower following the exercise intervention. The findings of the study above demonstrate that DTH responses are a suitable measure of cell mediated immune responses to recall challenged in studies with either between, or within group designs.

Following the same methodology as Harper-Smith et al. (2011) regarding sensitisation and elicitation to DPCP, Davison et al. (2016) and Jones et al. (2017) have both reported success using DPCP as an effective model of *in vivo* immunity. Davison et al. (2016) undertook the first *in vivo* immune study using DPCP alongside a nutritional intervention. They reported significant differences in DTH responses between exercising and non-exercising controls, but not between the exercising groups supplemented with carbohydrate or placebo. Jones et al. (2017) reported no overall differences in DTH responses to bovine colostrum supplementation following exercise. However, following examination of the dose response curves for each conditions (bovine colostrum or placebo) they discovered that the dose of DPCP required to bring about an immune response was lower in the bovine colostrum group compared to placebo, indicating that CHS sensitivity is related closely to host defence.

Although the majority of the population will not have been exposed to DPCP (it is commonly used in the treatment of alopecia; Buckley & du Vivier, 2001) and this may raise cause for concern, this particular method is an attractive one as it provides both control over sensitisation and the elicitation phase of the DTH response. All studies to date have relied upon sub-toxic doses of DPCP (generally in concentrations between 0.002% and 0.032%) and only one of the abovementioned studies reported any systemic reactions which affected only one participant.

The host's ability to cope with, and the host's response following exposure to a pathogen (i.e. mortality and morbidity rates) are considered to provide the most clinically relevant indication of the host's ability to cope with common pathogens (Albers et al., 2005 & 2013). In an ideal world, natural exposure to pathogens would provide the most accurate

picture of 'normal' exposure patterns and responses but this would be both impossible to control, and extremely unpredictable making it difficult to employ to clinical studies, and therefore an unsuitable method to implement in studies where the mechanisms need to be considered and/or assessed. It is possible, however, to expose subjects to vaccines which contain either inactivated or attenuated micro-organisms which trigger an *in-vivo* immune response. Information can then be gathered through the monitoring of these responses as to how subjects respond to "model infections". Different types of vaccines will provoke different immune responses (Murphy, 2012). Proteins based vaccines, for example, are T-cell dependent and can be used to initiate and measure immune memory responses. Polysaccharide vaccines, however, initiate T cell-independent responses and increase transient responses of IgM and IgG2 but neither induces the development of immunological memory, nor provokes an increase in antigen titres even with repeated exposures (Delves et al., 2011). The conjugation of a polysaccharide based antigen to a carrier protein (which acts as a T-cell epitope), however, has been shown to convert the mechanisms from that of a T-independent antigen to a T-dependent antigen. As a result, researchers can target specific immune responses based on the type of vaccine administered.

Primary exposure to restricted use vaccines (such as Hepatitis B) elicit primary T cell-dependent responses. Secondary, or subsequent exposure to vaccines of frequently occurring infections (such as influenza) can be used to initiate recall memory responses, with the exception of polysaccharide antigens (such as pneumococcal vaccinations) which do not provoke a secondary immune response due to nature in which they are processed at the point of primary exposure (Cohen et al., 2001).

Stress can have an impact on how well the host responds to vaccination. Long-term, following vaccination, stress can reduce the size of the antigen specific Th2 lymphocyte pool, reducing the speed and magnitude of a response following secondary exposure. Long-term, circulating number of antigen specific, serum IgG can be reduced (Burns et al., 2003*b*). However, the efficacy of a vaccination can be influenced by stress at the point of administration. If subjects are experiencing stress when the vaccine is administered, the clonal expansion and maturation of T-lymphocytes to Th2 primed effector, and memory lymphocytes can be reduced, as can the initial clonal expansion of B lymphocytes and the lymphoid tissue's ability to produce IgM from short-lived plasma cells (Burns et al., 2003*b*). A large number of studies have investigated the effects of psychological, or

psychosocial stress on vaccination responses (Burns et al., 2003*a*; Burns et al., 2003*b*; Burns et al., 2002; Burns & Gallagher, 2010), but the majority of studies that have used exercise alongside vaccination have been researching the potential to use exercise as an adjunct to vaccination within at risk populations (Bachi, et al., 2013; Grant et al., 2008; Keylock et al., 2007; Kohut et al., 2002). However, vaccinations are used as a model in sport and exercise immunological research (Campbell et al., 2010) but are not without limitation.

The use of vaccinations within sport and exercise research, usually relies upon antigens such as influenza or tetanus. The problem with these antigens is that the majority of the population will have experienced these antigens before through day to day contact with circulating pathogens, or through vaccination (Campbell et al., 2010; Edwards et al., 2010; Edwards et al., 2007). This results in a mixture of primary, secondary, and tertiary antibody responses, from which it is very difficult to: measure IgM (i.e. group changes in isotopes) as very little IgM is expressed in secondary and tertiary responses; compare concentrations of antigen specific IgG as secondary and tertiary exposure will typically result in increased IgG; and, investigate the underlying mechanisms of any responses observed as primary, secondary, and tertiary exposure will result in different signalling pathways being activated (Walsh et al., 2011*a*).

Despite these limitations, Bruunsgaard et al. 1997 successfully used an *in vivo* vaccination model in their study investigating the effects of a training competition (3 km swim, 130 km biking, and 21 km running) on 22 male triathletes on vaccination responses to a pneumococcal polysaccharide vaccine (T-cell independent) and two toxoids (tetanus and diphtheritis) (T cell dependent). However, they did not identify any differences in vaccine responses between the exercising subjects and controls, despite the fact that the exercise group displayed a lower skin test response to a skin test (Multitest). The authors concluded that cell mediated immunity was impaired following the training competition, whereas antibody production was not affected. However, subjects may have previously come into contact with the antigens and toxoids administered via vaccination, and therefore the limitations with the technique (discussed above) may have influenced results.

It is a combination of techniques that provide the most comprehensive overview of immune function, and therefore the studies in this thesis have, where possible, used a combination of subjective, *in vitro* and *in vivo* research techniques.

1.4 The use of Supplements and Nutraceuticals

There are a growing number of herbs and botanicals used to boost immune function, but only a few, predominantly echinacea and sambucol, have made the cross over from traditional supplements, to something used by a growing number of athletes. A growing number of athletes report using nutraceuticals as part of their training strategies. Indeed, one study which assessed the use of supplements in elite swimmers reported that 98% of athletes used at least one supplement during training or competition (Baylis et al., 2001). This figure is rather high compared to the majority of research in the area, however, with most studies reporting values of between fifty and seventy percent (Herbold et al., 2004; Krumbach et al., 1999; Krushkall & Johnson, 2001; Nieper, 2005; Slater et al., 2003; Ziegler et al., 2003), and this may be due to the fact that a large number of studies have taken place in young and collegiate athletes as the percentage of athletes using supplements tends to be higher among elite athletes (Sobal & Marquart, 1994). Female athletes are more likely to use supplements than males (Krumbach et al., 1999; Nieper, 2005; Slater et al., 2003; Ziegler et al., 2003). The maintenance of good health (Froiland et al., 2004; Herbold et al., 2004; Ziegler et al., 2003), and immune function (Froiland et al., 2004; Krumbach et al., 1999; Nieper, 2005; Ziegler et al., 2003) are the most common reasons athletes use supplements. Multivitamins are the most commonly used supplement taken on a regular basis (Baylis et al., 2001; Nieper, 2005; Ziegler et al., 2003), and up to 61% of athletes reported using herbal or botanical supplements all, or some of the time (Baylis et al., 2001; Froiland et al., 2004; Herbold et al., 2004). The nutraceuticals market as a whole was valued at $142.1billion globally in 2011, with this figure expected to reach $204.8billion by 2017 (Transparency Market Research, 2017).

1.5 Thesis aims and objectives

The studies contained within this thesis investigate the immune responses to various training, and nutritional interventions. A variety of *in vitro* and *in vivo* immune techniques are used, and it is hoped the findings of these studies will build upon existing research in the field.

Specifically, this thesis aims to further explore the use of salivary EBV DNA as a marker of *in vivo* immunity within sport and exercise through the investigation of its responses to

acute exercise, and the relationship it has with SIgA, and the incidence of URTI. The time sequence for the onset of some episodes of URTI and the involvement of EBV has been reported previously by Gleeson et al. (2002). Ahead of some episodes of URTI in elite swimmers, Gleeson et al. (2002) observed a suppression of SIgA, followed by a detection of EBV-DNA in saliva, ahead of the appearance of URS. Yamauchi et al. (2011) also reported that, within rugby-football players engaged in a month long training camp, URS increased along with a decrease in SIgA levels and the reactivation of EBV. This followed the same time-sequence which had previously been reported by Gleeson et al. (2002). The findings of these studies suggest that the viral shedding from a reactivated EBV infection may be linked to both the development of URTI and the subsequent mucosal immune response (e.g. a decrease in SIgA). It is acknowledged, however, that the detection of salivary EBV-DNA in the abovementioned studies may have been a reflection of the subclinical immune dysregulation associated with intensive training and not directly involved in the onset of URS. Based on research published to date, we hypothesised that the concentration of EBV DNA detected in saliva following prolonged exercise would increase, owing to the immune-modulation that occurs following exercise, however we also hypothesised that the nutritional interventions built into the studies contained in chapters 4, 5, and 6 would alter the amount of viral DNA shed by EBV into saliva either by reducing the amount of amount of EBV DNA shed into saliva, or by depressing any exercise-induced increases in the concentration of EBV DNA. We further hypothesised that a rise in salivary EBV DNA concentration would be associated with an increased incidence of URTI and be linked to changes in SIgA (e.g. decreases in SIgA secretion rate). Chapter 3 of this thesis details the design of 2 assays for the detection of EBV DNA in saliva. One of these assays is used in chapters 4, 5, and 6 to investigate the response of EBV to exercise. Chapter 4 aims to investigate the utility of EBV DNA as a marker of *in vivo* immunity within the context of acute exercise, by comparing responses with the well established marker of *in vivo* immunity, contact hypersensitivity. In addition, the chapter aims to investigate the role of acute carbohydrate (CHO) supplementation on the expression of EBV DNA in saliva following exercise. We hypothesised that there would be a relationship between the strength of the *in vivo* immune response to DPCP, and the concentration of salivary EBV DNA, and further hypothesised that CHO would blunt any increases in the concentration of EBV-DNA following exercise. Chapters 5 and 6 aim to investigate the influence of *Chlorella pyrenoidosa* (CHL) supplementation on immune responses to 2 days of intensified training. Research to date has shown that CHL has the potential to attenuate decreases in SIgA responses to exercise (Otsuki et al., 2011 & 2012)

and has shown promising potential in *in vitro* immuno-modulatory studies (e.g. Ewart et al., 2007; Kwak et al., 2012; Pugh et al., 2001). Based on these studies, we hypothesised that supplementation with CHL would prevent the increased shedding of EBV DNA into saliva that we expected to observe following exercise. We also expected CHL supplementation to attenuate SIgA responses to exercise, and result in fewer incidences of URTI. Within the general discussion, the data from all eligible studies are combined to explore the relationships between EBV DNA, URTI and the mucosal immune responses to exercise, as well as the potential of salivary EBV DNA to be used as a marker of *in vivo* immune function following acute exercise.

Chapter 2

General Methods

2.1 URTI Questionnaires

URTI data was collected using validated daily health questionnaires (Jackson et al., 1958). These questionnaires asked subjects to record data regarding the symptoms of illness (presence and severity), visits to the doctor, and use of medications (un-prescribed or prescribed). Participants were asked to report on any of the following symptoms associated with a URTI (sore throat, catarrh in the throat, runny nose, cough, repetitive sneezing, fever, persistent muscle soreness, joint aches and pains, weakness or fatigue, and headache), symptoms which are associated with gastrointestinal upset (loss of appetite, stomach upset, vomiting, abdominal pain, and diarrhoea), and any loss of sleep or disruption to training (Gleeson et al., 2011). Participants were asked to rate any symptoms on a scale of light (L), moderate (M), or severe (S) which were later given a score of 1, 2, or 3 for quantitative data analysis (Gleeson et al., 2012; Fricker et al., 2005). One episode of illness was counted when a subject reported one or more symptom associated with URTI on two or more consecutive days when the severity was rated as either M or S. If symptoms of URTI were separated by two days or less, they were considered to be a recurrence or continuation of the initial episode of illness and were counted as part of the original episode (Fricker et al., 2005). When an episode of URTI was counted, the type, duration (number of days), peak severity, and total illness score (sum of duration of days × peak severity) was recorded (Fricker et al, 2005). A cumulative score was also awarded to each episode which was calculated by awarding a daily score, based on the post-hoc numerical rating of symptoms, and by adding the daily scores from all days associated in one episode together (Gleeson et al., 2012). GI upsets were counted as an episode where a participant reported one or more symptom associated with GI upset on one or more consecutive day when the severity was rated as either M or S. The same principle was applied when participants reported a loss of sleep or an inability to train with both total 'illness' scores and cumulative scores being calculated.

2.2 Saliva sample collection

Saliva samples were collected using the passive drool technique. To reduce the risk of blood contamination, prior to pre-exercise samples being collected, subjects were asked to refrain from eating or brushing their teeth for at least 1 hour, and to have avoided dental work within 24 hours of sample collection. To remove any oral debris, subjects thoroughly rinsed their mouth with water 10-15 minutes before sample collection. The delivery of

fluid boluses during exercise was timed as such to allow the last bolus to be delivered within this timeframe. Unstimulated, whole saliva samples were collected by dribbling into sterile, pre-weighed 30 mL specimen collection tubes for at least 2 minutes, or until such time as approximately 1 mL of saliva had been collected. Subjects were asked to passively drool, depositing any saliva into the collection tube while they remained seated, leaning slightly forward, chin tilted towards their chest, with minimal orofacial movement. Immediately prior to sample collection, subjects were asked to empty the oral cavity by swallowing. At this point, a stopwatch was started, and the sample collection began. Following collection, the sample collection tubes containing the samples were weighed immediately to the closest milligram, and the volume of saliva calculated (assuming saliva density to be 1 g/mL). Saliva samples were centrifuged for 5 minutes at 5 °C and 1,700 × *g* to remove debris (AccuSpin Micro 17R, Fisher Scientific, Hampton, New Hampton, U.S.A.). The supernatant was aliquotted into 1.5 mL micro centrifuge tubes and stored at -80 °C ahead of analysis.

2.3 Blood samples

Blood samples were collected from an antecubital vein into Vacutainer (Becton-Dickinson, Oxford, U.K.) tubes (containing K_3EDTA, heparin, or silicone coated tubes for the separation of serum) using standard venepuncture techniques. K_3EDTA and heparin samples were centrifuged for 10 minutes at 1,500 × *g* at 4 °C (Eppendorf 5702R, Eppendorf Hamburg, Germany). K_3EDTA and heparin samples were processed as soon as possible following collection, once analyses on the whole blood had been undertaken. Serum samples were allowed to clot at room temperature for one hour before being centrifuged. The serum and plasma samples were stored in aliquots at -80 ºC until use.

2.4 SIgA analysis

Concentration of SIgA was analysed using an in-house, sandwich ELISA method based on the work of Leicht et al. (2011).

96 well immunoplates (Nunc Immunoplate, Life Technologies) were coated with a coating buffer containing 5 $\mu g.mL^{-1}$ mouse anti-human (IgA) secretory component capture antibody in a 0.05 M $NaHCO_3/Na_2CO_3$ solution (pH 9.6), covered, and incubated overnight at 4 °C. Plates were washed 4 times (200 µl/well) with a wash buffer (PBS, 0.3

M NaCl, 0.1% Tween 20), blocked with 2% BSA (100 µl/well) (Fraction V, Sigma-Aldrich, St. Louis, Missouri, U.S.A.), covered, and incubated at room temperature for 1 hour.

Defrosted saliva samples were spun in the microcentrifuge at 1,700 × g at 4 °C for 2 minutes. Saliva samples were then diluted 750x in PBS, and working standards prepared. A top working standard of 1 µg.mL^{-1} IgA (IgA from Human Colostrum (Sigma-Aldrich, St. Louis, MO, U.S.A.) was prepared in PBS, and diluted serially to give working standards with concentrations of 1 µg.mL^{-1}, 0.5 µg.mL^{-1}, 0.25 µg.mL^{-1}, 0.125 µg.mL^{-1}, 0.0625 µg.mL^{-1}, 0.03125 µg.mL^{-1}, 0.015625 µg.mL^{-1}, and 0 µg.mL^{-1} (PBS only). Plates were washed 4 times. 50 µl of standard or sample were loaded in duplicate, covered, and incubated at 4 °C overnight. The intraassay coefficient of variation (CV) = 2.5%.

Plates were washed 4 times (200 µl/well) with wash buffer and loaded with 50 µl/well of HRP conjugated polyclonal anti-IgA / PBS solution (1:2000 dilution of Polyclonal Rabbit Anti-Human IgA/HRP (Dako, Glostrup, Denmark) in PBS). Plates were covered, and incubated at room temperature for 90 minutes. Plates were washed 4 times (200 µl/well) with wash buffer and loaded with 50 µl/well of chromogenic substrate (1.25 µl of 30% H_2O_2 per 6 mL OPD substrate made from 1 OPD tablet (Dako, Glostrup, Denmark) per 3 mL dH_2O). Plates were covered, and incubated in the dark for 5 minutes. 75 µl stop solution (1M H_2SO_4) was then applied to each well and plates were read immediately at 490 nm and 630 nm on an automated absorbance plate reader (ELx808 Absorbance Reader, BioTek, Winooski, VT, U.S.A.).

Background readings at 630 nm were then subtracted from the readings at 490 nm and the mean optical density (OD) of duplicate wells calculated. A graph was then plotted with target IgA concentrations of the standards plotted on the x axis and the mean OD readings of standards on the y axis. A polynomial standard curve was fitted and the IgA concentration of samples calculated. The correct IgA concentration of samples were the calculated by multiplying sample concentration by the dilution factor (750) to give the final concentration (mg.L^{-1}). Secretion rate was calculated using the following equation:

$$sIgA\ Secretion\ Rate\ (\mu g.min^{-1}) = Flow\ rate\ (ml.min^{-1}) \times\ sIgA\ Concentration\ (mg.L^{-1})$$

The specificity (i.e. ruling out any interference from the sample matrix, and the assay's ability to only detect secretory specific IgA) of this method was validated in our laboratory. To rule out any interference from the sample matrix (i.e. whether there was anything in the sample which may interfere with the detection, result, or assay performance for the target analyte), the ELISA was validated by preparing samples and the ELISA plates as outlined in the method above. Dilution medium and saliva samples were spiked with known concentrations of IgA standard (80 - 160 $mg.L^{-1}$). Spiked and un-spiked samples were run on the plate concurrently. Un-spiked concentrations ($mg.L^{-1}$) were subtracted from the corresponding spiked sample concentration ($mg.L^{-1}$) and this was compared to the known concentration of IgA standard added to the sample prior to loading. The recovery rates were similar between the spiked diluents and saliva samples (83.8 - 82.5% for diluent and 88.6 - 100.6% for saliva samples). This indicates that the capture antibody successfully binds to the plate and that the majority of SIgA proteins added to the plate, successfully bind to these capture antibodies. A good level of recovery is present, indicating that there are no interfering substances (that affect this assay) in the saliva samples.

The specificity of the ELISA was validated through the addition of plasma IgA (non-secretory specific) into saliva diluent and samples before being loaded onto the plate. Final SIgA concentrations were not different from non-spiked samples, or affected by the addition of plasma IgA, indicating that the ELISA is specifically detecting SIgA, and not additional, non-specific proteins.

2.5 EBV Serostatus

EBV serostatus was determined by ELISA (Epstein Barr Virus (VCA) IgG ELISA; catalogue number: EIA-3475; DRG Instruments GmbH, Marburg, Germany). Serum samples were analysed in duplicate, following the test procedure recommended by the manufacturer. Standard curves and assay results were prepared in Microsoft Excel. Samples were considered seropositive when the mean absorbance value was more than 10% above the cut-off control for the respective microtiter plate. The intraassay CV = 1.2%.

2.6 Blood Cell Counts

Unless otherwise stated, Cell counts in whole blood (collected into a K_3EDTA vaccutainer) were analysed using an automated haematology analyser (HA-670 Auto-Haematology Analyser; Hawksley & Sons, Lancing, West Sussex, U.K.) which had been maintained following manufacturer guidelines.

2.7 Vitamin D

25-hydroxy vitamin-D2 and 25-hydroxy vitamin-D3 in serum were sent off for analysis by Sandwell and West Birmingham Hospitals NHS Trust. Vitamin D levels were determined using a Waters AQUITY® ultra performance liquid chromatography (UPLC) and tandem quadrupole detector mass spectrometry (TQD) system with an electro-spray ionisation interface following standardised hospital procedures. Low vitamin D status was classified as a baseline value below 50 nmol.L^{-1} for total 25-hydroxy vitamin D.

2.8 Bike Ramp Protocol

Maximal oxygen uptake ($\dot{V}O_2$max) was estimated using a ramped exercise test on a cycling ergometer (Excalibur Sport, Lode, Groningen, the Netherlands). Height and weight were recorded, and subjects were fitted with a heart rate (HR) monitor (Polar Electro, Kempele, Finland), and facemask (Cortex Biophysik, GmbH, Leipzig, Germany) connected to a breath-by-breath gas analyser (MetaLyser 3BR2, Cortex Biophysik, GmbH, Leipzig, Germany). Subjects completed 3 minutes of unloaded cycling before initiation of a continuous increment of workload by 30 W/min until volitional exhaustion. HR and subjects' rating of perceived exertion (RPE; Borg, 1970) were recorded at the end of each minute. The breath by breath analyser was calibrated prior to use according to the manufacturer's guidelines using a calibration gas of known composition and a three-litre syringe (Hans Rudolf Inc, Kansas, USA). Subjects' $\dot{V}O_2$max were estimated using the highest $\dot{V}O_2$ (l.min^{-1}) recorded using the mean of 30 seconds. Subjects' gas exchange thresholds (VT1) were estimated from the test data collected. Twenty-five percent of the difference (Δ) between the VT1 at VT1 and $\dot{V}O_2$max was calculated using a cluster of measures, including the V-slope method and the ventilatory equivalent method, and used for the prolonged endurance bout the next day (see below). The corresponding work-rate

was calculated using a regression equation with Watts plotted on the *x* axis, and VO_2 on the *y* axis. A ramp rate correction of two thirds was applied to allow for $\dot{V}O_2$ lag (i.e. -20 W).

2.9 Prolonged endurance ride (90 minutes steady state)

Subjects were fitted with a heart rate monitor (Polar Electro, Kempele, Finland) and a facemask (Cortex Biophysik GmbH, Leipzig, Germany) connected to a breath by breath gas analyser (MetaLyzer 3BR2, Cortex Biophysik GmbH, Leipzig, Germany). Subjects cycled for 90 minutes at 25% Δ, the intensity of which was validated within the first 10 minutes by monitoring $\dot{V}O_2$ responses, with the intensity (W) adjusted accordingly. $\dot{V}O_2$ was recorded at 20, 30, 60, and 90 minutes using the Douglas bag collection method detailed below. HR and RPE (Borg, 1970) were recorded every 10 minutes.

2.10 Douglas Bag Collection Method

Expired gas was collected into Douglas bags (Plysu Industrial, Ltd., Milton Keynes, UK) for 30 seconds every 20 minutes following validation of the relative intensity (see above). F_EO_2 and F_ECO_2 were analysed using a dry gas analyser (Servomex, West Sussex, UK) and volume measured using a dry gas meter (Harvard Apparatus, Kent, UK) to determine gas exchange variables as described by Hopker et al. (2012). Douglas bags were vacated of air prior to testing commencing, and the gas analyser calibrated following the manufacturer's instructions.

2.11 High Intensity Interval Exercise (HIIE) Sessions

A weighted cycle ergometer (Monark Erogomedic 874e; Monark Exercise AB, Vansbro, Sweden) was prepared and the basket loaded with 7.5% of the subject's body weight. Subjects performed a five minute warm-up at 70 rpm with the basket suspended, incorporating a five second sprint against resistance three minutes in. After two minutes' passive recovery seated on the bike, subjects performed three, thirty second sprints with ninety seconds' recovery. Subjects were allowed to bring their cadence up to 70 rpm before the basket was released. Lowest, peak, and mean power were recorded where possible (there were some technical difficulties with equipment for some of the tests, resulting in a loss of performance data).

Chapter 3

The development of PCR, and qPCR assays to detect salivary Epstein-Barr Virus DNA

Central to the techniques employed by a number of the projects featured in this thesis, was the development of a polymerase chain reaction (PCR) assay to detect EBV-DNA in saliva. After commencing the first study, access to quantitative real-time PCR (qPCR) machine became available which not only allowed for the detection of EBV-DNA in saliva, but also quantification. The data presented in this thesis, therefore, have been analysed using qPCR. However, both methods have been employed by researchers in the past and, as access to qPCR may not be available to all as it remains more expensive than traditional PCR. The development of both methods are presented hereof.

3.1 Introduction to PCR

PCR was developed in the mid 1980s by Kary Mullis (Saiki et al., 1985) who, along with Michael Smith, was awarded the 1993 Nobel Prize in Chemistry. The development of PCR has completely revolutionised the way in which microbiologists study gene function and gene sequencing, as well as the diagnosis of genetic and infectious disease. The technique allows a short region of a DNA molecule to be copied many times by a DNA polymerase enzyme, resulting in an amplification of the chosen region (National Laboratory of Enteric Pathogens, Bureau of Microbiology, Laboratory Centre for Disease Control, 1991). Generally, DNA fragments up to 10 kilobases (10 kb) can be amplified through PCR, but it is possible to use specialised protocols which allow the amplification of DNA fragments up to 40 kb.

Essentially, PCR relies on five key chemical components:

- A small amount of DNA that serves as the initial template, and contains the target sequence - PCR is very sensitive so only a small amount of target DNA needs to be added for the experiment to be successful;

- A pair of primers (oligonucleotides) that are complimentary to the 3' ends of each the sense and anti-sense stand of target DNA (i.e. each strand of the double helix);

- A DNA polymerase that will copy the template DNA (this enzyme is usually from the bacterium *Thermus aquaticus* which lives in hot springs and is thermostable, meaning it does not denature in response to heat treatment);

- Four deoxynucleotide triphosphates which are the building blocks from which the DNA polymerase synthesises new DNA strands;

- A buffer solution which provides stability of the DNA polymerase and often contains magnesium or manganese ions, and sodium or potassium ions.

These five chemical components, when mixed in an appropriate reaction volume and put through heating and cooling cycles, mimic the natural DNA replication which occurs in cells.

Amplification usually involves three main steps:

- Denaturisation, which involves the reaction mix being heated to 90-95 °C for a few seconds, allowing the hydrogen bonds between the two strands of DNA to break, separating the double helix into two single strands of DNA;

- Annealing, which involves the cooling of the reaction mix down to around 50-60 °C for around 30 - 60 seconds, allowing the primers to attach (anneal) to the target DNA templates;

- Extension, which involves the heating of the reaction mix up to 75 °C (the exact temperature will be dictated by the polymerase being used) for between 30 seconds and a few minutes (the length of time will depend upon the size of the fragment being amplified), which allows the DNA polymerase to bind with primer/template hybrid in the 5' to 3' direction, allowing a new DNA strand which is complimentary to the original DNA template being synthesised (Brown, 2010).

This three step cycle is then repeated 30-40 times, with each repeated cycle exponentially increasing the number of new copies of the DNA sequence being amplified.

It is not as simple as inserting bodily fluid or bodily tissue into a reaction, however. The quality and purity of template DNA can influence the efficacy of the PCR reaction. DNA, therefore, needs to be extracted from the target source before being incorporated into the reaction. There are many protocols published, and commercial kits available which offer extraction of DNA from different starting materials (e.g. saliva, muscle, blood, soil).

When designing a PCR, therefore, it is important that the extraction method is also carefully considered in the planning stages. PCR is, in theory, very simple to set up but the intentional, or unintentional, introduction of some chemical elements can influence the outcome significantly. The choice of buffers for DNA extraction is, therefore, extremely important.

However, it is not only chemical contaminants that can cause a PCR to fail. Foreign nucleic acids can be introduced to reactions very easily if appropriate workflow, and clean laboratory techniques are not followed. Problems can also occur if the primers are not specific for the target sequence (which can result in the formation of spurious products); if the annealing temperatures of the primers are not well matched; or if the primers bind to each other instead of the target DNA (known as primer dimer). The design of the primers is, therefore, a crucial step in any new PCR design, which is discussed in more detail below. The key stages and considerations for PCR design are discussed in further detail, before the optimisation process and methodologies of the PCR and qPCR adopted by this thesis are presented.

3.2 PCR Design

3.2.1 Primer Design

PCR experiments are reliant upon the design of the primers selected. The amplification of a single target DNA fragment within a target gene can only be achieved when primers are designed correctly. Primers should generally meet seven criteria (Brown, 2010):

- Be between 18 - 25 nucleotides long
- Have a GC content of approximately 40 - 60%
- Not contain runs of 4 or more consecutive G or C
- Contain fewer than 4 G and C in the last 5 (3') bases
- Not contain complementary sequences, in order to avoid primer-dimer
- Span or flank an intron to prevent, or at least identify, potential amplification of genomic DNA
- Not contain significant secondary structure within the amplicon or primer region

To help with achieving optimal PCR conditions, the primers should also have a similar melting temperature (T_m) (see below).

3.2.2 PCR Conditions

Each step of the PCR process (denaturisation, annealing, extension, and the number if cycles) needs to be carefully considered and tested.

Denaturation allows double stranded DNA to be broken into single stranded DNA in anticipation of the next stage of DNA synthesis in which the primers will bind to the target regions, initiating extension. In addition to facilitating the split in double stranded DNA, the high temperatures reached during this stage helps inactivate proteases and nucleases present in the sample, while protecting the DNA polymerases, which are thermostable (see below). The temperatures associated with denaturation are typically 94 - 98 °C. The temperature and time held at this step varies depending on the salt concentrations of the buffer (a high salt content will typically require a higher denaturation temperature to separate double stranded DNA), and the type of DNA being amplified (mammalian DNA is relatively complex and big which means it typically requires a longer incubation period).

After primer annealing (see below), the 3' end of the primers need to be extended, complimentary to the template. During this step, the DNA polymerase synthesises daughter strands of DNA by incorporating dNTPs through the 5' to 3' polymerase activity. The temperature of this step, typically between 70 - 75 °C, is dictated by the optimal temperature of the enzyme (e.g. taq polymerase).

3.2.3 Annealing Temperature

Getting the annealing temperature correct is the most important, and crucial stage of PCR development. The temperature of the reaction is lowered to allow the binding of the primers to the target DNA. The T_m of the primers are used to determine what annealing temperature should be used. T_m is estimated using the number of nucleotides present in the DNA oligo, while taking into consideration the salt concentration in the reaction using the following formula:

$$T_m = 81.5 + 16.6(\log[Na^+]) + 0.41(\%GC) - 675/\text{primer length}$$

During PCR optimisation, it is advisable to start with an annealing temperature 3 - 5 °C below the lowest T_m of the primers. However, if the temperature is too low, amplification of non-target sites is likely to occur as incorrect base pairs are able to form and, crucially, are tolerated which results in an increased number of potential hybridisation sites within the template molecule (Brown, 2010). Vice versa, if the annealing temperature is too high, no hybridisation can occur. Getting the temperature of this step correct, therefore, is critical. One of the easiest methods to develop this is to run the same PCR reaction on a gradient of different temperatures.

3.2.4 DNA Polymerase

Every PCR reaction is dependent on enzymes to synthesise new strands of DNA complimentary to the template DNA. These are known as DNA polymerases, and there are four types typically used in genetic studies. However, only the *Taq* DNA polymerase from the bacterium *Thermus aquaticus* is used in PCR studies. *Thermus aquaticus* lives in hot springs which results in the enzymes being thermostable meaning they are not denatured by heat treatment. This makes them ideal for PCR as they can withstand the high temperatures required during denaturation steps, without being inactivated.

All DNA polymerases make mistakes, but one potential problem with *Taq* polymerase is that it does not possess a proofreading function meaning it cannot, like other polymerases, correct its errors. For most applications, this does not present a problem as the errors are distributed randomly, and the error rate, in comparison to fragments of the correct sequence, are insignificant. Within the context of this thesis, the mistakes associated with *Taq* polymerase are not important as the cloned PCR product is not being used in further experimentation, merely the amplified DNA is being loaded directly onto an electrophoresis gel for gene detection.

3.2.5 PCR Cycling

Each of the PCR stages above (denaturation, annealing, and extension) are cycled over and over again in order to amplify the target DNA. The number of cycles in any PCR design will vary depending on the amount of template DNA in the reaction, and the required yield from the PCR reaction. The PCR product doubles during each cycle, until such time as the

components of the reaction become depleted. After 30 cycles, each starting molecule in the reaction will have generated over 130,000,000 short PCR products from the target DNA. Typically between 25 and 35 denaturation - annealing - extension cycles will be carried out but, if the DNA input is fewer than 10 copies, up to 40 cycles may be carried out. The greater the number of cycles, the lower the efficiency of the PCR and therefore anything over 45 cycles is avoided as reaction components start to deplete, and the accumulation of undesired by-products have been observed. This results in a plateau of the PCR amplification curve (figure 3.1).

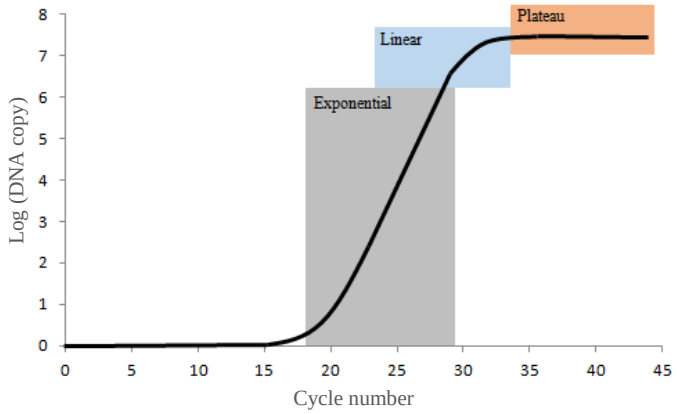


Figure 3.1 PCR amplification curve demonstrating product accumulation in relation to the number of cycles (adapted from ThermoFisher, 2018)

3.3 Downstream application of PCR

Once PCR is complete, gel electrophoresis is typically used to separate, identify and purify DNA. The PCR product is loaded onto an agarose gel containing a stain, such as ethidium bromide (EtBr), and an electrical current is run through the gel. The sugar-phosphate backbone of nucleic acid is negatively charged, which means it is attracted towards a positive charge. The electrophoresis tank is designed to run electricity from the negative to the positive electrode. The negatively charged DNA therefore migrates through the gel towards the positive electrode. The larger the DNA fragment, the slower it moves through the agarose gel. The incorporation of EtBr into the gel, results in EtBr intercalating between the bases of DNA meaning that staining occurs during electrophoresis, allowing visualisation of the DNA fragment. The use of a DNA ladder alongside the stained samples allows the size of the fragment to be determined. The density of the gel, voltage, and time run, all influence the efficacy of the procedure.

3.4 Introduction to qPCR

qPCR is a modification of traditional PCR whereby the amount of product generated by the PCR following each cycle is measured over time. Traditional PCR is only semi-quantitative as it is dependent on a comparison between relative values as opposed to absolute amounts. qPCR, however, provides information on the absolute amount of PCR product as the PCR is run.

Since the development of qPCR in the mid-nineties, instruments combining the rapid thermal cycling required for PCR, along with a real-time fluorescence measurement of the PCR product accumulating have been developed, making the routine quantification of nucleic acids in small biological samples practicable (Heid et al., 1996).

3.5 qPCR Design

3.5.1 qPCR Primers

The design of primers for qPCR do not vary from traditional PCR. However, the primers selected for qPCR usually result in a smaller product than traditional PCR and the efficiency is more important as it is much more sensitive

3.5.2 qPCR Marker of Fluorescence

One difference between traditional PCR and qPCR is the addition of a marker of fluorescence in addition to the 5 chemical components required for PCR (see section 3.1). Probably the most common method relies on SYBR Green 1 which is used for the detection of double stranded DNA. A second method uses a sequent specific, fluorescent probe for the target gene product. The probe contains a reporter dye for which the fluorescence emission is quenched by a secondary dye until the 5'-nuclease activity of the polymerase cleaves the probe and releases the reporter. Using this method, fluorescence increases as more and more PCR product is accumulated during the amplification process (Holland et al., 1991). This method ensures that only the specific PCR product is detected as the fluorescent signal is only produced when the probe hybridises with the correct target sequence. It is the shape of curve produced (i.e. the quantification value, Cq) compared to

a standard curve which determine the precise concentration of the target (e.g. template) in the original sample.

3.5.3 qPCR Conditions

qPCR relies upon the same denaturation - annealing - extension cycles as described above. Like PCR, qPCR conditions should be optimised in order to ensure the experiment runs efficiently and accurately.

3.6 Target Gene: Epstein-Barr Virus (EBV)

EBV is one of two γ-herpesviruses, a subfamily of herpesviruses, distinguished by their preference for infecting lymphocytes (Longnecker & Neipel, 2007). γ-herpesviruses can further subdivided into *lymphocryptoviridae* (including EBV) and *rhadinoviridae* (Longnecker & Neipel, 2007). Infection with EBV is prevalent in both child and adult populations with approximately 95% of the population infected by the time they reach adulthood (Jenson, 2011). Primary infection usually occurs in childhood which typically results in an asymptomatic infection. Where primary infection occurs later in life, the result is normally infectious mononucleosis (also known as glandular fever). Following primary infection, the host usually remains a carrier of the latent virus for the remainder of their life (Rickinson et al., 2014; Young et al., 2007). It is currently accepted that the latent virus resides in CD19+ B-lymphocytes, periodically shedding virus through the induction of lytic replication in these B-lymphocytes. The number of infected cells is, however, extremely low, and cellular and humoral immune responses keep the viral load low. A loss of balance within these cellular and humoral immune responses leads to an increase in viral load.

The EBV genome (accession number NC_007605) is approximately 172,000 kb in length, and is composed of double stranded DNA (Jenson, 2011). BALF5 (NCBI gene ID: 3783681) is one of the early lytic EBV genes mediated in viral replication (Young et al., 2007) and is located between 153,241 and 156,288 bp within the EBV genome, consisting of 3,048 bp. During viral replication, it is one of the earlier genes expressed as it is the catalytic component of the viral DNA polymerase (Young et al., 2007). As such, the detection of BALF5 is an early indicator that the cellular and humoral immune responses have lost balance and that the virus has started to replicate and reactivate.

3.7 DNA Extraction

DNA extraction is a routine procedure which isolates and purifies the DNA from a sample using both physical and chemical methods. Following collection of the sample of interest (in the case of this thesis, the sample of interest is always saliva, unless otherwise stated), the DNA from the sample needs to be isolated from within the cell membrane and nucleus, or the sample of interest (e.g. cell free DNA in saliva supernatant). This can be achieved using a number of methods:

- Acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC) (Chomczynski & Sacchi, 2006)

- Spin column-based nucleic acid purification

- Boom method (Boom et al., 1990)

- Synchronous coefficient of drag alteration (SCODA) DNA purification (Marziali et al., 2005)

The length of DNA to be extracted, purity of DNA required, and time available will influence the method selected. For the purposes of this thesis we have selected spin column-based nucleic acid purification which is a quick, solid phase extraction method. Spin column-based nucleic acid purification relies on the fact that, under certain conditions, nucleic acid will bind to the solid phase of silica. The four stages of any spin column-based nucleic acid purification method are:

- Lyse: If required, the cell's membrane and nucleus are broken to release the nucleic acid.

- Bind: A buffer solution is added to the lysed cells, transferred to a spin column and centrifuged. The solution is pushed through a silica gel membrane inside the spin column to which the nucleic acid binds.

- Wash: Impurities and residual buffers are removed by adding a wash-buffer to the spin column and centrifuging. The nucleic acid remains attached bound to the silica gel.

- Elute: The bound nucleic acid is removed from the silica gel membrane by adding a wash buffer (or MQdH_2O). The column is centrifuged again and nucleic acid is collected in the bottom of the column ready for use in downstream applications.

There are a large number of commercial extraction kits available based on this method. Kits are designed to extract a specific type, or range of target DNA (e.g. genomic, mitochondrial, viral) from a target source (e.g. cells, tissue, blood, urine, soil, water), and are designed to extract DNA up to a certain size.

3.8 Positive and Negative Controls

As with any biological assay, there needs to a positive and negative control

3.8.1 Bacterial Transformation

Gene cloning allows for recombinant DNA to be replicated within a host organism. Bacteria, specifically *E. coli*, are commonly used as host cells for making copies of DNA as they are easy to grow in large numbers, and create a new generation of genetically identical bacteria every 20 minutes. Prior to the vector being inserted, however, *E. coli* cells need to be treated to facilitate the uptake of the vector. The most common method for this is a method is known as the Hanahan method. The Hanahan method essentially involves the treatment of *E. coli* cells with $CaCl_2$ and water which causes the cells to swell which is necessary for the uptake of the vector. The swollen cells are known as competent cells. It is possible to create competent bacteria in house, but for the purposes of this thesis, commercially prepared cells were purchased.

3.8.2 Purification of DNA

The resulting product from a bacterial transformation will contain both the plasmid DNA, and the bacterial chromosomal DNA present in the cell. The presence of bacterial chromosomal DNA in gene cloning experiments can impact on the integrity of results and

therefore the plasmid DNA needs to be isolated and purified before further experimentation occurs (Brown, 2010). Typically, a plasmid specific spin column-based nucleic acid purification method is used (as detailed above in section 3.7). The concentration and purity of the DNA should then be assessed which is measured by ultraviolet (UV) absorbance spectrophotometry at 260 and 280 nm. The concentration of DNA is determined by the UV absorbance at 260 nm (A_{260}) as the amount of UV absorbed by the sample at A_{260} is directly proportional to amount of DNA in the sample (Brown, 2010). The purity of the sample is determined by the ratio of the UV absorbance of the sample at 260 and 280 nm (A_{260}/A_{280}). A ratio of 1.8 is considered to be a pure sample. A ratio below 1.8 suggests that the sample is contaminated with either protein or phenol (Brown, 2010).

3.9 Methods

3.9.1 Positive Control

Bacterial transformation

A pcDNA3.1^{+}-BALF5-V5/His construct (Catalogue number: K4800-01: Life Technologies Corporation, Carlsbad, California, U.S.A.) was obtained from a research group at the University of Birmingham, U.K. One µl (50 ng) of pCDNA3.1^{+}-BALF5 was added to an aliquot containing 25 µl Subcloning Efficiency DH5α Competent E. coli cells (catalogue number: 18265017: ThermoFisher Scientific, Waltham, Massachusetts, U.S.A.) and stirred gently using a pipette tip. This was incubated on ice for 30 minutes, after which, the aliquot was promptly transferred to a water bath set at 42 °C for 30 seconds, then incubated once again on ice for a further 2 minutes. 250 µl of SOC outgrowth medium (Catalogue number B9020S; New England Biolabs, Ipswich, Massachusetts, U.S.A.) was added to the tube before being placed in a shaking incubator at 37 °C and 225 rpm for 45 - 60 minutes. Following this incubation, 100 µl of the media containing pCDNA3.1^{+}-BALF5 was spread onto an LB agar plate containing 200 mg.L^{-1} ampicillin (Catalogue number: 11593027: ThermoFisher Scientific) (LB-amp) and placed in a bacterial incubator at 37 °C overnight. The following morning, the LB-amp plate was removed from the incubator. In a 30 mL universal tube, 5 µl of ampicillin (1:1000 dilution; working solution of 100 mg.L^{-1}) was added to 5 mL of LB broth. The antibiotic broth was inoculated with a small, distinct colony from the LB-amp plate and placed in a shaking incubator at 37 °C and 225 rpm

overnight. The following morning, a 15% glycerol stock of the DH5α cells containing pcDNA3.1^{+}-BALF5 was prepared and frozen at -80 °C. The rest of the culture was taken forward into plasmid purification.

Purification of DNA

Plasmid purification was undertaken using a commercially available MiniPrep kit (QIAprep Spin Miniprep Kit, catalogue number: 27106; QIAGEN, Hilden, Germany). The manufacturer's protocol for Plasmid DNA Purification using the QIAprep Spin Miniprep Kit and a Microcentrifuge was followed, with the exception that MQdH2O heated to 70 °C was used instead of buffer EB during step 10 (elution).

Stock solutions

A working stock with a concentration of 1 ng.µl^{-1} was prepared in MQdH_2O and used as a positive control in all PCR assays, and to prepare standard the curves for all qPCR assays.

3.9.2 Negative Control

MQdH_2O was used in place of template DNA to form a negative control for all PCR reactions.

3.9.3 DNA concentration and purity

The concentration and purity of samples were tested using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, U.S.A.). The concentration in ng.µl^{-1}, and the A_{260}/A_{280} is reported for each sample tested.

3.9.4 DNA Extraction

Our laboratory, historically, had used the AmpliSens DNA-sorb-AM, Nucleic acid extraction kit, (Cat #: K1-11-50-CE; Ecoli s.r.o., Bratislava, Slovakia) for the extraction of DNA from saliva. This is a commercially available pre-made extraction kit, with accompanying PCR and electrophoresis kits. However, the specificity of the extraction process, and target gene are undisclosed by the manufacturer. The development of an in-

house method was sought, therefore, in order to allow for the extraction of known genetic material (e.g. all DNA), and to allow for the confident use of alternative PCR assays on extracted samples. The results from the AmpliSens DNA-sorb-AM, Nucleic acid extraction kit, (Cat #: K1-11-50-CE; Ecoli s.r.o., Bratislava, Slovakia) were compared to an alternative, commercially available kit (Quick-DNA Universal Kit (Cat #: D4068; Zymo Research, Irvine, California, U.S.A.) which uses a spin-column method. The manufacturer guidelines were followed in all cases (specifically the Biological Fluids & Cells protocol for the Quick-DNA Universal Kit).

3.9.5 PCR Primers

Design

In earlier experiments, primer selection for BALF5 amplification was based on those used by Yamauchi et al., 2011 which targets EBV at 305 bp (sense: 5'-CCC TGT TTA TCC GAT GGA ATG AC-3'; antisense: 5'-CTT CTG AAA AAG CCT GAC AAG GAG-3') (Yamauchi primers). These primers were diluted with $MQdH_2O$ resulting in 10M solutions (15.9 µl forward primer with 159 µl $MQdH_2O$ and 13.8 µl reverse primer with 138 µl $MQdH_2O$ respectively). Following preliminary experimentation using this methodology, the Yamauchi primers were run *in-silico* and did not meet a high enough criteria to be carried forward into further experiments (appendix A). The primers and plasmid were changed to the methodology detailed below.

Table 3.1 BALF5 PCR Primers

Primer	Length	Position	Tm	%GC	Sequence	Amplicon (nt)
Forward1	20	155168-155187	59.4	55	ATC TCC ACG CTG AGG TCT CT	452
Reverse1	20	155619-155600	59.4	55	CAC GGG TGT CGG ATC TTT GA	
Forward2	20	154909-154928	59.4	55	GGC TAC TGT GTC CAG CTT GT	275
Reverse2	20	155183-155164	59.4	55	ACC TCA GCG TGG AGA TTG TG	

New primers (BALF5 primers) were designed using NCBI's Primer-BLAST (Basic Local Alignment Search Tool) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and checked using the Bioinformatics Sequence Manipulation Suite's Primer Stats software (www.bioinformatics.org/sms2/pcr_primer_stats.html). The primers targeting BALF5, detailed in table 3.1, were selected following checks.

Both sets of primers were ordered from Eurofins Scientific (Eurofins GSC LUX, Luxembourg). Primers were reconstituted using $MQdH_2O$ to give a concentration of 100 $pmol.\mu l^{-1}$ (100 µM).

Primer Stock Solutions

A 1:10 dilution was prepared from these to provide a working stock of 10 $pmol.\mu l^{-1}$ (10 µM) (i.e. 10 µl primer into 90 µl $MQdH_2O$).

Table 3.2 PCR Design for DreamTaq Polymerase

Component	**Volume (µl)**
DreamTaq Polymerase	12.5
EBV Forward Primer	2.5
EBV Reverse Primer	2.5
Sample/Control	2.5
$MQdH_2O$	5
TOTAL	**25**

3.9.6 PCR Design

PCR Reactions

The AmpliSens EBV-EPh PCR Kit (Cat #: V0-100_R0,5-CE; Ecoli s.r.o., Bratislava, Slovakia) was known to successfully amplify EBV DNA (although the specific target gene was unknown). Samples were therefore amplified using both the AmpliSens EBV-EPh PCR Kit, and an in house method. The AmpliSens EBV-EPh PCR Kit manufacturer's recommendations were followed giving a final reaction volume of 25 µl which contained 10 µl of template DNA and amplified a 290 bp amplicon of EBV. The in house method used ThermoScientific DreamTaq Green PCR Master Mix (x2) (DreamTaq; ThermoScientific, Loughborough, UK) as a polymerase. DreamTaq is a ready to use solution containing the Taq polymerase, buffer solution, $MgCl_2$, and dNTPs. Following

the manufacturer's recommendations, 2.5 µl each of the diluted forward and reverse primers, 2.5 µl of template DNA (either extracted saliva, plasmid, or negative control) and 5 µl or MQdH_2O were added to 12.5 µl of DreamTaq (Table 3.2).

Cycling conditions

During all analysis, samples were amplified in flat-cap 0.2 mL tubes (Cat #: AB062; ThermoFisher Scientific, Waltham, Massachusetts, U.S.A.) using an Eppendorf Mastercycler EP Gradient S Thermal Cycler (Eppendorf, Hamburg, Germany).

The AmpliSens EBV-EPh PCR Kit had amplification programme settings already established which were followed as per the manufacturer guidelines. The annealing temperature for the abovementioned kit was 65 °C. The T_m for both set of custom BALF5 primers was 59.4 °C. As such, samples were run on a gradient from 55 °C to 65 °C (table 3.3) to establish the optimal annealing temperature for the DreamTaq reaction. For consistency, denaturation and extension phases of the AmpliSens EBV-EPh PCR Kit were maintained for both reactions set-ups.

Table 3.3 Thermal PCR Cycling Conditions

Step	Temperature °C	Time	Number of Cycles
Initial denaturation	95	5 min	1
Denaturation	95	15s	
Annealing	55 → 65	25s	42
Extension	72	25s	
Final extension	72	1 min	1

3.9.7 Downstream application

Gel

A 1.6% agarose gel was prepared using 0.5% TAE buffer, and stained with EtBr. For a 50 mL gel, this would require 0.8 g agarose, 50 mL 0.5% TAE buffer, and 0.5 µl EtBr.

23 µl of each amplified PCR product (sample or control) were loaded into individual wells alongside a DNA ladder.

DNA Ladder

An Invitrogen Low DNA Mass Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) was used to help visualise approximate sizing of double-strand DNA in the range of 100 - 2,000 bp with bands at 100, 200, 400, 800, 1,200, and 2,000 bp. 10 µl of Invitrogen Low DNA Mass Ladder was mixed with 2 µl of DNA Gel Loading Dye (Cat #: R0611; Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.). 12 µl of the DNA ladder, prepared as outlined above, were loaded into one or two wells per row, per gel.

Electrophoresis power supply

During all analysis, electrophoresis was powered by a Consort EV265 Electrophoresis Power Supply delivering 100 V for 1.5 hours (depending on the size of the gel - shorter gels required less run time and were monitored for the run off of preceding dyes).

Gel Imaging

Gels were imaged using a Syngene G:Box Gel Documentation Imaging System and complimentary software (Syngene, Cambridge, U.K.).

3.9.8 qPCR Primers

Primers were designed using the Roche Universal Probe Library (UPL) (https://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html). The UPL automatically undertakes a BLAST search to ensure that the primers do not miss-prime with any other gene. It also runs the PCR in silico to rule out primer dimer. The primers targeting BALF5, detailed in table 3.4, were selected from the UPL.

Table 3.4 qPCR Primers

Primer	**Length**	**Position**	**Tm**	**%GC**	**Sequence**
Forward	20	1065-1084	60	60	GGA GCT GGA CAT GCT CTA CG
Reverse	20	1105-1124	60	55	ACA ATC TCC ACG CTG AGG TC

These primers produce an amplicon that is 60 nt (GGAG CTGG ACAT GCTC TACG CCTT CTTC CAGC TCAT CAGA GACC TCAG CGTG GAGT TTGT) and meet all the criteria (i.e. there are no risks of miss-priming or primer dimer). These custom primers were ordered from Eurogentec (Liège, Belgium) and prepared following the manufacturer's guidelines to give working concentrations of 400 nM each.

3.9.9 qPCR Probe

These primers were used in combination with probe 11 (cat. no. 04685105001) from the UPL (Roche, Basel, Switzerland). The probe was prepared following the manufacturer's guidelines to give a working concentrations of 200 nM.

3.9.10 qPCR Design

0.2 µl of each primer, 0.2 µl of probe, and 2.5 µl of template DNA was added to 1.9 µl MQdH_2O and 5.0 µl of PCR mix prepared from the FastStart Essential DNA Probes Master (Cat #: 06402682001; Roche, Basel, Switzerland) following the manufacturer's guidelines. Reactions were prepared in a 96 well plate and analysed using a LightCycler 96 (Roche, Basel, Switzerland). Fluorescence was measured every cycle to allow for Cq of samples and standards to be calculated. The Cq and baseline settings were automatically calculated by the LightCycler 96 Application Software, applying a positive/negative filter based on end point fluorescence, maximum relative slope, and deviation from linearity.

In accordance to the recommendations made by Roche, the following amplification programme was run:

Table 3.5 qPCR Thermal Cycling Conditions

Run Editor				
Detection format		**Reaction volume (µl)**		
FAM		20		
Programme				
Step	**Temperature °C**	**Time**	**Number of Cycles**	**Acquisition mode**
Initial denaturation	95	10 min	1	None
Denaturation	95	10s		None
Annealing	55	30s	50	Single
Extension	72	1s		None

3.10 Results and discussion

3.10.1 Development of a positive control: Bacterial transformation

The concentration of the purified pcDNA3.1^{+}-BALF5 was 630.5 ng.µl^{-1}. The purity, as accepted by the A_{260}/A_{280}, was 1.86. The concentration and purity of this transformation are indicative of a successful experimental process. A 1:100 dilution, followed by a subsequent 1:6 dilution were performed to provide a working stock with a concentration of 1 ng.µl^{-1}.

3.10.2 DNA Extraction

When extracting DNA, both the yield and quality of the DNA extracted are important to subsequent experimentation. In the case of the present study, viral DNA needed to be extracted from the supernatant of saliva. It was likely that the yield, in most cases, would be relatively low, and therefore the total quantity and quality of the DNA extracted needed to be of a good standard. In order to identify the best kit for our purpose, two commercial extraction kits were used to extract total DNA from the same saliva sample. The concentration and purity of the extracted DNA from each kit were then analysed, before a final decision could be made on which extraction method should be taken forward.

Table 3.6 Comparison of DNA concentration and purity using 2 commercially available extraction kits

Kit	**Sample**	**Concentration (ng/µl)**	**A_{260}/A_{280}**
Quick-DNA Universal Kit	Fresh	12.4	1.16
	-20	42.2	1.23
	-80	37.8	1.16
AmpliSens DNA-sorb-AM	Fresh	56	2.96
	-20	162.1	2.2
	-80	99.8	2.91

A fresh saliva sample from an EBV-seropositive subject was collected, centrifuged for 5 minutes at 1,700 × g, and the supernatant removed. The supernatant was aliquotted into

four 1.5 mL microcentrifuge tubes. With both kits, one aliquot was extracted immediately (fresh); one aliquot was frozen at -20 °C and thawed prior to extraction (-20); and one aliquot was frozen at -80 °C and thawed prior to extraction (-80).

Following extraction, the concentration and purity of the DNA extracted was analysed as described above. The results of this analysis are presented in Table 3.6.

A ratio of 1.8 at A_{260}/A_{280} is optimal, but a purity between 1.7 and 1.9 is expected for double-stranded DNA. A low A_{260}/A_{280} ratio is indicative of contamination with phenol, protein, or guanidine isothiocyanate which may prevent the DNA quantification from the A_{260} measurement (Lee at al., 2010). Alternatively, it can be indicative of RNA contamination. A high A_{260}/A_{280} ratio is not indicative of an issue per say, but may be the result of a measurement issue (i.e. the solution used as a blank measurement during the nanodrop pedestal calibration should have the same pH as the solution to be measured) (Wilfinger et al., 1997). Measuring the purity, and concentration, of DNA samples using spectrophometry, however, is not perfectly precise because of these issues mentioned above. There may have been an efficient extraction of DNA, but because of contamination, a low A_{260}/A_{280} ratio may be reported. The only way to effectively check if an extraction has worked, is to subject the sample to further experimentation, such as a qPCR. Three samples extracted using the Quick-DNA Universal Kit were compared to the same three samples extracted using an alternative, spin column based commercial kit (QIAamp DNA Blood Mini Kit, cat #: 51104; QIAGEN, Hilden Germany). The qPCR only detected EBV DNA in 2 out of three samples extracted using the QIAamp DNA Blood Mini Kit (mean EBV DNA concentration 5.99 ± 5.20 ng.µl^{-1} $\times 10^{-6}$) compared to three out of three extracted using the Quick-DNA Universal Kit (mean EBV DNA concentration 63.60 ± 58.84 ng.µl^{-1} $\times 10^{-6}$). The subsequent qPCR testing confirmed that the Quick-DNA Universal Kit was extracting an adequate concentration and purity of DNA for downstream application and it was therefore taken forward into the final methodology.

3.10.3 Optimisation of the PCR

Both the primers for 452 bp and the primers for 275 bp successfully amplified their corresponding regions with an annealing temperature of 65 °C for 42 cycles (table 3.8) using the BALF5 plasmid from Birmingham. There was no amplification when the

primers were run independently of the plasmid, and vice versa, indicating there are not any underlying issues with the amplification, such as primer dimer. As such, both sets of primers were accepted as a viable methodology for the detection of EBV in saliva samples.

Table 3.7 Final PCR Thermal Cycling Conditions

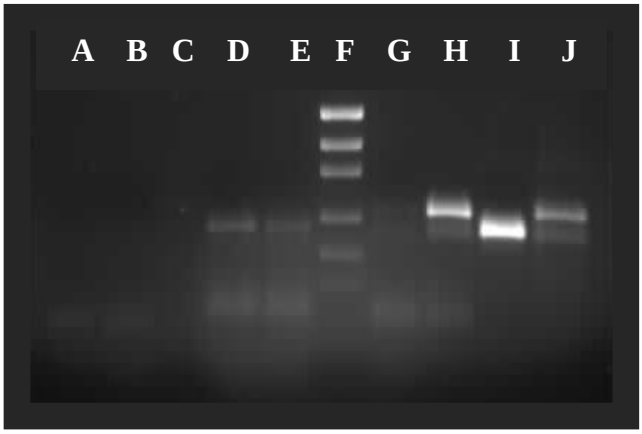
Step	Temperature °C	Time	Number of Cycles
Initial denaturation	95	5 min	1
Denaturation	95	15s	
Annealing	65	25s	42
Extension	72	25s	
Final extension	72	1 min	1

However, the amplification of extracted saliva samples had, as yet, been unsuccessful but it was unclear if this was due to a fault with the assay, or due to the samples lacking EBV DNA. One way to check if a PCR assay has amplified successfully is to use an internal control, or reference gene. An internal control can act as an indicator of nucleic acid extraction, the quality of samples, and the quality of the PCR. The internal control, and target gene will be co-amplified, meaning it is important that the primers do not out compete, and should result in two clear, separate bands during subsequent analysis by electrophoresis. In the present study, β-globin was selected as an internal control. Primers GH20 and GH21 targeting the β-globin gene at chromosome 11 were selected from Takara Bio (sense: 5'-GAA GAG CCA AGG ACA GGT AC-3'; antisense: 5'-GGA AAA TAG ACC AAT AGG CAG-3') which amplified a region which was 408 bp in length (Saiki et al., 1988). Extracted saliva samples that tested positive for EBV using the AmpliSens DNA-sorb-AM kit and AmpliSens EBV-EPh PCR kit, respectively, were taken forward into experimentation. Extracted saliva samples and controls were prepared and amplified using both the new BALF5 primers for 275 bp and the β-globin primers for 408 bp. Reaction volumes for each gene were prepared separately in order to confirm the success of the assay before attempts at multiplexing were made. Preparations were made using the reaction volumes reported above (table 3.2) and cycled using the final 65 °C cycle (table 3.7). Initial experimentation worked (figure 3.2) with positive results for both EBV and β-globin for extracted saliva samples and positive controls. The extracted saliva samples did provide faint, but positive results for EBV (Lanes D and E), despite the fact they had been extracted using the AmpliSens DNA-sorb-AM kit. The previous failure of the BALF5 assay to detect EBV in saliva may, therefore, have been to the number of starting copies in

the saliva. If these were not present, or too low, there may not have been enough of the gene amplified to create subsequent banding on the gel (Yamauchi et al., 2011).

Based on the success of this experiment, attempts were made to multiplex the assay with the primers for BALF5 at 275 bp and β-globin at 408 bp. The resolution of the gel was not very clear, but the assay does not appear to have been efficient at amplifying any material other than the BALF-5 plasmid (figure 3.3). In this, and all subsequent PCR multiplexed reactions, β-globin failed to amplify effectively. This may have been due to degradation of the primers or, most likely, there may have been a need for an alternative PCR Master Mix (e.g. ThermoScientific Phusion U Multiplex PCR Master Mix in replacement of the ThermoScientific DreamTaq Green PCR Master Mix (x2); both ThermoScientific, Loughborough, UK). However, in the interests of time, attempts to multiplex the assay were abandoned.

Figure 3.2 Results of EBV (BALF5 275 bp) and β-globin (408 bp)



Lane A: -ve EBV control; **Lane B:** -ve β-globin control; **Lane C:** BLANK; **Lane D:** +ve sample A with EBV primers; **Lane E:** +ve sample B with EBV primers; **Lane F:** Low Mass DNA Ladder; **Lane G:** +ve sample A with β-globin primers; **Lane H:** +ve sample B with β-globin primers; **Lane I:** +ve EBV control; **Lane J:** +ve β-globin control. Ladder bands are visible, from top to bottom, at 2,000, 1,200, 800, 400, 200, and 100 bp.

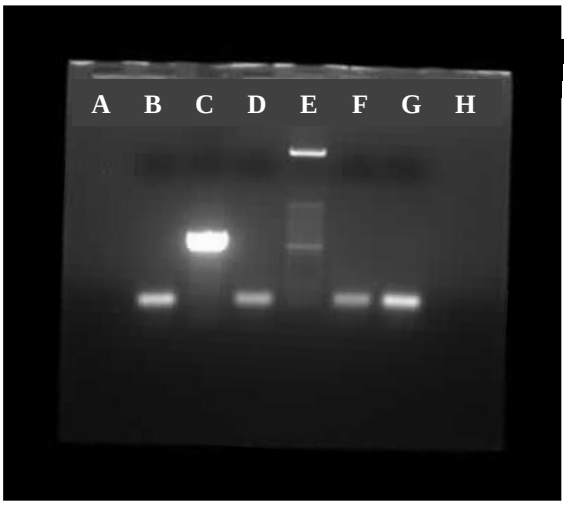
3.10.4 Downstream application: Agarose gel electrophoresis

Agarose gel electrophoresis separates DNA molecules by size. The agarose gel is porous and the density of the gel should be adjusted to suit the size of molecule on which the experiment is being performed. A 1.6 % agarose gel is optimal for the resolution of linear

DNA that are 200 - 3,000 bp which is why it was selected for the present experiment. 100 V for 1.5 hours in a relatively large gel (~20 cm) worked well in the

present experiment, but the voltage and time can be varied however the lower the voltage, the longer the run time needs to be.

Figure 3.3 Results of EBV (BALF5 275 bp) and β-globin (408 bp) multiplex PCR assay



Lane A: BLANK; **Lane B:** multiplexed reaction with saliva sample extracted using the Quick-DNA Universal Kit; **Lane C:** multiplexed EBV plasmid; **Lane D:** β-globin primers with saliva sample extracted using the Quick-DNA Universal Kit; **Lane E:** Low DNA Mass Ladder; **Lane F:** multiplexed negative control; **Lane G:** EBV primers with saliva sample extracted using the Quick-DNA Universal Kit; **Lane H:** BLANK. Ladder bands are visible, from top to bottom, at 2,000, 1,200, 800, 400, 200, and 100 bp (although the clarity of the image here is poor).

3.10.5 Traditional PCR design conclusions

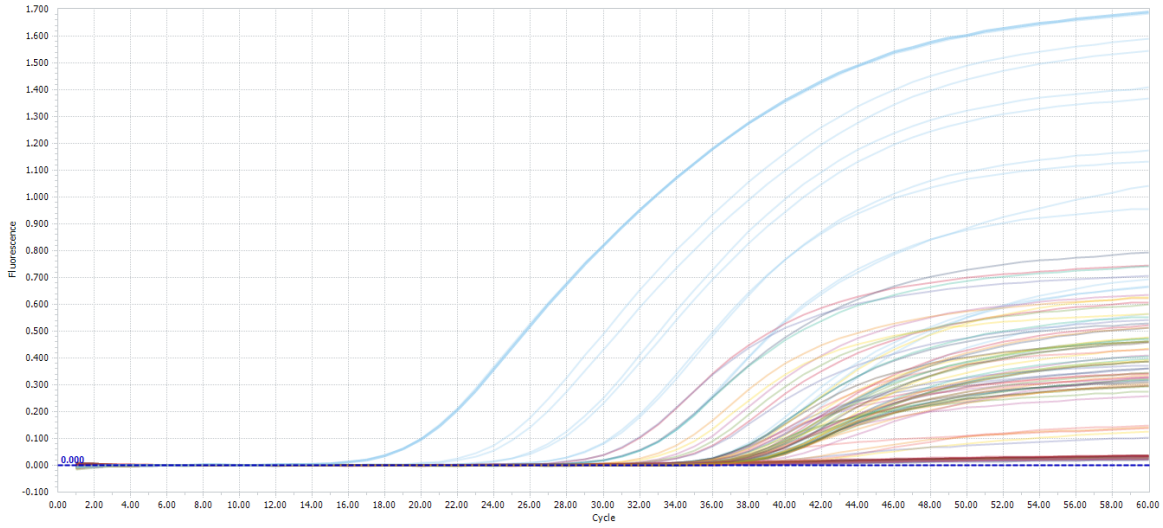
To conclude, either of the two sets of primers tested (table 3.1) are suitable for use in a traditional PCR assay for the detection of EBV in saliva targeting the BALF5 gene. Which primer pair is selected will depend on the size of the amplicon required. Reaction volumes should be prepared as described in table 3.2, and amplified using the programme described in table 3.7. Future work should look to multiplex the assay with an internal control, such as β-globin to confirm the efficiency of the amplification and improve the accuracy of the assay. Alternatively, each reaction (i.e. EBV and β-globin) could be run separately.

3.10.6 qPCR

In many ways, analysing samples using qPCR is simpler, methodologically, than traditional PCR. Reactions volumes are prepared in a similar way, but the analysis is automated, removing the need for downstream analysis, such as electrophoresis. The sensitivity of qPCR is greater than that of traditional PCR which allows for a greater accuracy of quantification, but it does mean that the risks of contamination interfering with the samples are greater. Owing to the real time nature of qPCR analysis, the need for opening amplified PCR material is eliminated, however, which means the risk of contaminated laboratory space or equipment, is reduced greatly.

The majority of the design of the qPCR for the present study was done using the online facilities provided by Roche. Primers, probes, and MasterMix were all designed and/or sourced using Roche. As such, there was an increased likelihood that components would be compatible, and the assay a success.

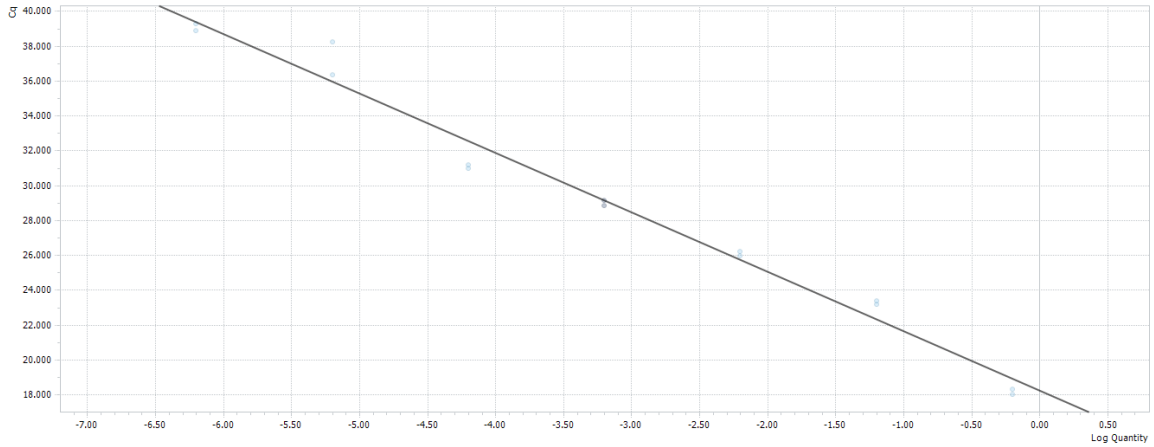
Figure 3.4 Amplification Curves for EBV BALF5 qPCR



The assay was first tested using a serial dilution of BALF5 plasmid. This was followed up using BALF5 plasmid, and a number of saliva samples that had been extracted using the Quick-DNA Universal Kit. A quantification cycle value (C_q) was calculated for each sample as the number at which fluorescence exceed a threshold limit (determined as 10 × SD of the baseline fluorescence calculated from cycles 3 - 10). All samples were analysed in duplicate, and samples were classified as negative if the C_q exceeded that

which had been calculated automatically by the LightCycler 96. The accepted criteria for a qPCR's success is a standard curve slope between -3.1 and -3.6 which will result in an amplification efficacy of 90 - 110%. In the present experiment, the mean standard curve slope value was -3.4661 ± 0.1350, which gave a mean efficiency of 1.95 ± 0.05 (94.60%). In addition, the assay reported a mean r^2 of 0.99 ± 0.01, and a mean *y* intercept of 18.39 ± 0.27 (figures 3.4 and 3.5).

Figure 3.5 EBV BALF5 qPCR Standard Curve



Repeated experiments demonstrated that the methodology was robust and amplified and quantified EBV efficiently in samples where target DNA was detectible. As such, the reported methodology is accepted as a reliable and valid method for the detection of EBV DNA in saliva, and has been used for the detection of EBV in saliva throughout the remainder of this thesis. Samples were run in duplicate, and the intraassay CV = 6.0%.

Chapter 4

The influence of acute carbohydrate supplementation on salivary EBV DNA expression in athletes, and the comparison of two *in vivo* measures of immune function

Related publication:

Davison, G., Kehaya, C., Diment, B., & Walsh, N. (2016). Carbohydrate supplementation does not blunt the prolonged exercise-induced reduction of in vivo immunity. *European Journal of Nutrition*, 55(4): 1583-1593.

Abstract

Background A recent study demonstrated that, despite the fact carbohydrate (CHO) supplementation blunts *in vitro* immunoendocrine responses to prolonged exercise, it does not influence the decrease in *in vivo* immune induction using experimental contact hypersensitivity with the novel antigen diphenylcyclopropenone (DPCP).

Purpose To investigate the relationship between the ingestion of CHO before, during and after exercise on URTI incidence, the *in vivo* immune measure of saliva EBV DNA expression, and saliva SIgA responses. In addition, the interactions between URTI, saliva EBV DNA expression, and saliva SIgA with cutaneous responses to DPCP are explored.

Methods In a double-blind design, 24 healthy males were matched as closely as possible according to speed at their lower ventilatory threshold, age, and speed at $\dot{V}O_2max$, and randomly assigned to 120 min of treadmill exercise at 60% $\dot{V}O_2max$ with CHO (n = 12) or placebo (PLA) (n = 12) supplementation. Standardised diets (24 hours pre trial) and breakfasts (3.5 hours pre trial) were provided. Subjects received a primary DPCP exposure 20 minutes after trial completion, and exactly 28 days later the strength of immune reactivity was quantified by magnitude of the cutaneous response (skin-fold thickness and erythema) to a low dose-series DPCP challenge. EBV serostatus, Saliva SIgA, saliva EBV concentration, stress hormones, leucocyte trafficking and URTI incidence in the 2 weeks after trial were also monitored.

Results CHO supplementation blunted the cortisol and leucocyte trafficking responses but did not influence in incidence of URTI (PLA n = 1, CHO n = 2; P = 0.571), or the concentration of EBV DNA in saliva 1 hour post exercise (EBV concentration (median (IQR): PLA 1.88 (2.02), CHO 1.68 (3.49) $ng.\mu l^{-1} \times 10^{-6}$; P = 0.231). There were no overall changes in EBV DNA concentration 1 hour post exercise compared to baseline (P = 0.929). EBV DNA in saliva was not correlated with SIgA concentration or secretion rate, oedema and erythema responses to DPCP, or the incidence of URTI (P = 0.537, 0.942, 0.809, 0.374, and 0.969 respectively).

Conclusions Acute CHO supplementation does not influence the incidence of URTI or the expression of EBV DNA; nor is there an interaction between EBV DNA and SIgA, cutaneous responses to DPCP, or the incidence of URTI. The effects with more stressful, or fasted exercise remain to be determined. However, there appears to be no benefit under the conditions of this, and a previous study, of CHO supplementation on *in vivo* immunity.

4.1 Introduction

Athletes engaged in regular training which is prolonged in duration and/or strenuous have a higher than normal incidence of URTI (Gleeson, 2007; Walsh at al., 2011*a*). It is thought that this is related to an exercise-induced immunodepression (Gleeson, 2007; Walsh et al., 2011*a*) which is caused by an increase in exercise-induced stress responses (e.g. the release of stress hormones), and the redistribution of leucocyte subsets (Davison & Gleeson, 2005; Lancaster et al., 2005; Robson et al., 1999;). As a result, immunological and nutritional sports studies have investigated the use of various sports supplements with a view to attenuating this response. One supplement that has repeatedly been found to be particularly efficacious in blunting the abovementioned exercise-induced stress responses is the acute ingestion of carbohydrate (CHO), however, its effects on infection risk within an athletic population have not yet been established (Davison & Gleeson, 2005; Gleeson, 2006*a*; Gunzer et al., 2012; Henson, et al., 1999; Lancaster, et al., 2005; Moreira, et al., 2007; Scharhag et al., 2006; Scharhag, et al., 2002; Walsh et al., 2011*a*;). The effects of CHO supplementation on *in vitro* immune markers (e.g. increased cortisol, cytokine and leucocyte redistribution responses, decreased neutrophil degranulation and oxidative burst functions, lymphocyte proliferation and function, and natural killer cell function) have been the main assessment outcome of research to date (Davison & Gleeson, 2005; Henson et al., 1999; Lancaster et al., 2005; Scharhag et al., 2006; Scharhag et al., 2002). The effects of CHO on URTI incidence has seldom been reported (Nieman et al., 2002), and there is a lack of research investigating the effects of CHO on *in vivo* immune markers.

The DTH response to DPCP is an established, well reported technique which measures a T-cell mediated dermatological response to a novel allergen. We previously reported that the ingestion of a CHO drink before, during, and after endurance exercise did not affect the *in vivo* immune response to DPCP (Davison et al., 2016). An alternative measure of *in vivo* immunity is the reactivation, or expression of, EBV. EBV infected B-cells, when the host's immune system is working optimally, are regulated by T-cells which maintain the latent status of the virus (Cruchley et al., 1997). When the immune system is exposed to stress (i.e. exercise), there is an opportunity for the virus to reactivate which can be detected by the presence of EBV-DNA in saliva. Numerous studies have reported the presence of salivary EBV-DNA in athletes engaged in intensified periods of training (Gleeson et al., 2002; Mehta et al., 2000; Payne et al., 1999) but we are not aware of any

studies to date which have investigated the effects of CHO supplementation on the expression of EBV-DNA.

The effects of exercise, and the effects of CHO during exercise on the *in vivo* immune response to DPCP, have been reported previously. Harper-Smith et al. (2011) reported that the *in vivo* immune response to DPCP was significantly reduced following 2 hours of treadmill running, a finding that was supported by a later study by this group (Davison et al., 2016; Diment et al., 2015). This reduction is observed following both the induction of the immune system in subjects with no prior exposure, and the elicitation of immunity in subject with a well-established immunological memory to DPCP (Diment et al., 2015). Furthermore, support has been provided to the assumption that the observed decrease in *in vivo* immune induction to DPCP is T-cell mediated, as similar effects have not been observed in response to croton oil (which is an irritant that stimulates a non T-cell mediated inflammatory response after a single exposure, similar in nature to the cutaneous erythema response to DPCP, but with no sensitising properties), following the same exercise stimulus (Diment et al., 2015).

The aim of this study was to investigate the relationship between the ingestion of CHO before, during and after exercise on URTI incidence, saliva EBV DNA expression, and saliva SIgA secretion rate and concentration. In addition, the interaction between URTI, EBV DNA expression, and SIgA were investigated with the established *in vivo* response to DPCP.

4.2 Methods

All subjects provided informed consent before participation and completed a pre-exercise screening questionnaire (Physical Activity Readiness Questionnaire: PAR-Q) before participating in each exercise test. Ethical approval for the study was granted from the University of Kent's Ethics Committee.

4.2.1 Subjects

Twenty four physically active males aged 31 ± 8 years (mean ± SD) volunteered for the study (height 1.78 ± 0.05 m; weight 76.68 ± 10.49 kg; maximal aerobic capacity ($\dot{V}O_2max$) 58.4 ± 6.8 $mL^{-1}.kg.min^{-1}$). Subjects were excluded from participation if they were using

nutritional supplements or medication or if they had given blood, received vaccinations, or suffered an infection within 1 month of the study commencing. All subjects were familiar with treadmill running and had not had any previous exposure to DPCP. Subjects were asked to avoid strenuous exercise for 24 h before, and 48 h following each trial (exercise/sensitisation and elicitation) in addition to abstaining from the consumption of alcohol or caffeinated drinks 48 h ahead of each trial.

4.2.2 Determination of maximal oxygen update ($\dot{V}O_2$max)

Subjects' $\dot{V}O_2$max was assessed using an incremental treadmill test. Subjects were fitted with a HR monitor (Polar Electro, Kempele, Finland), and facemask (Cortex Biophysik GmbH, Leipzig, Germany) which was connected to a breath by breath gas analyser (MetaLyzer 3BR2, Cortex Biophysik GmbH, Leipzig, Germany) that had been calibrated with gas of a known composition prior to use, following the manufacturer's guidelines. Subjects completed a 5 minute warm-up at 5 $km.h^{-1}$ with a 1% treadmill gradient (h/p/cosmos Saturn treadmill, h/p/cosmos Sports and Medical gmbh, Nussdorf, Germany). Immediately following the warm-up, treadmill speed increased by 1 $km.h^{-1}$ every minute until 18 $km.h^{-1}$ at which point speed remained stable but treadmill gradient increased by 1% every minute until volitional exhaustion. Subjects' $\dot{V}O_2$max was determined as the highest value calculated from rolling 30 s averages. Breath-by-breath gas, HR, and subjects' RPE (Borg, 1970) were collected throughout. Speed ($km.h^{-1}$) was plotted against oxygen uptake ($\dot{V}O_2$) ($L.min^{-1}$) and speed at 60% $\dot{V}O_2$max was estimated using linear regression. Following this session, subjects were split into pairs, matched according to speed at VT1 (first ventilatory threshold), age, and speed at $\dot{V}O_2$max (the aim being to achieve as close a match as possible for speed at VT1, followed by age, followed by speed at $\dot{V}O_2$max) and randomly assigned to either 120 min of treadmill running at 60% of $\dot{V}O_2$max with CHO supplementation (n = 12), or 120 min treadmill running at 60% $\dot{V}O_2$max with placebo (n = 12).

4.2.3 Familiarisation

Approximately one week following their $\dot{V}O_2$max test, and one week ahead of their main trial, subjects attended the laboratory to complete a familiarisation session. Subjects completed a warm up for 5 min at 5 $km.h^{-1}$ and 1% gradient on the same treadmill used for the $\dot{V}O_2$max test. Immediately following this warm up, subjects ran on the same treadmill

at a speed equivalent to 60% $\dot{V}O_2$max for 60 minutes. If necessary, minor adjustments to running speed were made during the first 15 minutes to attain the target 60% $\dot{V}O_2$max. Subjects were provided with a fluid bolus containing 5 ml.kg^{-1} body mass water 20 minutes before, and upon completion of the trial, an additional 2 ml.kg^{-1} water was provided every 15 minutes of the trial to familiarise subjects with the fluid provision schedule. HR and RPE were recorded every 5 minutes, and subjects were familiarised with the blood and saliva collection methods.

4.2.4 Main Trial

Approximately one week following familiarisation, subjects reported to the laboratory at 07:00 following an overnight fast. Dietary intake was controlled for the 24 h before the main trial by providing subjects with prescribed water intake (35 mL.kg^{-1}.day^{-1}), and food from a standardised list which met their estimated daily energy requirement (11.4 ± 1.5 MJ day^{-1}) which comprised of 15, 60, and 25% of energy from protein, CHO and fat, respectively (equivalent to ~1.3, 5.4, and 1.0 g.kg^{-1}.body mass, respectively, Cunningham, 1991). At 7:30am, subjects were provided with a standardised breakfast providing ~0.03 MJ kg^{-1} (equivalent to 2.4 ± 0.3 MJ, comprised of ~0.2, 1.0, and 0.2 g.kg^{-1}.body mass of protein, carbohydrate, and fat, respectively) and provided with a standardised volume of water (~6.6 ml.kg^{-1}, proportional to their calculated requirement for the prior 24 h) which they could drink ad-libertum between 07:30 and 10:30, ahead of the trial's start at 11:00. Subjects were allowed to undertake light activity during this period, such as reading or using a computer. They were then required to sit for 10 minutes with minimal movements before a venous blood sample was collected from a superficial anterior vein using standard venepuncture techniques detailed in chapter 2, and an unstimulated saliva sample was collected via passive drool technique detailed in chapter 2. Subjects received a fluid bolus (5 ml.kg^{-1}.body mass) of their respective drink (CHO or PLA) 20 minutes before, and immediately after exercise. In addition, subjects were provided with a fluid bolus containing 2ml.kg^{-1}.body mass of their respective drink every 15 minutes during exercise. Starting at 11:00, subjects ran at 60% $\dot{V}O_2$max for 120 minutes with a 1% treadmill grade. Within the 15 minutes that followed the trial, blood and saliva samples were collected before subjects showered and returned to the laboratory. Exactly 20 minutes post-exercise (13:20), subjects were sensitised with DPCP (see below). Further blood and saliva samples were collected 1 hour post-exercise. During exercise, 60s steady state gas collections were analysed during minutes 10, 20, 40, 50, 70, 80, 100, and 110 using the

Douglas bag method detailed in chapter 2. HR and RPE were recorded every 15 minutes during exercise, and nude body mass (NBM) was recorded pre and post-exercise. DPCP elicitation was then performed exactly 28 days following this exercise trial (see below).

4.2.5 Drink composition

A lemon flavoured CHO-based sports drink (Go Energy, Science in Sport, Nelson, U.K.) was prepared according to the manufacturer's guidelines (with the exception that 100 mL of water was replaced with 100 mL sugar-free, artificially sweetened lemon-flavoured drink concentrate (Tesco, Dundee, U.K.) to give a CHO concentration of 10% w/v. This was provided to subjects in the CHO condition. The PLA drink was CHO-free, taste-matched as closely as possible, and made using a sugar-free, artificially sweetened lemon-flavoured drink concentrate (Tesco, Dundee, U.K.) which was diluted 4 parts water to 1 part concentrate with an additional ~1.25 g NaCl. An independent laboratory technician was responsible for preparing drinks ahead of the main trial in order to maintain double blind procedures. Experimental conditions and groupings were validated at the end of the study by checking drink osmolality using an osmometer (Osmocheck, Vitech Scientific Ltd, Horsham, UK).

4.2.6 Induction of contact hypersensitivity (sensitisation)

In line with previous studies, subjects were sensitised to DPCP at 13:20, exactly 20 minutes post-exercise (Harper-Smith et al., 2011; Diment, et al., 2015)to ensure that cutaneous blood flow had an opportunity to return to baseline (Kenny et al., 2008). An 11-mm filter paper disk was placed on a 12-mm aluminium Finn chamber (Epitest Oy, Tuusula, Finland) on Scanpor hypoallergenic tape. The disc was soaked in 22.8 µl of 0.125% DPCP in acetone and allowed to air dry for 5 minutes before being applied to the skin of the subjects' lower back. Subjects were required to keep this patch in place for exactly 48 hours. This method provided a dose of 30 $\mu g.cm^{-2}$ DPCP.

4.2.7 Elicitation

In accordance with the methods of Harper-Smith et al. (2011) and Diment et al. (2015), the magnitude of an *in-vivo* response was elicited by secondary exposure to DPCP. Exactly 28 days following the main trial and sensitisation to DPCP, subjects returned to the laboratory

and received a challenge with a dose-series of low-concentration DPCP (0.000, 0.0048, 0.0076, 0.0122, 0.0195 and 0.03125% DPCP). Six individual 7-mm filter paper disks were placed on six individual 8-mm aluminium Finn chambers (Epitest Oy, Tuusula, Finland) on Scanpor hypoallergenic tape. Each disk was soaked in 10 µl of the appropriate concentration of DPCP, and allowed to air dry for 5 minutes before being applied to the volar aspect of the subject's upper arm. This method provided a dose of: 0.00; 1.24; 1.98; 3.17; 5.08; and 8.12 $\mu g.cm^{-2}$ DPCP per patch, respectively. The location of the patches remained the same between subjects, but their allocation order varied to minimise any anatomical variability in responses. The allocation order was randomly assigned, but for every allocation pattern used in one group, there was an equivalent, identical pattern used in the other (i.e. this pattern was matched between subjects in each of the groups). Subjects were required to keep this patch in place for exactly 6 hours. The strength of *in-vivo* immune reactivity was assessed as cutaneous responses exactly 48 hours post-application.

4.2.8 Assessment of cutaneous responses

Triplicate measures of both skin oedema and skin erythema were taken for each of the six elicitation sites (CV for measures of oedema and erythema = 1.9% and 1.5% respectively). Skin oedema is the key measure of contact hypersensitivity (CHS) elicitation responses (Harper-Smith et al., 2011). Modified spring loaded callipers (Harpenden Skin-fold Calliper, British Indications, England) were used to measure skin-fold thickness at each elicitation site to the nearest 0.1 mm. Skin-fold thickness was measured by the same investigator on all occasions by holding the callipers at a 90 degree angle to the skin, and placing the jaws of the callipers at the outer diameter of each elicitation site (measuring skin-fold only, not subcutaneous fat) (Harper-Smith et al., 2011; Diment et al., 2015). An erythema meter (ColorMeter DSM11, Cortex Technology, Hadsund, Denmark) was used to determine skin erythema at each elicitation site (Harper-Smith et al., 2011; Diment et al., 2015). The mean value of the triplicate measures was calculated for each site and, in order to determine the true increase in skin oedema and erythema, the value from the acetone only site was subtracted from each patch site value. The dose-response for all of the sites minus acetone were summed to give the overall reactivity of each subject to DPCP (∑-oedema, and ∑-erythema) (Harper-Smith et al., 2011; Diment et al., 2015).

4.2.9 Upper respiratory tract infections

URTIs were recorded by subjects for 2 weeks following the main trial using the method detailed in chapter 2.

4.2.10 Saliva sample collection

Saliva samples were collected via passive drool and processed as described in chapter 2 pre, post, and 1 hour post exercise.

4.2.11 Blood sample collection

Blood samples were collected and processed as described in chapter 2. Blood samples were collected pre, post and 1 hour post exercise. A full study's worth of blood samples were only available for 14 of the 26 subjects.

4.2.12 Determination of saliva SIgA

Saliva SIgA concentration and secretion rate were measured using an in house ELISA as described in chapter 2.

4.2.13 Saliva osmolality

Saliva osmolality was measured using a freezing point osmometer (Osmomat 030 Series M, Gonotec, Berlin) which had been calibrated prior to use following the manufacturer's guidelines.

4.2.14 Adrenaline and noradrenaline

Plasma epinephrine and norepinephrine concentrations in K_3EDTA plasma were determined using a commercially available CatCombi ELISA (IBL International, Hamburg, Germany). Samples were measured in duplicate (CV for epinephrine and norepinephrine = 5.4% and 4.0% respectively).

4.2.15 Cortisol

Plasma cortisol concentration in heparinised plasma was determined using a commercially available ELISA kit (DRG, Germany; Biomercia, California, U.S.A.). Samples were measured in duplicate with an intraassay CV of 2.3%.

Table 4.1 Subject characteristics and physiological response data

	Placebo (*n* = 12)	**Carbohydrate (*n* = 12)**	***P* value**
Age (years)	32 (9)	30 (8)	0.724
Height (m)	1.78 (0.05)	1.78 (0.05)	0.752
Weight (kg)	76.31 (10.73)	77.08 (10.69)	0.859
$\dot{V}O_2$max (L.min^{-1})	4.2 (0.6)	4.7 (0.5)	**0.030**
$\dot{V}O_2$max (ml.kg^{-1}.min^{-1})	55.61 (6.69)	61.58 (5.60)	**0.025**
VT1 (L.min^{-1})	2.1 (0.3)	2.3 (0.4)	0.388
$\dot{V}O_2$max during trial (%)	58.11 (3.88)	57.08 (4.87)	0.565
Mean HR (BPM)	136 (17)	135 (11)	0.876
Mean RPE (Borg, 1970)	11 (2)	11 (2)	0.533

Mean (SD)

4.2.16 Determination of serostatus

EBV serology was determined from samples collected at baseline, using the methodology detailed in chapter 2.

4.2.17 EBV Reactivation

The concentration of EBV pre, post and 1 hour post exercise was determined by qPCR using the methodologies detailed in chapter 3.

4.2.18 EBV Status and Quantification

The majority of sport and exercise studies that have previously investigated EBV have simply classified athletes as positive or negative for EBV (Gleeson, et al., 2017), or reported percentages of samples with detectible EBV and those which were positive for EBV (Cox et al., 2004). These data are presented in the present study, but in addition, the absolute change in EBV concentration pre, to 1 hour post exercise was calculated.

To investigate the relationship between EBV expression and DPCP responses, DPCP responders were classified as high and low (i.e. the greater the ∑-oedema and/or ∑-erythema the higher the response) by splitting the groups down the middle. The absolute change in EBV concentration was explored between these two groups.

4.2.19 Statistical analysis

All results are presented as mean (SD) unless otherwise stated. For data that was not normally distributed (WBC, granulocytes, monocytes, adrenaline, noradrenaline, haematocrit, and EBV) the median and interquartile range (IQR) are reported. A significance level of 0.05 was pre-set for all statistical analyses. Normal distribution within the data was analysed using the Shapiro-Wilk test. For normally distributed variables, means of subject characteristics and exercise trial physiological responses were compared using Independent Samples T-Tests. Illness episodes were compared using the Chi Squared Test with the Fisher's Exact Test statistic reported. The log transformed and square-roots of data for which normal distribution could not be assumed were first tested using the Shapiro-Wilk test before non-parametric tests were undertaken. Adrenaline, noradrenaline, and monocytes could be normalised through the log transformation or the square-root of the data. The means, and changes over time for these variables were compared using two-way mixed ANOVAs. Mann Whitney U tests were undertaken for WBC, granulocyte, haematocrit, EBV concentration, and EBV concentration change in relation to the ∑ of oedema and erythema. Where post-hoc tests were required, Wilcoxon tests were undertaken to identify the effects of time. Pearson correlations were used to analyse the correlations between EBV, SIgA, and URTI. All other means and changes over time were compared using two-way ANOVAs. Significant differences were identified using the Holm-Bonferonni Test. All tests were carried out using SPSS Version 24.0 (IBM Corp, Armonk, NY, USA).

4.3 Results

4.3.1 Subject characteristics and physiological response data

There were no significant differences in subjects' characteristics or measures of physiological response (table 4.1).

Table 4.2 Acute responses to 1.5 hours treadmill running at 60% $\dot{V}O_2max$

	Baseline	Post	1H Post
SIgA concentration (mg.L^{-1})			
PLA (*n* = 12)	279 (76)	274 (85)	262 (86)
CHO (*n* = 10)	329 (106)	361 (103)	282 (93)
SIgA secretion rate (µg.min^{-1})			
PLA (*n* = 12)	104.8 (60.5)	113.7 (86.3)	122.4 (62.8)
CHO (*n* = 10)	129.3 (54.6)	167.6 (91.0)	142.5 (73.4)
SIgA concentration : osmolarity			
PLA (*n* = 12)	3.64 (0.10)	-	3.60 (1.12)
CHO (*n* = 10)	4.65 (1.29)	-	4.29 (1.40)
Adrenaline (pg.mL^{-1})			
PLA (*n* = 7)	46 (66)	795 (50)	62 (24)
CHO (*n* = 7)	68 (42)	91 (139)	62 (24)
Noradrenaline (pg.mL^{-1})		*	
PLA (*n* = 7)	643 (171)	784 (419)	707 (203)
CHO (*n* = 7)	368 (297)	**869 (360)** †	516 (255)
Cortisol (ng.mL^{-1})			
PLA (*n* = 7)	256 (124)	368 (186)	374 (141)
CHO (*n* = 7)	379 (134)	**224 (104)** †	**209 (85)** †
WBC (×10^9 cells.L^{-1})		*	**
PLA (*n* = 8)	5.07 (2.46)	**9.48 (4.71)**†	**10.54 (5.94)**†
CHO (*n* = 6)	5.16 (0.94)	**6.53 (0.88)**†	6.09 (0.82)
Lymphocytes (×10^9 cells.L^{-1})			
PLA (*n* = 8)	1.24 (0.40)	1.55 (0.61)	1.26 (0.52)
CHO (*n* = 6)	1.55 (0.63)	1.52 (0.33)	1.42 (0.43)
Granulocytes (×10^9 cells.L^{-1})		*	**
PLA (*n* = 8)	3.25 (1.89)	**6.86 (3.56)** †	**8.14 (3.99)** †
CHO (*n* = 6)	2.98 (0.94)	**4.27 (0.48)** †	**3.85 (0.58)** †
Monocytes (×10^9 cells.L^{-1})			
PLA (*n* = 8)	0.66 (0.46)	0.84 (1.02)	0.97 (0.90)
CHO (*n* = 6)	0.80 (0.49)	0.69 (0.45)	0.75 (0.40)
GLR		*	**
PLA (*n* = 8)	2.97 (1.20)	5.61 (3.15)	**6.37 (2.21)** ††
CHO (*n* = 6)	2.32 (1.26)	2.91 (0.76)	**3.07 (1.27)** †
Haematocrit (%)			
PLA (*n* = 8)	40.0 (3.4)	40.6 (2.8)	40.3 (2.1)
CHO (*n* = 6)	40.2 (2.9)	41.0 (1.9)	39.3 (3.8)
Haemoglobin (g.L^{-1})			*
PLA (*n* = 8)	153.9 (8.8)	154.0 (6.8)	151.8 (7.5)
CHO (*n* = 6)	154.0 (13.4)	154.8 (12.5)	150.8 (13.3)

Mean (SD), except for WBC, granulocytes, monocytes, adrenaline, noradrenaline, and haemoglobin whereby data are presented as Median (Interquartile Range (IQR)

* ($P < 0.05$) indicate significant change from baseline (data grouped as there was a main effect of time)

** ($P \leq 0.001$) indicate a significant change from baseline (data grouped as there was a main effect of time)

† ($P < 0.05$) indicate significant change from baseline (post post hoc follow-up for time: each group analysed separately due to significant group × time-point interaction).

†† ($P \leq 0.001$) indicate significant change from baseline (post post hoc follow-up for time: each group analysed separately due to significant group × time-point interaction).

4.3.2 URTI Incidence

Three subjects (13%) suffered a URTI during the two weeks following the exercise trial. Episodes lasted an average of 4 (1) days, had a peak severity of 3 (1), an illness score of 9 (1), and a cumulative score of 14 (6). Data were then grouped into CHO and PLA conditions. There were no significant differences in the number of URTI episodes in the CHO (n = 2) and PLA (n = 1) groups (P = 0.571). Episodes lasted an average of 4 (1) days in the CHO group and 3 days in the PLA group, had a peak severity of 3 (1) in the CHO which remained unchanged in the PLA group (peak severity = 3), an illness score of 10 (1) in the CHO group compared to 9 in the PLA group, and a cumulative score of 12 (7) compared to 18 in the PLA group.

4.3.3 SIgA responses

SIgA data were grouped into CHO and PLA conditions. SIgA concentration showed a main effect of time (P = 0.035), but not group (P = 0.134). SIgA secretion rate did not show a main effect of time (P = 0.176) or group (P = 0.274). There was not a group × time interaction for either SIgA concentration or secretion rate (P = 0.169 and 0.353 respectively). Post-hoc analysis did not reveal any changes in SIgA concentration at any time point from baseline. SIgA concentration relative to saliva osmolarity did not change over time (P = 0.438), nor was there an effect of group (P = 0.523), nor was there a group × time interaction (P = 0.168) (see table 4.2).

4.3.4 Catecholamines and cortisol

Adrenaline and noradrenaline showed a main effect of time (P = 0.006 and <0.001 respectively), but not a main effect of group (P = 0.963 and 0.310 respectively). There was not a group × time interaction for adrenaline (P = 0.762), but there was for noradrenaline (P = 0.026). Post-hoc analyses did not reveal any changes in adrenaline from baseline, however noradrenaline was significantly higher immediately post-exercise compared to baseline (P = 0.002). Noradrenaline showed no main effect of time in the PLA group (P = 0.298), but there was a main effect of time in the CHO group (P = 0.002) with noradrenaline significantly higher immediately post exercise compared to baseline (P = 0.004). There was not a main effect of time (P = 0.811) or group (P = 0.242) for cortisol, but there was a significant group × time interaction (P = 0.004). Post-hoc analysis did not

identify a main effect of time in the PLA condition (P = 0.281), but there was a main effect of time in the CHO condition (P = 0.008) with a significant decrease in adrenaline from baseline both immediately post (P = 0.001) and 1 hour post (P = 0.042) (see table 4.2).

4.3.5 WBC Counts

WBCs and granulocytes were not normally distributed, and therefore non-parametric tests were conducted. There were no significant differences in WBC counts at baseline between groups (P = 0.755) but there was immediately post, and 1 hour post the exercise trial (P = 0.013 and 0.003 respectively). WBCs were higher in the PLA condition compared to CHO both immediately, and 1 hour post exercise (P = 0.012 and P = 0.012 respectively), but an increase in WBCs from baseline was only evident immediately post exercise in the CHO group (P = 0.046) There was not a significant difference in the granulocyte count between groups at baseline (P = 0.755) but there was immediately post (P = 0.001), and 1 hour post exercise (P = 0.001). Post-hoc tests identified a significant increase in circulating granulocytes from baseline in both the PLA and CHO conditions immediately post (P = 0.012 and 0.028 respectively) and 1 hour post exercise (P = 0.012 and 0.027 respectively) (table 4.2).

There was not a main effect of time (P = 0.089), group (P = 0.580), or a group × time interaction (P = 0.158) for lymphocytes. There was not a main effect of time (P = 0.168) or group (P = 0.362) in monocytes, but there was a time × group interaction (P = 0.026). Post-hoc analysis identified a main effect of time in the PLA group (P = 0.009) but not the CHO group (P = 0.794). There was a trend for an increase in monocytes immediately post (P = 0.059) but not 1 hour post exercise (P = 0.137) compared to baseline. There was a main effect of time (P <0.001), group (P = 0.032) and a time × group interaction (P = 0.002) for the GLR. Post-hoc tests identified a significant increase in the GLR from baseline immediately post (P = 0.031) and 1 hour post exercise (P <0.001). There was a main effect of time in both the PLA (P = 0.001) and CHO group (P = 0.039) with a significant increase in the GLR at 1 hour post (P = 0.001 and 0.036 respectively) but not immediately post (P = 0.061 and 0.319 respectively) compared to baseline in both groups (table 4.2).

There was a main effect of time (P = 0.005) for haemoglobin, but not group (P = 0.993) or a group × time interaction (P = 0.771). Post-hoc analysis identified a significant decrease in haemoglobin 1 hour post (P = 0.016) but not immediately post (P = 1.000). There were

no significant differences in haematocrit at baseline, immediately post, or 1 hour post (P = 0.491, 0.573, and 1.000 respectively) (table 4.2).

4.3.6 EBV Serology

Nineteen (83%) of 23 subjects tested were seropositive for EBV (bloods were not available for 1 subjects). Of the nineteen EBV-seropositive subjects, 15 had also provided saliva samples (five in the placebo group and 10 in the carbohydrate group). Ten out of fifteen subjects (67%) had detectible levels of EBV DNA in their saliva at baseline (table 4.3).

Table 4.3 EBV detection in saliva

	Baseline	**Post**	**1 Hour post**
% of samples positive for EBV			
PLA (n = 5)	80 (4/5)	50 (1/2)	100 (5/5)
CHO (n = 10)	60 (6/10)	100 (3/3)	63 (5/8)
Median EBV concentration (ng.µl^{-1}) ×10^{-6} (IQR)			
PLA (n = 5)	0.79 (1.00)	3.09 (0.00)	0.49 (2.86)
CHO (n = 10)	0.94 (5.0)	0.31 (-)	1.27 (7.66)

Within the 20 minute window post-exercise, ahead of DPCP sensitisation, the priority was given to subjects showering ahead of the timely application of the DPCP patch. Any delay in obtaining saliva samples (e.g. reduced saliva flow rate) lead to this specific sample collection time-point being abandoned. Owing to a lack of data immediately-post exercise, therefore, these data were excluded from further analysis. A further 3 subjects' data were also excluded as they were missing a sample from the required time-points pre, or 1-hour post exercise. The following analyses were therefore undertaken on the data of 12 subjects (four in the placebo group and eight in the carbohydrate group).

There was not a significant effect of time (P = 0.929), nor were there differences in EBV concentration at baseline between groups (P = 0.732) or 1 hour post (P = 0.231) (see table 4.4). There were no significant differences between any of the four time points (P = 0.753).

Table 4.4 Median EBV concentration ($ng.\mu l^{-1}$) $\times 10^{-6}$ (IQR)

	Pre	1 hour post
PLA (*n* = 4)	0.72 (0.52)	1.88 (2.02)
CHO (*n* = 8)	1.78 (2.66)	1.68 (3.49)

There was no significant correlation between the change in SIgA concentration or secretion rate pre to 1 hour post exercise and the change in EBV concentration pre to 1 hour post exercise (r = 0.222 and 0.027 respectively; P = 0.537 and 0.942 respectively).

There was no significant correlation between the ∑-oedema, or the ∑-erythema 48 hours following elicitation and the EBV concentration 1 hour post exercise (r = 0.088 and 0.316 respectively; P = 0.809 and 0.374 respectively). There was not a significant difference between the change in EBV concentration pre, to 1 hour post exercise when subjects were grouped by DPCP ∑-oedema responses 48 hours following elicitation (P = 0.690), nor when EBV change was grouped by DPCP ∑-erythema responses 48 hours following elicitation (P = 0.841). The relationship between EBV expression and URTI could not be investigated as there were too few incidences of URTI in subjects for whom EBV analysis was undertaken (n = 1) for effective statistical analysis to be conducted.

4.4 Discussion

The aim of the present study was to investigate the relationship between the ingestion of CHO before, during and after exercise on URTI incidence, EBV DNA expression, and SIgA secretion rate and concentration. In addition, the interaction between URTI, EBV DNA expression, and SIgA secretion rate and concentration were investigated with the established *in vivo* response to DPCP. The main findings were that, despite CHO ingestion blunting the cortisol and leucocyte trafficking responses to prolonged treadmill running, there was no effect on the *in vivo* response of EBV reactivation, and no associated response between the CHS response to DPCP and the incidence of URTI, SIgA secretion rate, SIgA concentration, or EBV DNA expression. Previous research has demonstrated that, following prolonged treadmill exercise, *in vivo* immunity is reduced when assessed by CHS to DPCP (Davison et al., 2016; Diment et al., 2015; Harper-Smith et al., 2011), however we are not aware of any research that has been published to date detailing the acute reactivation responses of EBV following an acute bout of exercise.

In the present study, 83% of subjects were seropositive for EBV which is in line with estimates in the general population (Pottgiesser et al., 2006). 67% of seropositive subjects had detectible levels of EBV DNA in their saliva ahead of the exercise trial. This is very similar to the findings of Gleeson et al., 2002 where 64% of swimmers studied presented with evidence of latent EBV viral shedding. Both the present study, and Gleeson et al. (2002) have found a greater number of subjects to be shedding EBV viral DNA into saliva compared to previous studies, where the proportion of subjects presenting signs of a latent EBV infection reactivating typically range from 17 - 21% (Mehta et al., 2000; Payne et al., 1999).

EBV reactivation is an emerging marker of *in vivo* immune function and is believed to be a sign of an upset immune balance which has been linked to the occurrence of URTI (Gleeson et al., 2002). Given the findings of Gleeson et al. (2002) we hypothesised that there would be a link between EBV reactivation and the incidence of URTI within the present study. However, contrary to our original hypothesis, there was not a discernible relationship between EBV expression and SIgA responses, or EBV expression and the CHS responses to DPCP. This may be due to the acute nature of the present study. The majority of subjects had detectible levels of EBV in their saliva before the start of the trial and therefore it may not have been a reactivation, as has been observed by longitudinal monitoring studies, such as Gleeson et al. (2002), but rather an increase in viral load.

One established marker of *in vivo* immunity is CHS which measures the DTH response to a novel allergen. A T-cell mediated immune response at a local dermal level results in an immune response (Delves et al., 2011). In line with previous studies, the novel allergen DPCP was used (Davison et al., 2016; Harper-Smith et al., 2011; Jones et al., 2017; Sleijffers et al., 2001). Exercise has been shown to result in a diminished DPCP response (Davison et al., 2016), and the ingestion of CHO was not effective at preventing the exercise-induced decrease of *in vivo* immune function (Davison et al., 2016). CHO ingestion in the current study also appears to be ineffective in improving the *in vivo* immune response of viral EBV DNA shedding into saliva (i.e. decreasing the amount of viral DNA shed into saliva) despite CHO ingestion blunting the cortisol and leucocyte trafficking responses, as would be expected. Both CHS and the shedding of EBV-DNA from B-cells into saliva are T-cell mediated (Delves et al., 2011; Diment et al., 2015) and there may, therefore, be a shared explanation as to why CHO supplementation does not

appear to influence these *in vivo* immune responses. We recognise that a limitation of the present study is that we did not investigate the under laying mechanisms of the *in vivo*, T cell mediated immune responses. However, there does not appear to be a relationship between the strength of DTH response and the amount of EBV DNA that was shed from infected B-cells into saliva, therefore, we speculate that the mechanistic pathways, although T-cell dependent, do not interact, although both appear to remain unaffected by the ingestion of CHO. One further limitation of the present study is that a non-exercising control group was not present. The differences in overall *in vivo* immune responses following primary exposure to DPCP have, to date, only been observed between exercising subjects, and non-exercising controls (Davison et al., 2016; Harper-Smith et al., 2011). It may be, therefore, that the utility of *in vivo* markers of immune function may only be of benefit when contrasts are being made between exercising and non-exercising controls. However, as discussed earlier, differences in host defence can be detected through the use of *in vivo* immune techniques, without the need for a non-exercising control group (Jones et al., 2017) and this therefore requires further investigation.

URTIs can prove troublesome for athletes engaged in both training and competition (Pyne, et al., 2000; Pyne & Gleeson, 1998) and their cause has, therefore, come under a lot of scrutiny in the past. Longitudinal studies have identified EBV reactivation, in addition to other herpes group viruses such as cytomegalovirus, to be associated with long-term fatigue, poor performance and, in some endurance sports, URS (Gleeson et al., 2002; Reid et al., 2004; Walsh et al., 2011*a*). Not all cases of URTI in athletes can be attributed to EBV reactivation, however (Cox et al., 2004), with anything from 22 - 64% of athletes' URS being linked (Gleeson et al., 2002; Reid et al., 2004). In the present study, when the change in EBV concentration pre- to post exercise is correlated with URTI, there is not an association. There are obvious differences in the methodologies employed by this study compared to others which may be why the findings of this study do not agree with the findings of previous research. In the present study, 14% of subjects reported a URTI in the 2 weeks following the exercise trial. Although the current rates of URTI are not as high as those reported by some studies (Peters et al., 2010; Peters & Bateman, 1982), the findings of the present study align closely with the findings of Nieman et al. (1990) who reported that 12.9% of subjects suffered with a URTI in the 2 weeks following their participation in the Los Angeles Marathon. However, it should be noted that the Los Angeles Marathon is a competitive, mass participation event whereby the study subjects' exposure to other athletes and, subsequently, the number of circulating pathogens they came into contact

with would have been greater than that of subjects participating in a study conducted within a controlled laboratory setting. In the present study, there wasn't a statistically significant difference between the number of subjects reporting a URTI in the PLA (n = 1) and CHO (n = 2) groups. Nieman et al., 2002, also found no difference in URTI incidence between subjects who had been supplemented with PLA or CHO during a marathon race (n = 98). This suggests that CHO supplementation may not be beneficial for preventing URTI in the 2 weeks that follow an intensive bout of exercise.

The effects of CHO ingestion on *in vivo* immunity has been previously reported (Davison et al., 2016) in a novel study undertaken by members of this research group. Davison et al. (2016) reported that CHO supplementation did not benefit *in vivo* immune function. It appears that the *in vivo* marker of EBV reactivation, as employed by the present study, is also unaffected by CHO supplementation. However, without a rested control group, it is not possible to draw conclusions as to the acute effects of exercise on EBV expression. The findings of the present study, that CHO supplementation was not beneficial to *in vivo* immunity post exercise, are not only supported by our previous findings in to the effect of CHO supplementation on the CHS response to DPCP (Davison et al., 2016), but also to those of Nieman et al. (2002). Nieman et al. (2002) compared the effects of PLA or CHO supplementation during a marathon race on various physiological and immunological markers. Despite the fact that CHO supplementation resulted in a lower post-race cortisol concentration, there was no difference between the groups in terms of SIgA responses (an established marker which relates to URTI risk (Albers et al., 2013 & 2005) or URTI incidence in the 15 days following the race. The findings of the present study do agree with the data reported by Nieman et al. (2002). However, previous research has shown CHO supplementation to be of benefit to other immune markers such as leucocyte trafficking and anti-inflammatory cytokines such as IL-6, IL-10 and IL-1ra (Chen, et al., 2008; Davison & Gleeson, 2005; Gleeson, 2007; Nieman, 1998), owing to the maintenance of blood glucose during exercise, and the blunting of exercise-induced increases in stress hormones, such as cortisol (Walsh, et al., 2011*a*). From the research to date, there does not appear to be an effect of CHO supplementation on SIgA responses (Walsh et al., 2011*a*). In line with previous research, CHO supplementation during the present study resulted in a lower post-exercise cortisol concentration and the attenuation of the leucocytosis observed in the CHO group, but did not affect SIgA responses.

Neither SIgA concentration or secretion rate in the present study were affected by CHO ingestion. This finding is in line with previous research (Li & Gleeson, 2005; Nieman et al., 2002). However, SIgA concentration and secretion rates were also unaffected by the exercise stimulus. There is, typically, a depression in SIgA immediately following intensive exercise (Gleeson & Pyne, 2000) in addition to a delayed period of time following exercise (up to 18 hours) in which SIgA concentration returns to baseline (Peters and Bateman, 1983). The same acute, and delayed effects of exercise on mucosal immunity were not observed in the present study which may be due to the duration and/or intensity of the exercise stimulus. A post-exercise decrease in SIgA has been a consistent observation made by many studies within an athletic population (Gleeson et al., 2002; Gleeson et al., 1996; Gleeson et al., 1995; MacKinnon et al., 1989). Furthermore, Gleeson et al. (2002) presented the time-sequence of SIgA suppression, detection of EBV-DNA, followed by the appearance of URS. This same pattern was not observed in the present study and there did not appear to be an association between SIgA, URTI, or EBV-DNA. None of the subjects in the present study presented with an SIgA concentration below 40 $mg.L^{-1}$, or experienced a 40% drop in SIgA secretion rate from baseline, which have been previously stated as the thresholds at which an athlete's risk of developing a URTI significantly increases (Gleeson et al., 1999*a*; Neville et al., 2008). If the exercise stimulus employed by the current study was not enough to cause a drop in SIgA severe enough to leave subjects vulnerable to URTI, it may also not have been severe enough to cause an increase in the expression of EBV DNA into saliva, as detailed by Gleeson et al. (2002). EBV infected B-cells are normally controlled by cytoxic T-cells which maintain the suppression of the virus (Cruchley et al., 1997). If the immune system is put under stress (e.g. following intensive exercise), there is a transient suppression of cytotoxic T-cells (Gabriel & Kindermann, 1997) and it is this diminished T-cell control that is thought to be responsible for the expansion of the latent EBV infected-B-cells (Gleeson et al., 2002). The response of cytotoxic T cells, observed following aerobic exercise lasting up to, but not more than 2 hours, is much more dependent on the exercise intensity, however, with the greatest responses being observed at anaerobic threshold (Gabriel & Kindermann, 1997). None of the athletes in the present study were working at intensity above their anaerobic threshold; however, the T-cell responses to the exercise stimulus were not measured in the present study. This means that the reasons behind the lack of change in EBV-DNA concentration cannot be established, but it is likely owing to the exercise intensity and duration.

4.5 Conclusion

In conclusion, CHO supplementation does not appear to be of benefit to the *in vivo* immune measure of salivary EBV DNA reactivation. This is despite CHO supplementation blunting the exercise induced cortisol response, and attenuating the leucocyte responses following exercise. In the present study, there is not a correlation between DPCP responses and the change in EBV concentration, nor is there a correlation between the change in EBV concentration and SIgA. The effects with more stressful, or fasted exercise remain to be determined. However, there appears to be no benefit under the conditions of this, and the previous study by Davison et al. (2016), of CHO supplementation on *in vivo* immunity.

Chapter 5

The effect of 4 weeks *Chlorella pyrenoidosa* supplementation on immune responses to 2 days intensified training: A between groups, double blind, placebo-controlled study

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Abstract

Background It is widely reported that athletes engaged in regular prolonged activity and/or strenuous exercise have a higher than normal incidence of upper respiratory tract infection (URTI) which may be related to an exercise-induced impairment of immune function, One food supplement that has been suggested to have beneficial effects on immune function is *Chlorella pyrenoidosa* (CHL). However, there are relatively few studies of humans *in vivo*, and fewer still on athletes, and those with high physical activity levels.

Purpose To investigate the effects of supplementation with CHL on leucocyte, mucosal immune responses, and EBV reactivation to two days intensified training.

Methods Twenty-six subjects (age 29 ± 9 years; $\dot{V}O_2$max 53.7 ± 11.7 mL.kg^{-1}.min^{-1}) provided resting blood and saliva samples for determination of leucocytes, SIgA, and salivary EBV-DNA at baseline, and following 4, 5 and 6 weeks of daily supplementation with CHL (n = 13) or placebo (n = 13). During week 4, a 2-day intensified training period was undertaken [morning and afternoon sessions each day, respectively: $\dot{V}O_2$max test, high-intensity interval exercise (HIIE, 3 × 30 s Wingate sprints); 90 min at ~60% $\dot{V}O_2$max; 3 × 30 s HIIE].

Results CHL increased resting SIgA secretion rate (trial × time, P = 0.016: no change with PLA but increases with CHL at week 4 and 5, P = 0.034 and 0.032 respectively. PLA vs. CHL: week-0 = 53.8 ± 33.2 vs. 57.2 ± 36.9 µg.min^{-1}; week-4 = 54.3 ± 34.5 vs. 83.1 ± 57.0 µg.min^{-1};, and week-5 = 62.7 ± 45.8 vs. 98.0 ± 47.1 µg.min^{-1} respectively. Minimal acute changes in SIgA were seen in response to individual exercise bouts, but it was higher at some times in the CHL group (for bouts 2 and 3).

Conclusions Supplementation with CHL has beneficial effects on resting SIgA, which might be beneficial during periods of intensified training.

5.1 Introduction

During, and in the hours and days following periods of intensified training, athletes are at a greater risk of developing URTI and/or URS compared to their sedentary peers (Kakanis et al., 2010). Athletes' increased risk of infection is believed to be due to immune disturbances which arise as a result of the training intensity and duration, endured by both some recreational, and elite athletes alike (Gleeson, 2007; Walsh et al., 2011*a*). Recently it has been shown that large variations in training intensity, particularly acute increases in training load, have been shown to be a good predictor of the development of URS in

athletes (Svendsen et al., 2016). This supports previous research in the area which demonstrates a relationship between training load and URTI risk (Dias et al., 2011; Fahlman & Engels, 2005; Klentrou et al., 2002). There has been an interest in the use of nutritional interventions to help manage and attenuate the immune disturbances that are observed in athletic populations, but there is often limited evidence available (Bermon et al., 2017; Davison et al., 2014; Walsh et al., 2011*b*). One nutritional supplement which has been shown to be beneficial for immune function, is *Chlorella pyrenoidosa* (CHL).

Chlorella pyrenoidosa is a freshwater cyanobacteria, single-celled, freshwater microalgae, of which there are approximately 8,000 species in the genus. Many claims have been made with regard to chlorella's beneficial effects on health including: boosting immune function and fighting infections (Halperin et al., 2003; Merchant & Andre, 2001); reducing high blood pressure and lowering cholesterol (Merchant & Andre, 2001); helping to control blood glucose in diabetes (Cherng & Shih, 2006); preventing the spread of cancer and slowing the growth of tumours (Merchant et al., 1990); and helping to relieve the symptoms of fibromyalgia (Merchant et al., 2001). Although not all of the abovementioned claims are supported by an abundance of literature, there is a substantial and growing body of evidence to support chlorella's use as an immunostimulant, and its safety has been confirmed through a number of *in-vivo* studies.

Despite the substantial pool of research investigating the effects of chlorella on a variety of chronic illnesses and ailments, there is limited research within the field of sport and exercise science. The research that does exist in the wider, non-sports specific fields has shown that chlorella supplementation can improve Th1 immune responses *in vitro* (Ewart et al., 2007) and *in vivo*; (Kwak et al., 2012) and mucosal immunity *in vivo* (Otsuki et al., 2011 & 2016); increase IFN- γ *in vitro* (Ewart et al., 2007) and *in vivo* (Kwak et al., 2012), TNF-α *in vitro* (Ewart et al., 2007, Pugh et al., 2001), IL-1β *in vitro* (Kwak et al., 2012, Pugh et al., 2001), IL-12 *in vitro* (Kwak et al., 2012), IL-2 in mice (An et al., 2008), and NK-cell activity *in vivo* (Kwak et al., 2012). In addition, Hsu et al. (2010) reported the activation of IL-1 mRNA expression; proIL-1 protein expression; and IL-1 secretion from macrophages in addition to the up regulation of LPS induced TNF-α and IL-1β secretion in response to hot-water-soluble polysaccharides from chlorella pyrenoidosa (CWSP) *in vitro*. Ewart et al. (2007) reported that the beneficial effects of chlorella appeared to be limited to Th-1 immune responses *in vitro*, with no effects observed in IL-4 or IL-13 in mice (An et al. 2008). Increases in both SIgA levels and secretion rates (Otsuki et al., 2011; Otsuki et

al., 2012) through short-term use (4-8 weeks) in healthy, human populations consuming 5-6 g of chlorella per day have been reported. It has also been shown, through studies in mice with acquired immunodeficiency syndrome, to boost immune responses to both bacterial and viral infections through increasing the number of $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}\alpha\beta$ T cells (Hasegawa et al., 1995); and to accelerate superoxide generation and chemokinesis in polymorphonuclear leucocytes (Tanaka et al., 1986) in murine species.

Only one study to date has measured immune responses to chlorella supplementation in athletes. Ten female kendo athletes' diets were supplemented with either chlorella or a placebo for 4 weeks ahead of a 5 day training camp. SIgA concentration and secretion rates maintained near-baseline levels throughout the duration of the training camp in the experimental group. Athletes' SIgA concentrations and secretion rates demonstrated a significant reduction from baseline in the control group (Otsuki et al., 2012). The findings of this study, although in only a limited cohort of athletes, demonstrate that chlorella may help attenuate the suppression in immune function observed following both acute bouts of exercise and during intensified periods of training.

The problem with a large number of these studies, however, is that they are conducted in murine species, often with *in-vitro* analysis using CWSP as opposed to direct supplementation *in vivo*. For those studies conducted in humans, they are within populations with underlying health conditions, and do not consider any wider effects the supplementation may be having. The purpose of this study was to investigate the effects of CHL supplementation (SunChlorella Corporation, Kyoto, Japan) on immune responses to a two day intensified training period in trained cyclists.

5.2 Methodology

5.2.1 Subjects

Twenty six trained cyclists (twenty-one males and five females) aged 29 ± 9 years (mean ± SD) volunteered for the study (height 173.3 ± 7.4 cm; weight 71.2 ± 11.1 kg; maximal aerobic capacity ($\dot{V}O_2max$) 53.65 ± 11.69 $mL.kg.min^{-1}$). Subjects spent an average of 8 hours training per week, and were excluded from participation if they were using nutritional supplements or medication, or if they had given blood, received vaccinations, or suffered an infection within one month of the study commencing. All subjects provided

informed consent before participation. Ethical approval for the study was granted from the University of Kent's Ethics Committee.

5.2.2 Supplements

In line with previous research, the final dose of 6 g per day (30 tablets) of CHL tablets (Sun Chlorella 'A' tablets, Sun Chlorella Corporation, Kyoto, Japan) or placebo (Otsuki et al., 2011, 2012 & 2016) (see table 5.1 for nutritional composition). The placebo tablets were also provided by Sun Chlorella Corporation and were indistinguishable from the CHL tablets. Supplements were provided in sealed foil pouches with a blinding code that was held by the manufacturer and not revealed until after all analyses had been completed.

Table 5.1 Nutritional values of placebo and Sun Chlorella 'A' tablets

	Placebo	**Chlorella**
Energy, kcal.6g^{-1}	24.36	23.22
Moisture, g.6g^{-1}	0.19	0.29
Protein, g.6g^{-1}	0.12	3.28
Fat, g.6g^{-1}	0.35	0.71
Total carbohydrate, g.6g^{-1}	5.21	1.28
Sugar, g.6g^{-1}	5.14	0.53
Dietary fibre, g.6g^{-1}	0.07	0.75
Ash, g.6g^{-1}	0.13	0.43

5.2.3 Study Design

Subjects reported to the laboratory between 06:30 and 08:30 hours following an overnight fast. Baseline blood and saliva samples were collected from subjects using the standard sampling methods detailed in chapter 2. Subjects were randomly assigned to either the experimental (CHL) or control (PLA) group and provided with the first four weeks of supplementation (either CHL or a PLA tablets), along with daily illness questionnaires (see chapter 2). Subjects were advised to swallow their tablets with water, consuming half alongside their breakfast and half alongside their evening meal for the duration of the study. The dose was gradually increased over the first 3 days starting with a total of 10

tablets per day (2 g) on day one; 20 tablets per day on day two (4 g); and finishing with to 30 tablets per day (6 g) which was then maintained for the duration of the study. Subjects were advised to take their tablets with water, consuming half alongside their breakfast, and half alongside their evening meal.

Subjects collected weekly saliva samples at home for 4 weeks and stored these at -20 ºC. After 4 weeks, subjects reported to the laboratory between 06:30 and 08:30 hours following an overnight fast. Height and weight were recorded and subjects completed a ramped $\dot{V}O_2$max test until exhaustion, as detailed in chapter 2. Blood and saliva samples were collected pre, post, and one-hour post exercise using the methodology detailed in chapter 2.

Subjects were allowed to return home following sample collection, and then reported to the laboratory again between 16:00 and 18:00 hours in a euhydrated state having refrained from eating within the hour preceding the test to complete a HIIE session (detailed in chapter 2) (HIIE1). Saliva samples were collected pre, post, and one hour post exercise. The following morning, subjects once again reported to the laboratory between 06:30 and 08:30 hours following an overnight fast. Baseline blood and saliva samples were collected, and subjects' NBM was recorded. Subjects were then fitted with a HR monitor (Polar Electro, Kempele, Finland) and a facemask (Cortex Biophysik GmbH, Leipzig, Germany) connected to a breath by breath gas analyser (MetaLyzer 3BR2, Cortex Biophysik GmbH, Leipzig, Germany) before undertaking a 90 minute steady state cycle (detailed in chapter 2). A water bolus containing 2mL.kg^{-1}.bm was provided every 15 minutes. Blood and saliva samples were collected immediately post and one hour post testing. Subjects NBM was recorded as soon as post samples had been collected. Following testing, subjects were once again allowed to return home and returned to the laboratory later the same day between 16:00 and 18:00 hours in a euhydrated state having refrained from eating in the hour preceding exercise. Subjects completed an identical HIIE session as undertaken the previous day (see chapter 2) (HIIE2). Saliva samples were once again collected pre, post, and one hour post.

Subjects continued to take their supplement, record daily illness diaries, and collect weekly saliva samples for a further 2 weeks following the intensified training period.

5.2.4 Biological samples

Blood samples, cell counts, vitamin D status, saliva samples, SIgA, and EBV serology were conducted using the methodology detailed in chapter 2
The concentration of EBV DNA in saliva samples was analysed by qPCR using the methods detailed in chapter 3.

5.2.5 Statistics

All results are presented as mean (SD) or, in the case of age, mean RPE for 90 minute cycle, severity of illness, all leucocyte responses, GLR, haematocrit, haemoglobin, and EBV, the median and interquartile range (IQR) are reported. A significance level of 0.05 was pre-set for all statistical analyses. Normal distribution within the data was analysed using the Shapiro-Wilk test. For normally distributed variables, means of subject characteristics, exercise trial performance, and illness duration, illness score, and symptom score were compared using Independent Samples T-Tests. Illness episodes were compared using the Chi Squared Test with the Fisher's Exact Test statistic reported. The log transformed and square-roots of data for which normal distribution could not be assumed were first tested using the Shapiro-Wilk test before non-parametric tests were undertaken. All leucocyte, haemoglobin, haematocrit, GLR, and EBV data could be normalised with log transformation or square roots. As such, two-way ANOVAs were conducted on the transformed data for these variables. Mann Whitney U tests were undertaken for age; mean RPE of the 90 minute trial; and severity of illness. All other means and changes over time were compared using two-way mixed ANOVAs. Significant differences were identified using the Holm-Bonferonni Test. All tests were carried out using SPSS Version 22.0 (IBM Corp, Armonk, NY, USA).

5.3 Results

5.3.1 Subject characteristics

There were no significant differences in subjects' physical characteristics and physiological responses between groups (tables 5.2 and 5.3), nor were there any significant

Table 5.2 Subject Characteristics

	Chlorella		**Placebo**		***P*-Value**
	(n = 13)		***(n = 13)***		
	Mean (SD/IQR)	**Range**	**Mean (SD/IQR)**	**Range**	**2 Tailed**
Age (yr)	23 (14)	19 - 43	29 (13)	19 - 45	0.204
Height (cm)	175.2 (7.3)	165.0 - 188.2	171.2 (7.0)	154.0 - 181.0	0.170
Weight (kg)	71.82 (9.38)	58 - 91.8	70.76 (12.52)	52.4 - 89.8	0.809
$\dot{V}O_2$max (mL.kg^{-1}.min^{-1})	57.68 (13.21)	39.70 – 79.67	50.93 (9.86)	34.19 – 69.14	0.153
No. (%) of females	1 (8)		4 (31)		

Table 5.3 Physiological responses to the $\dot{V}O_2$max Test on Day 1 of the Training Intervention

	Chlorella (*n* = 13)		**Placebo (*n* = 13)**		***P*-Value**
	Mean (SD)	**Range**	**Mean (SD)**	**Range**	
$\dot{V}O_2$max (L.min^{-1})	4.1 (0.7)	3.1 – 5.0	3.6 (0.7)	2.5 – 4.9	0.082
$\dot{V}O_2$max (ml.kg^{-1}.min^{-1})	57.7 (13.2)	39.7 – 79.7	50.9 (9.9)	34.2 – 69.1	0.153
Work rate (% max)	56.1 (7.7)	47.1 - 66.6	59.9 (6.0)	52.8 - 72.3	0.979
VT1 (L.min^{-1})	1.8 (0.4)	1.1 – 2.4	1.6 (0.3)	1.1 – 2.2	0.314
VT1 (ml.kg^{-1}.min^{-1})	25.3 (6.9)	15.5 – 36.4	23.6 (5.5)	15.4 – 33.5	0.497
VT1 (% max)	44.0 (8.0)	34.2 – 62.7	46.3 (5.5)	38.7 – 55.1	0.410
W_{max} (watts)	341 (55)	254 - 425	303 (61)	187 - 404	0.109
Time to exhaustion (min)	14.4 (1.8)	11.5 – 17.2	13.1 (2.0)	9.2 – 16.5	0.109

Table 5.4 Physiological responses to 90 Minute Cycling on Day 2 of the Training Intervention

	Chlorella (*n* = 13)		**Placebo (*n* = 13)**		***P*-Value**
	Mean (SD/IQR)	**Range**	**Mean (SD/IQR)**	**Range**	
Watts (W)	155 (28)	105 – 200	129 (30)	78 – 180	0.310
Target $\dot{V}O_2$ (L.min^{-1})	2.4 (0.5)	1.6 – 3.0	2.1 (0.4)	1.5 – 2.8	0.980
Mean HR (BPM)	138 (17)	110 – 170	133 (12)	115 – 153	0.378
Mean RPE	13 (2)	12 – 16	13 (2)	12 – 18	0.762
Mean $\dot{V}O_2$ (L.min^{-1})	2.4 (0.5)	1.6 – 3.1	2.1 (0.4)	1.6 – 2.9	0.344
Mean RER	0.9 ± (0.0)	0.9 – 1.0	0.9 (0.1)	0.8 – 1.1	0.190

Table 5.5 Change in NBM (kg) 90 Minute Cycling on Day 2 of the Training Intervention

	Chlorella (*n* = 13)		**Placebo (*n* = 13)**		***P*-Value**		
	Mean (SD)	**Range**	**Mean (SD)**	**Range**	**Group**	**Time**	**Trial × Time**
NBM Pre (kg)	71.5 (9.5)	56.4 - 92.0	70.6 (12.4)	52.0 - 89.8	0.872	**0.001**	0.097
NBM Post (kg)	70.9 (9.3)	55.8 - 90.4	70.4 (12.2)	51.6 - 89.3			

differences in subjects performance during the 90 minute steady state cycling trial (table 5.4). There was, however, a main effect of time in NBM pre to post exercise, but not a main effect of group, or a group by time interaction. Post hoc analysis identified a significant decrease in NBM over time (table 5.5).

5.3.3 Illness incidence

Four subjects in each group (31%) suffered with a URTI in the 2 weeks following the exercise intervention. There were no significant differences between the number of episodes (P = 1.000) (illness duration (PLA = 5 ± 2, CHL = 8 ± 5); peak severity (PLA [median] = 2 [IQR = 1], CHL [median] = 2 [IQR = 2]); illness score (PLA = 9 ± 5, CHL = 15 ± 11); symptom score (PLA = 11 ± 7, CHL = 22 ± 13).

5.3.4 Cell counts

There was no significant main effect of group for total WBC (P = 0.245), lymphocyte (P = 0.962), or monocyte numbers (P = 0.233), nor was there significant time × group interactions for the same (P = 0.938, 0.482, and 0.997 respectively). There were, however, significant effects of time observed in total WBC, lymphocyte, and monocyte numbers (P <0.001, <0.001, and <0.001 respectively) with post-hoc tests showing a significant increase in total WBCs, lymphocyte, and monocyte numbers from baseline (pre supplementation) to immediately post 90 minutes (P <0.001, P = 0.022, 0.001 respectively). Total WBCs also showed a significant increase from baseline to 1 hour post 90 minutes (P <0.001). There was a significant main effect of group on total circulating granulocyte numbers (P = 0.025) and a main effect of time (P <0.001). Post-hoc tests showed no significant effect of group at any time point. For both the placebo and chlorella groups, however, a significant increase in circulating granulocyte numbers was observed from baseline (pre supplementation) to immediately post exercise (P = 0.031 and 0.008 respectively) and 1 hour post exercise (P = 0.010 and 0.015 respectively). There was no significant main effect of group (P = 0.078 and 0.251 respectively) or time (P = 0.295 and 0.112 respectively) for haemoglobin or haematocrit. There was no significant main effect of group for the granulocyte to lymphocyte ratio (GLR) (P = 0.080). There was, however, a significant main effect of time (P <0.001) with post-hoc tests showing a significant increase from baseline (pre supplementation) to 1 hour post 90 minutes (P <0.001) (table 5.6).

Table 5.6 Acute Blood Responses Compared to Baseline during 90 min Steady State Endurance Ride on Day 2

			Bout 3 (Day 2 morning): 90 min SS		
	Baseline	**Pre- $\dot{V}O_2max$**	**Pre-Ex**	**Post-Ex**	**1h-Post-Ex**
Total WBC (×10^9 cells.L^{-1})				**	**
PLA (*n* = 9)	4.80 (2.77)	5.59 (2.12)	5.73 (2.77)	7.08 (4.81)	8.67 (4.98)
SC (*n* = 10)	6.07 (2.27)	6.13 (2.68)	6.53 (3.07)	9.58 (4.88)	10.31 (5.65)
Lymphocytes (×10^9 cells.L^{-1})				*	
PLA (*n* = 9)	1.30 (0.99)	1.76 (1.13)	1.15 (0.78)	1.98 (1.72)	0.97 (0.81)
SC (*n* = 10)	1.40 (0.49)	1.43 (0.82)	1.26 (0.56)	1.72 (1.11)	1.36 (0.88)
Monocytes (×10^9 cells.L^{-1})				**	
PLA (*n* = 9)	0.63 (0.49)	0.65 (0.54)	0.72 (0.37)	1.01 (0.57)	0.81 (0.29)
SC (*n* = 10)	0.79 (0.80)	0.82 (0.38)	1.02 (1.10)	1.34 (0.91)	0.91 (0.46)
Granulocytes (×10^9 cells.L^{-1})				*	*
PLA (*n* = 9)	3.14 (1.86)	2.91 (0.83)	3.16 (1.58)	4.17 (9.86)	6.28 (4.55)
SC (*n* = 10)	3.72 (1.40)	3.79 (2.31)	4.22 (2.45)	5.97 (3.97)	8.56 (5.34)
Haemoglobin (g.L^{-1})					
PLA (*n* = 9)	148 (27)	152 (18)	147 (19)	151 (38)	148 (25)
SC (*n* = 10)	156 (13)	157 (22)	158 (20)	163 (19)	162 (20)
Haematocrit (%)					
PLA (*n* = 9)	40.3 (5.7)	40.0 (3.5)	38.1 (2.8)	39.3 (3.7)	39.8 (5.1)
SC (*n* = 10)	40.5 (4.0)	39.5 (4.4)	40.4 (3.8)	42.2 (5.4)	42.4 (5.0)
Granulocyte:Lymphocyte				**	
PLA (*n* = 9)	2.22 (1.14)	2.10 (1.15)	2.53 (0.98)	2.60 (3.08)	6.62 (3.55)
SC (*n* = 10)	2.53 (0.82)	2.49 (1.47)	2.91 (2.99)	3.10 (1.80)	4.95 (7.54)

Values are median (IQR)
90 min SS: steady state endurance ride.
* ($P < 0.05$) and ** ($P < 0.01$) indicate significantly lower than baseline (post hoc follow-up for time: both groups pooled due to no group × time-point interaction).

5.3.5 Vitamin D status

Blood for vitamin D analysis was available for 20 subjects (10 in each group). There was not a significant main effect of group (P = 0.542) or time (P = 0.445). Nor was there a significant time × group interaction (P = 0.567) for total vitamin D. There was a significant main effect of group (P = 0.021) but no main effect of time (P = 0.062), nor was there a significant time × group interaction (P = 0.102) for subject who started the trial with insufficient vitamin D levels (<50 nmol.L). There was no significant main effect of group (P = 0.923), but a significant effect of time (P = 0.048) and no time × group interaction (P = 0.595) for subjects who started with adequate vitamin D levels (>50 nmol.L) (table 5.7).

When D_2 was analysed for all subjects, there was a main effect of group (P <0.001), a significant time × group interaction (P <0.001), and a significant main effect of group (P <0.001). Post-hoc analysis showed a significant increase in D_2 post-supplementation with chlorella (P =<0.001). When D_3 was analysed for all subjects, there was no main effect of group (P = 0.125), but a significant time × group interaction (P = 0.028). There was not a significant main effect of time (P = 0.056). Post-hoc analysis, although non-significant, showed a trend for D_3 to decrease with chlorella supplementation but not placebo (P = 0.056) (table 5.7).

Forty percent of subjects had a vitamin D total <50.00 nmol.L^{-1} at baseline (n = 8; 4 × placebo, 4 × chlorella). D_2 in subjects with low starting vitamin D status showed a significant main effect of group (P = 0.001), a significant time × group interaction (P <0.001), and a significant main effect of time (P <0.001). Post-hoc analysis showed a significant increase in D_2 post-supplementation with Chlorella (P <0.001) but not placebo (P = 0.176) in subjects with low starting total vitamin D. D_3 in subjects with low starting vitamin D status showed no significant main effect of group (P = 0.377), nor a significant time × group interaction (P = 0.270), nor a significant main effect of time (P = 0.151) (table 5.7).

Table 5.7 Vitamin D Responses to Supplementation

	Baseline (ALL)	**Post Supplementation (ALL)**	**Baseline (low starters)**	**Post Supplementation (low starters)**	**Baseline (adequate starters)**	**Post Supplementation (adequate starters)**
	(PLA *n* = 10; CHL *n* = 10)		(PLA *n* = 2; CHL *n* = 2)		(PLA *n* = 8; CHL *n* = 8)	
Total Vitamin D (nmol.L^{-1})				**		
PLA	65.92 (36.85)	66.80 (33.17)	35.40 (3.03)	34.03 (9.94)	86.27 (34.60)	88.65 (22.10)
SC	56.29 (20.01)	62.36 (10.28)	36.15 (3.96)	57.10 (8.10)	69.72 (13.06)	65.87 (10.67)
Vitamin D_2 (nmol.L^{-1})						
PLA	4.23 (2.71)	3.69 (1.89)	3.25 (0.83)	2.80 (0.00)	4.88 (3.39)	4.28 (2.32)
SC	3.14 (1.08)	**27.53 (9.78)** ‡	3.65 (1.70)	**33.55 (12.72)** ‡	2.80 (0.00)	**23.52 (5.16)** ‡
Vitamin D_3 (nmol.L^{-1})						
PLA	61.69 (36.52)	63.11 (32.24)	32.58 (2.36)	31.23 (9.94)	81.38 (35.10)	84.37 (21.36)
SC	53.15 (20.50)	**34.83 (12.92)** †	32.50 (5.40)	23.55 (8.77)	66.92 (13.06)	**42.35 (9.21)** †

Values are mean (SD)

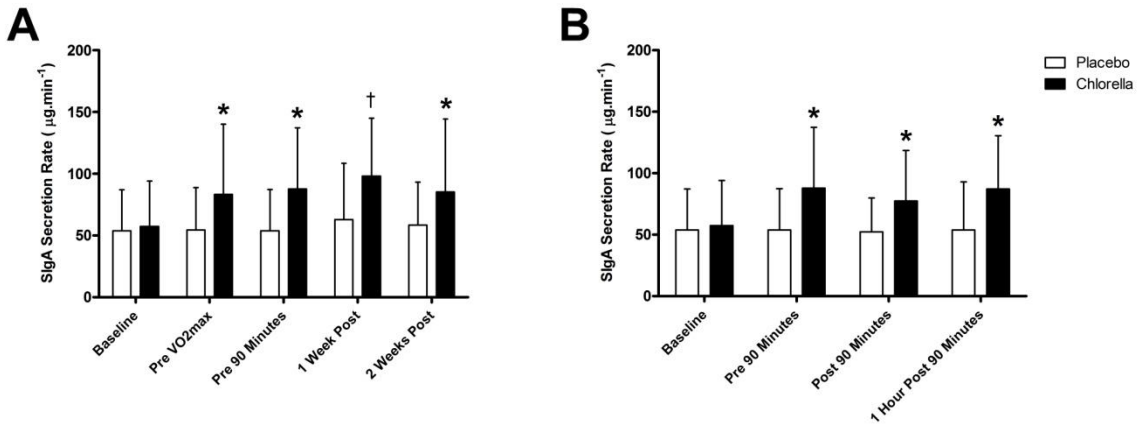
Low starters are subjects with a starting Total Vitamin D of <50.00 nmol.L^{-1}; Adequate starters are subjects with a starting Total Vitamin D of >50.00 nmol.L^{-1}

† ($P < 0.05$) and ‡ ($P < 0.01$) indicate significant change from baseline (post post hoc follow-up for time: each group analysed separately due to significant group × time-point interaction).

5.3.6 SIgA

There was no significant main effect of group for SIgA concentration or secretion rate between resting time-points throughout the trial (P = 0.248 and 0.124 respectively). A significant effect of time (P = 0.015 and 0.002 respectively) and a significant time × group interaction (P = 0.022 and 0.016 respectively) was observed for both concentration and secretion rate. Post-hoc analysis did not show any significant time effects for SIgA concentration in the placebo group (P = 0.782), however, there was a significant effect of time in the chlorella group (P <0.001) although no changes in SIgA concentration were indentified one week, and two weeks post the training intervention (P = 0.078 and 0.056 respectively) (table 5.8). Chlorella supplementation resulted in an increase of SIgA secretion rate from baseline to pre $\dot{V}O_2$max (P = 0.020), pre 90 minutes (P = 0.006), 1 week (P <0.001) and 2 weeks post exercise intervention (P = 0.016). No effect of time was observed in the placebo group (P = 0.557) (table 5.8 and figure 5.1).

Figure 5.1 SIgA secretion rates



A: SIgA secretion rates at rest compared to baseline, pre supplementation; B: SIgA secretion rates in response to 90 minutes steady state cycling, compared to baseline, pre-supplementation.
* (P < 0.05) and † (P < 0.01) indicate significantly higher than baseline (post post hoc follow-up for time: each group analysed separately due to significant group × time-point interaction).

Compared to pre-supplementation, there was no significant main effect of group for SIgA concentration in response to the $\dot{V}O_2$max test (P = 0.607). There was a significant main effect of time (P <0.001), but no time × group interaction (P = 0.066). Post-hoc analysis showed a significant decrease in SIgA concentration 1 hour post exercise compared to pre-supplementation (P = 0.008). When pre-supplementation values were excluded and exercise responses analysed in isolation, there was not a significant main effect of group in

SIgA concentration in response to the $\dot{V}O_2$max test (P = 0.745). There was a significant main effect of time (P <0.001) and a significant time × group interaction (P = 0.040). Post-hoc analysis identified a significant main effect of time in both the placebo and chlorella groups (P = 0.014 and <0.001 respectively) with no significant changes in SIgA concentration in the placebo group but a significant increase in SIgA concentration immediately post (P = 0.022) and a significant decrease one hour post $\dot{V}O_2$max test (P = 0.038) compared to pre $\dot{V}O_2$max test values in the chlorella group (table 5.9). However, SIgA secretion rate remained unaffected by supplementation (P = 0.349) or time (P = 0.107), and there was no significant time × group interaction (P = 0.066). When pre-supplementation values were excluded and exercise responses analysed in isolation, there were still no changes in SIgA secretion rate in response to the $\dot{V}O_2$max test (group, P = 0.267; time, P = 0.652; time × group interaction, P = 0.0587) (table 5.9).

Compared to pre-supplementation, there was no significant main effect of group for SIgA concentration or secretion rate in response to HIIE1 (P = 0.771 and 0.194 respectively). There was a significant main effect of time for both SIgA concentration and secretion rate (P <0.001 and 0.018 respectively). No time × group interaction was observed in SIgA concentration (P = 0.790) but a significant interaction was observed for SIgA secretion rate (P = 0.016). There was a significant decrease in SIgA concentration pre (P = 0.004) and 1 hour post exercise (P = 0.004).

When pre-supplementation values for SIgA concentration were excluded and exercise responses analysed in isolation, there was a significant main effect of time (P <0.001) but no main effect of group (P = 0.633) or a time × group interaction (P = 0.773) in response to HIIE1. Post-hoc analysis identified a significant decrease in SIgA concentration pre-HIIE1 (P = 0.005) and 1 hour post HIIE1 (P = 0.003) compared to pre-$\dot{V}O_2$max test. SIgA secretion rate significant decreased immediately post HIIE1 (P = 0.044) in the chlorella condition. There was not a significant effect of time in the placebo group (P = 0.075), however there was a significant effect of time (P = 0.003) in the chlorella group with a significant increase in SIgA secretion rate from baseline pre (P = 0.009), and immediately post exercise (P = 0.010). When pre-supplementation SIgA secretion rate values were excluded and exercise responses analysed in isolation, there was a significant main effect of time (P = 0.047) but no main effect of group (P = 0.142) or a time × group interaction (P = 0.099) however no significant changes in SIgA secretion rate were identified in either group (PLA, P = 0.064; CHL = P = 0.111) (table 5.9).

There was no significant main effect for group in SIgA concentration or secretion rate in response to 90 minutes steady state cycling (P = 0.771 and 0.102 respectively). There was a significant main effect of time for both concentration and secretion rate (P = 0.007 and 0.023 respectively). No time × group interaction was observed for SIgA concentration (P = 0.551), however as significant interaction was observed in SIgA secretion rate (P = 0.017). SIgA concentration significantly decreased 1 hour post exercise (P = 0.016). Post-hoc analysis showed a significant increase in the SIgA secretion rate from baseline compared to pre 90 minutes (P = 0.038), immediately post 90 minutes (P = 0.029), but no change was identified 1 hour post 90 minutes (P = 0.074) in the chlorella condition. There was no significant effect of time in the placebo group (P = 0.996). There was a significant effect of time (P <0.001) in the chlorella group with a significant increase in SIgA secretion rate from baseline pre 90 minutes (P = 0.023), immediately post 90 minutes (P = 0.005) and 1 hour post 90 minutes (P = 0.004) (table 5.9 and figure 5.1).

When pre-supplementation values were excluded and exercise responses analysed in isolation, there was a significant main effect of time (P <0.001) but no main effect of group (P = 0.438) or a time × group interaction (P = 0.291) for SIgA concentration. Post-hoc analysis identified a significant decrease in SIgA concentration 1 hour post 90 minutes (P = 0.009) compared to pre-$\dot{V}O_2$max test (table 5.9). No changes were observed in SIgA secretion rate (group, P = 0.060; time, P = 0.644; time × group interaction, P = 0.459) (table 5.9).

In response to HIIE2, there was no significant main effect of group (P = 0.564 and 0.244 respectively) or a time × group interaction (P = 0.554 and 0.181 respectively) for SIgA concentration or secretion rate. A main effect of time was observed for both SIgA concentration and secretion rate (P <0.001 and <0.001 respectively) Post-hoc analysis showed a significant decrease in SIgA concentration pre (P = 0.021) and 1 hour post exercise (P = 0.034) accompanied by a significant increase in IgA concentration 1 hour post exercise (P <0.001).

When pre-supplementation values were excluded and exercise responses to HIIE2 analysed in isolation, there was a significant main effect of time (P <0.001 and 0.005 respectively) but no main effect of group (P = 0.464 and 0.150 respectively) or a time × group interaction (P = 0.619 0.577 respectively) for SIgA concentration or secretion rate. Post-hoc analysis identified a decrease in SIgA concentration pre-HIIE2

Table 5.8 Resting SIgA Responses compared to baseline, pre supplementation

	Baseline	Pre Exercise Day 1	Pre Exercise Day 2	1 Week Post	2 Weeks Post
SIgA concentration ($mg.L^{-1}$)					
PLA (*n* = 13)	205 (106)	176 (90)	194 (101)	185 (103)	192 (57)
SC (*n* = 13)	193 (79)	199 (86)	192 (79)	261 (112)	368 (261)
SIgA secretion rate ($\mu g .min^{-1}$)					
PLA (*n* = 13)	53.8 (33.2)	54.3 (34.5)	53.8 (33.5)	62.7 (45.8)	58.3 (34.9)
SC (*n* = 13)	57.2 (36.9)	**83.1 (57.0) ***	**87.7 (49.6) ***	**98.0 (47.1) ****	**85.1 (59.2) ***

Values are mean (SD)
* ($P < 0.05$) and ** ($P < 0.01$) indicate significantly higher than baseline (post post hoc follow-up for time: each group analysed separately due to significant group × time-point interaction).

Table 5.9 SIgA Responses Compared to pre-exercise

		Bout 1 (Day 1 morning): $\dot{V}O_2max$ test			Bout 2 (Day 1 afternoon): HIIE			Bout 3 (Day 2 morning): 90 min SS			Bout 4 (Day 2 afternoon):HIIE		
	Baseline	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**
SIgA concentration ($mg.L^{-1}$)				**	**		**			*	*		*
PLA (*n* = 13)	205 (106)	176 (90)	197 (85)	148 (62)	131 (49)	201 (109)	141 (124)	194 (101)	168 (57)	126 (60)	135 (73)	197 (100)	147 (81)
SC (*n* = 13)	193 (79)	199 (86)	266 (128)	123 (65)	151 (53)	215 (194)	137 (79)	192 (79)	179 (69)	157 (60)	148 (66)	248 (104)	169 (114)
SIgA secretion rate ($\mu g.min^{-1}$)													
PLA (*n* = 13)	53.8 (33.2)	54.3 (34.5)	66.6 (80.6)	73.3 (73.1)	58.6 (41.1)	45.5 (36.9)	66.6 (73.3)	53.8 (33.5)	52.2 (27.6)	53.7 (39.2)	53.1 (32.8)	46.3 (21.7)	75.6 (41.5)
SC (*n* = 13)	57.2 (36.9)	83.1 (57.0)	88.7 (67.1)	82.2 (59.1)	**99.9 (82.1) ‡**	**84.3 (66.4) †**	92.6 (97.2)	**87.7 (49.6) †**	**77.3 (41.0) ‡**	**87.2 (43.3) ‡**	80.3 (37.3)	72.6 (62.9)	124.6 (128.9)

Values are mean (SD)
HIIE: High-intensity interval exercise session; 90 min SS: steady state endurance ride. Values are mean (SD)
* ($P < 0.05$) and ** ($P < 0.01$) indicate significantly lower than baseline (post hoc follow-up for time: both groups pooled due to no group × time-point interaction).
† ($P < 0.05$) and ‡ ($P < 0.01$) indicate significantly higher than baseline (post post hoc follow-up for time: each group analysed separately due to significant group × time-point interaction).

(P = 0.015) and 1 hour post HIIE2 (P = 0.018) accompanied by a significant decrease in SIgA secretion rate 1 hour post HIIE2 (P = 0.030) compared to pre $\dot{V}O_2$max. A significant increase in SIgA concentration immediately post HIIE2 (P = 0.039) compared to pre-$\dot{V}O_2$max test was also observed (table 5.9).

There was a main effect of time (P = 0.018), but no main effect of group (P = 0.348), or a time × group interaction (P = 0.119) for saliva flow rate at resting time points. Post-hoc tests identified a significant increase in saliva flow rate pre-90 minutes compared to baseline (P = 0.046) (table 5.10). There was a main effect of time (P = 0.031), not a main effect of group (P = 0.400), or a time × group interaction (P = 0.435) for saliva flow rate responses to the $\dot{V}O_2$max test compared to baseline. Post-hoc tests identified a significant increase in saliva flow rate 1-hour post the $\dot{V}O_2$max test compared to baseline (P < 0.001). When pre-supplementation values were excluded and saliva flow rate responses to the $\dot{V}O_2$max test analysed in isolation, there was a main effect of time (P < 0.001), but no main effect of group (P = 0.383), or a time × group interaction (P = 0.311). Post-hoc tests identified a significant increase in saliva flow rate 1 hour post $\dot{V}O_2$max test compared to pre $\dot{V}O_2$max test (P = 0.001). There was a main effect of time (P < 0.001), but no main effect of group (P = 0.227), or a time × group interaction (P = 0.171) for saliva flow rate responses to HIIE1 compared to baseline. Post-hoc tests identified a significant increase in saliva flow rate pre HIIE1 (P < 0.001) and 1 hour post HIIE1 (P = 0.001). When pre-supplementation values were excluded, and the saliva flow rate responses to HIIE1 analysed in isolation, there was a main effect of time (P < 0.001), but no main effect of group (P = 0.198), or a time × group interaction (P = 0.121). Post-hoc tests identified a significant decrease in saliva flow rate immediately post HIIE1 compared to pre HIIE1 (P < 0.001). There was a main effect of time (P < 0.001), but no main effect of group (P = 0.133) or a time × group interaction (P = 0.075) for saliva flow rates in response to 90 minutes steady state cycling. Post-hoc tests identified a significant increase in saliva flow rate from baseline pre (P = 0.027) and 1 hour post the 90 minute cycle (P = 0.002). When pre-supplementation values were excluded, and the saliva flow rate responses to the 90 minute steady state cycle analyses in isolation, there was a main effect of time (P = 0.001), but no main effect of group (P = 0.103) or a time × group interaction (P = 0.683). Post-hoc tests identified a significant increase in saliva flow rate 1 hour post the 90 minute cycle compared to pre 90 minutes (P = 0.004). There was a main effect of time (P < 0.001), but no main effect of group (P = 0.386), or a time × group interaction (P

Table 5.10 Resting saliva flow rate (ml.min^{-1}) responses compared to baseline, pre supplementation

	Baseline	Pre Exercise Day 1	Pre Exercise Day 2	1 Week Post	2 Weeks Post
			*		
PLA (*n* = 13)	0.30 (0.15)	0.35 (0.28)	0.27 (0.38)	0.26 (0.38)	0.24 (0.32)
SC (*n* = 13)	0.23 (0.33)	0.41 (0.37)	0.51 (0.42)	0.41 (0.40)	0.33 (0.38)

Values are median (IQR)
* ($P < 0.05$) indicates a significant change from baseline

Table 5.11 Acute saliva flow rate (ml.min^{-1}) responses to each exercise bout during the 2-day intensified training period (*n* = 13)

		Bout 1 (Day 1 morning): $\dot{V}O_2$max test			Bout 2 (Day 1 afternoon): HIIE			Bout 3 (Day 2 morning): 90 min SS			Bout 4 (Day 2 afternoon):HIIE		
	Baseline	Pre-Ex	Post-Ex	1h Post-Ex	Pre-Ex	Post-Ex	1h Post-Ex	Pre-Ex	Post-Ex	1h Post-Ex	Pre-Ex	Post-Ex	1h Post-Ex
				**††	**	††	*	*		*†	**	††	**†
PLA	0.30 (0.15)	0.35 (0.28)	0.27 (0.24)	0.45 (0.53)	0.31 (0.47)	0.22 (0.29)	0.33 (0.75)	0.27 (0.38)	0.26 (0.22)	0.48 (0.39)	0.39 (0.47)	0.21 (0.32)	0.53 (0.56)
SC	0.23 (0.33)	0.41 (0.37)	0.36 (0.37)	0.66 (0.32)	0.61 (0.46)	0.47 (0.48)	0.57 (0.56)	0.51 (0.42)	0.38 (0.23)	0.55 (0.32)	0.57 (0.34)	0.26 (0.27)	0.73 (0.75)

HIIE: High-intensity interval exercise session; 90 min SS: steady state endurance ride. Values are median (IQR)
* ($P < 0.05$) and ** ($P < 0.001$) indicates significant change from baseline
† ($P < 0.05$) and †† ($P < 0.001$) indicate significant change from pre exercise values

Table 5.12 Change in EBV concentration compared to baseline, pre supplementation

	Baseline	Pre Exercise Day 1	Pre Exercise Day 2
EBV Concentration (ng.µl^{-1} x10^{-6})			
PLA (*n* = 9)	0.70 (2.07)	0.54 (123.73)	0.85 (10.85)
SC (*n* = 6)	4.56 (15.35)	1.53 (2.23)	0.73 (1.91)
EBV Concentration (ng.µl^{-1} x10^{-6})			
No URTI (*n* = 7)	0.72 (8.10)	1.05 (2.05)	0.82 (5.80)
URTI (*n* = 8)	1.16 (15.77)	0.33 (123.80)	1.44 (8.04)

Values are median (IQR)

= 0.261) for saliva flow rate in response to HIIE2, compared to baseline. Post-hoc tests identified a significant increase in saliva flow rate pre ($P < 0.001$) and 1 hour post HIIE2 compared to baseline ($P < 0.001$). When pre-supplementation values were excluded, and the saliva flow rate responses to HIIE2 analysed in isolation, there was a main effect of time ($P < 0.001$), but no main effect of group ($P = 0.377$) or a time × group interaction ($P = 0.061$). Post-hoc tests identified a significant decrease in saliva flow rate immediately post HIIE2 compared to pre ($P < 0.001$), and a significant increase in saliva flow rate 1 hour post HIIE2 compared to pre ($P = 0.018$) (table 5.11).

5.3.7 EBV

15 of 20 subjects tested (75%) were seropositive for EBV (9 in the PLA group, and 6 in the CHL group). There was no significant main effect of group ($P = 0.735$), time ($P = 0.963$), or time × group interaction ($P = 0.087$) for EBV concentration from pre-supplementation, and the mornings of both exercise days. When subjects were grouped by those who had or hadn't suffered with a URTI in the 2 weeks following the exercise intervention, there was no significant main effect of group ($P = 0.805$), time ($P = 0.964$), or a group × time interaction ($P = 0.931$) (table 5.12).

5.4 Discussion

The aim of the current study was to assess the effects of chlorella supplementation on acute immune responses (leucocyte responses and salivary immune and stress response markers) and delayed immune responses (SIgA and illness incidence) in response to a two day intensified training period using a double blind, between groups design. This is the first time the influence of chlorella on immune function has been investigated in response to intensified training. The main findings of this study were that a daily dose of 6 g CHL for 4 weeks before (and for the 2 weeks following) the training intervention increased the resting SIgA secretion rate by week 4, accompanied by a trend for increased resting SIgA concentration by week 5. Neither SIgA secretion rate or concentration were acutely affected by any one individual exercise bout, but SIgA secretion rate in the CHL group appears to increase in response to some of the exercise bouts. The same response was not observed in the PLA group. None of these changes in SIgA could be attributed to changes in saliva flow rate. There were also no differences in the frequency, severity, or duration of self- reported URS between the PLA and CHL groups. WBCs, lymphocytes,

monocytes, and granulocytes increased from baseline immediately post the 90 minute cycle, with WBCs and granulocytes remaining elevated 1 hour post exercise, but there were no differences between the CHL and PLA groups. The GLR increased from baseline 1 hour after the 90 minute cycle on day 2 of the training intervention. Supplementation with CHL did not appear to effect haemoglobin or haematocrit levels in response to any of the exercise sessions, nor did it appear to have any effect on the *in vivo* immune marker of salivary EBV. Vitamin D increased in the CHL group following 4 weeks of daily supplementation, however, this appears to be due to an increase in D_2 alongside a reduction of D_3 in the CHL group. No changes were observed in the PLA group's levels of total vitamin D, D_2 or D_3.

The acute decreases in SIgA concentration after some of the training sessions are in line with some research studies that have looked at intensive training periods (Hall et al., 2007) but not others (Davison, 2011). However, the fact that no changes in SIgA secretion rate were observed following any of the exercise bouts differs from Hall et al. (2007) and agrees with Davison et al. (2011). There is very little research, however, that has tracked salivary SIgA responses to multiple exercise sessions over such a short period of time. Papacosta at al. (2013) and Otsuki et al. (2012) have shown decreases in resting SIgA over longer periods (i.e. 1-2 week training camps) which differs from the current study in which no differences in resting SIgA concentration or secretion rates were seen in responses to day 2 of the exercise intervention, however this may be due to the shorter intensified training period of the current study. Neither Papacosta et al. (2013) or Otsuki et al. (2012) assessed the response to individual training sessions in the training period, which a novel aspect of this the current study.

SIgA is one of the body's first lines of defence against pathogens entering through the oral cavity. A decrease in SIgA has been shown to be related to an increased risk of developing URTI and the development of associated URS. Because of this, it was hypothesised that any increase (or the avoidance of a decrease) in SIgA would result in a reduced risk of URS (i.e. subjects would report fewer symptoms). A decrease in the number of URS reported would be in line with previous research studies on intensified training periods/training camps and long-term monitoring of athletes (Fahlman & Engels, 2005; Gleeson et al., 2012; Gleeson et al., 2011; and Neville et al., 2008). Following an acute bout of moderate intensity exercise, SIgA normally drops below baseline immediately post-exercise and returns to normal within the following hour. For more intensive bouts of

exercise, or in periods of intensified or over training, SIgA levels can take much longer to return to baseline. Chlorella supplementation in the group studied, appears to have attenuated the post-exercise drops in SIgA compared to baseline.

Chlorella supplementation did not influence the exercise induced SIgA responses. However, chlorella supplementation significantly increased SIgA secretion rate from baseline at every resting time-point, while no changes in SIgA were observed in the placebo group. Based on previous research, subjects in the chlorella group, therefore, should have had an increased protection against URTI (Gleeson et al., 2012). However, no differences were observed between groups in the study, with a 31% incidence rate in both the placebo and chlorella groups. This is not surprising as CHL supplementation in the present study did not influence the expression of EBV DNA into saliva. Salivary EBV DNA expression is considered to be a good measure of *in vivo* immune function. The incidence of URTI is also indicative of how well the immune system is working at an *in vivo* level, and therefore the fact that neither salivary EBV DNA expression, or URTI incidence were affected by CHL supplementation suggest that the immune function as a whole, indicated by these two measures, is not improved following 4 weeks of CHL supplementation. This is supported by our finding that EBV concentration was not higher in subjects who went on to develop a URTI. Yamauchi et al. (2011) reported that salivary EBV DNA expression tended to increase in subjects who were suffering with a sore throat and cough. One limitation of the present study is that we did not manage to successfully collect all saliva samples during the 2 week period post the exercise intervention. One further limitation of the present study is the modest sample size (n = 26) which may have limited our ability to comprehensively determine the effects of chlorella supplementation on URTI/URS. Furthermore, resting SIgA levels remained relatively close to baseline in the placebo group following most, but not all, of the exercise sessions. The maintenance of SIgA levels close to baseline within this group means that URTI/URS risk was not adversely affected to a great extent, even with the intensified training period. Despite this, our findings, in combination with those of Otsuki et al. (2012), do show some benefit of CHL supplementation in terms of increases above baseline levels of salivary SIgA. Importantly, resting SIgA concentration, although not acutely affected by supplementation before or during the intensified training period, did appear to start increasing in the two weeks that followed the training period (i.e. weeks 5 and 6). It is possible, therefore, that the supplementation period used in the present study may not have been long enough to yield any beneficial effects on immune function and it would be feasible to suggest that,

had the training period commenced after 5 or 6 weeks of supplementation, rather than 4, that this may have translated into greater effects on URS reports. However, this will require further study.

The most likely mechanisms for the increase in salivary SIgA observed after 4-5 weeks of supplementation with CHL are via the immunostimulating properties of compounds found in CHL such as specific polysaccharides and glycoproteins or protein/polysaccharides complexes (Kralovec et al., 2007; Morris et al., 2008; Tanaka et al., 1998). In particular, Kralovec et al. (2007) identified relatively high molecular weight (> 100 kDa) protein/polysaccharide complexes and polysaccharides as being responsible for the immunostimulatory effects of CHL *in vitro*. They identified glutamic acid and aspartic acid as the major amino acid constituents of the protein/polysaccharide complexes (~22-26%) with galactose (~22-50%), rhamnose (~18-40%), and arabinose (~14-26%) as the main constituent monosaccharides found in the polysaccharides and protein/polysaccharide complexes with the greatest immune stimulating activity *in vitro*. This included B cell stimulation and proliferation, which could explain the beneficial effects seen in humans *in vivo*, including antibody response to influenza vaccination (Halperin et al., 2003) and aspects of mucosal immunity as observed in the present study and previous research (Otsuki et al., 2016, 2012, & 2011). However it is not possible to determine the exact mechanisms and we can only speculate at this stage. Indeed, CHL contains an abundance of nutrients, many of which could influence immunity in athletes. However, in line with previous suggestions (Otsuki et al., 2016, 2012, & 2011), the specific dose of each of these nutrients is unlikely to be responsible for the effects we have observed on SIgA we have observed in a healthy young adult population with no known dietary deficiencies. As such, we suggest it is the compounds such as polysaccharides and protein/polysaccharide complexes (Kralovec et al., 2007) that are responsible for the effects observed in the present study. This will require further study (including the determination of the bioavailability from orally consumed CHL and monitoring levels of these substances in plasma and/or immune cells after ingestion, but this was beyond the scope of the current investigation).

Although it is the polysaccharides (predominately galactose, rhamnose, and arabinose) and proteins in chlorella which are believed to be responsible for a large percentage of chlorella's immunostimulating activities (Kralovec et al., 2007), Chlorella also contains a number of vitamins (B1, B2, B6, B12, C, D_2, E, K1, niacin, pantothenic acid, folic acid and

biotin); minerals (calcium, iron, magnesium, zinc, potassium, sodium and phosphorus); and amino acids (isoleucine, leucine, lysine, methionine, cysteine, phenylalanine, tyrosine, threonine, tryptophan, valine, arginine, histidine, alanine, aspartic acid, glutamic acid, glycine, proline and serine); is rich in beta-carotene; and high in protein (Dam et al., 1965). Although most of the nutrients in 6 g of chlorella are provided in very low doses (and therefore unlikely to influence immune responses), one nutrient in which CHL is abundant is vitamin (He at al., 2013*a*) (SunChlorella contains 54 µg/6 g, equivalent to 2,160 IU). The primary outcome analysis for subjects' vitamin D levels in the present study showed that there was no difference in vitamin total Dbetween the CHL and PLA groups at baseline, or post-supplementation. When subjects were stratified by starting vitamin D status (either low <50 nmol.L^{-1}, or normal >50 nmol.l^{-1}) however, on face value it appears that CHL increases total vitamin D in subjects with a low level at baseline. However, when levels of ergocalciferol (D_2) and cholecalciferol (D_3) were analysed, there was a significant increase in circulating D_2 observed in this population, alongside a reduction in D_3 (the same, although non-significant observation can be made in the CHL group who started with adequate vitamin D (table 5.7). Both D_2 and D_3 function as prohormones and therefore have no biological effect. In order to become active compounds, they must first be converted to 25-hydroxyvitamin D (25(OH)D). Recent research has shown that vitamin D_3 increases serum 25(OH)D more efficiently than vitamin D_2 (Armas et al., 2004; Heaney et al., 2011; Oliveri et al., 2015; Trang et al., 1998). Low levels of 25(OH)D have been linked to an increased risk of developing URTI (Berry et al., 2011; Ginde et al., 2009; He et al., 2013*a*). Therefore, a rise in vitamin D_2 levels, in combination with a fall in vitamin D_3 as observed in the CHL group of the present study may not have been beneficial to any aspect of their immunity including URTI. 40% (n = 8) of subjects for whom blood was available had low total vitamin D (<50 nmol.L^{-1}) with n = 4 of these subjects were in the CHL group. Therefore 20% of subjects started with a low vitamin D status which would not have been corrected optimally via supplementation with CHL. This may go some way to explaining why there was not a decrease in the number of URTI/URS reported in the CHL group as these subjects would have been more susceptible to infection.

The leucocytosis observed in response to the training intervention and, specifically, the 90 minute prolonged cycle at 25% Δ, is in line with the findings of previous research (Robson et al., 1999). Typically, prolonged endurance exercise results in a delayed leucocytosis which usually sees a doubling of leucocytes in circulation but, in some instances, a four-fold increase has been observed (Eskola et al., 1978). The significant increase in

circulating lymphocytes following exercise is due to the redistribution of white blood cells already present. Typically, following intensive exercise lasting less than one hour, leucocytosis is caused by a significant increase in circulating neutrophils and lymphocytes. Following prolonged exercise, leucocytosis is predominately caused by neutrophilia. In this study, we were unable to differentiate granulocytes into neutrophils, eosinophils, basophils, and mast cells. Increases in eosinophils, basophils, and mast cells in response to exercise within a healthy population are unlikely, however, so we can be confident in our assumption that the significant increases in granulocytes observed in this study, are in fact neutrophils. Therefore, the cellular blood responses in the present study, where total WBC, lymphocytes, monocytes, and granulocytes (neutrophils) all significantly increased from baseline immediately following the 90 minute cycling on day 2 of the intervention, with granulocytes remaining elevated 1 hour post, are not atypical of the research currently in circulation (Blannin, 2006; Nieman et al., 1994, Pyne, 1994; Robson et al., 1999). Despite the fact a doubling of total WBCs from baseline was not observed (which would be typical of exercise sessions lasting in excess of one hour), the significant increases in WBCs do suggest that our training intervention provoked an inflammatory response. A limitation of this study is that we did not measure cortisol responses to each exercise session and therefore the individual stress response has not been measured. However, an increase in the GLR is also considered to be a good measure of immunological stress (Chen et al., 2017) and, in the present study, this increased immediately post-exercise and remained elevated one hour-post. The typical leucocyte responses to exercise observed in the present study, and fact that there were no differences observed in the responses between the CHL and PLA groups suggest that CHL supplementation does not blunt the stress response typically observed following endurance exercise.

5.5 Limitations

One limitation of this study is that we did not measure $\dot{V}O_2max$ pre-supplementation, so it is not possible to see the effect of CHL supplementation on performance parameters, such as $\dot{V}O_2max$, VT1, and W_{max}. Following four weeks of supplementation with Chlorella pyrenoidosa, Umemoto & Otsuki (2014) reported a significant, 9% increase in peak oxygen uptake during a maximal cycling test to exhaustion, attributed by the study's authors to be caused by branch chain amino acids (although the levels contained within the administered dose would be small compared to the amounts used in branch chain amino acid performance studies), the bioactive effects of some of the vitamins and nutrients, or to

do with the wide spectrum of nutrients available (as opposed to the bioactive effects). This built on the findings of a murine study 3 years previously which reported a two-fold increase in time to exhaustion when undertaking a swimming challenge in mice that were supplemented with CHL (Mizoguchi et al., 2011). However, the present study was not designed to measure cardiovascular or performance responses to supplementation, but rather the immunological responses to a controlled period of intensified training (i.e. with all subjects exposed to the same relative training demand). We also did not record physiological responses during HIIE sessions, owing to technical difficulties, to ensure that exercise intensity was comparable between PLA and CHL groups. However, we are confident that subjects produced a maximal effort (and hence the same relative demand) for these sessions based on the maximal RPE values expressed following all HIIE sprints.

5.6 Conclusion

The aim of the current study was to assess the effects of chlorella supplementation on acute and delayed immune responses to a two day intensified training period. In conclusion, daily supplementation with CHL was able to increase salivary SIgA secretion rate at rest. Supplementation did not reduce the acute stress responses to exercise, as observed by leucocyte responses and, although vitamin D increased in the CHL group for subjects with low vitamin D levels (<50 $nmol.L^{-1}$), it was an increase in vitamin D_2 as opposed to the more efficacious vitamin D_3 that provided the increase. This is the first study of its kind to investigate the effects of CHL supplementation on vitamin D status.

Together with previous research, there is now substantial evidence to show that CHL can enhance salivary SIgA; however, in the present study, it appears that a longer supplementation period may be required to translate to protection against URTI and reduced URS reports.

Chapter 6

The effect of 4 weeks Chlorella pyrenoidosa supplementation on immune responses to 2 days intensified training: A crossover, double blind, placebo-controlled study

Funding:

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Abstract

Background: It is widely reported that athletes engaged in regular prolonged activity and/or strenuous exercise have a higher than normal incidence of upper respiratory tract infection (URTI) which may be related to an exercise-induced impairment of immune function. The study detailed previously, in Chapter 5, reported that *Chlorella pyrenoidosa* (CHL) had beneficial effects on immune function at rest in trained cyclists.

Purpose: To investigate the effects of supplementation with CHL on leucocyte, cytokine, and mucosal immune responses, illness incidence, and EBV reactivation to two days intensified training using a double blind, crossover study design.

Methods: Fourteen subjects (age 31 ± 10 years; $\dot{V}O_2$max 54.44 ± 13.39 mL.kg^{-1}.min^{-1}) provided resting blood and saliva samples for determination of leucocytes, neutrophil function, SIgA, and salivary EBV-DNA at baseline, and following 4, 5 and 6 weeks of daily supplementation with CHL or placebo. During week 4, a 2-day intensified training period was undertaken [morning and afternoon sessions each day, respectively: $\dot{V}O_2$max test, high-intensity interval exercise (HIIE, 3 × 30 s Wingate sprints); 90 min at ~60% $\dot{V}O_2$max; 3 × 30 s HIIE]. Subjects continued supplementation for a further 2 weeks before undertaking a 12 week wash out period. The exercise responses of IL-4, IL-10 (stimulated in whole blood), salivary lysozyme, salivary lactoferrin, salivary cortisol, salivary α-amylase were also investigated.

Results: CHL increased resting SIgA concentration (time × trial, P = 0.024: no change with PLA but increase with CHL at week 5 (P = 0.040). PLA vs. CHL: week-0 = 185 ± 71 vs. 209 ± 163 mg.L^{-1} and week-5 = 168 ± 71 vs. 267 ± 123 mg/L^{-1}, respectively. Minimal acute changes in SIgA were seen in response to individual exercise bouts, but SIgA concentration was lower at some times in the PLA group (for bout 2).

Conclusions: Supplementation with CHL has beneficial effects on resting SIgA, which might be useful during periods of intensified training.

6.1 Introduction

In the previous study, we reported that daily supplementation with Chlorella pyrenoidosa (CHL) increased salivary SIgA concentration and secretion rate at rest. This study reports on a follow up, double blind crossover study investigating the effects of CHL supplementation on immune responses and illness incidence to two days intensified cycling training.

6.2 Methodology

6.2.1 Subjects

Fourteen subjects who took part in the original study (Chapter 5) agreed to return to the laboratory following a 12 week wash out period to volunteer for the crossover study. Eleven males and three females returned for the follow up study (age: 31 ± 10 years (mean ± SD); height 172.4 ± 4.3 cm; weight 71.01 ± 10.31 kg; maximal aerobic capacity ($\dot{V}O_2max$) 54.44 ± 13.39 mL.kg.min^{-1}) (table 6.1). Subjects spent an average of 7 hours training per week, and were excluded from participation if they were using nutritional supplements or medication, or if they had given blood, received vaccinations, or suffered an infection within one month of either phase of the study commencing. All subjects provided informed consent before participation. Ethical approval for the study was granted from the University of Kent's Ethics Committee.

Table 6.1 Subject Characteristics (*n* = 14)

	Chlorella		**Placebo**		***P*-Value**
	Mean (SD)	**Range**	**Mean (SD)**	**Range**	**2 Tailed**
Age (yr)	31 (10)	19 - 45	31 (10)	19 - 45	0.336
Height (cm)	172.3 (4.3)	165 - 181	172.4 (4.4)	165 – 181.7	0.190
Weight (kg)	71.34 (10.57)	58.4 – 89.8	71.64 (10.85)	58 – 93.4	0.629
$\dot{V}O_2max$ (mL.kg^{-1}.min^{-1})	53.62 (13.20)	34.19 – 78.69	54.97 (13.21)	35.10 – 79.67	0.117

6.2.2 Study Design

The study design remained the same for crossover as reported in Chapter 5, and subjects were provided with the opposite supplement to their first arm of the study (i.e. if a subject had been provided with placebo during phase one, they were provided with chlorella, and vice versa). 7 subjects started with CHL and 7 subjects started with PLA. The collection of blood and saliva samples remained at the same points and there was consistency of collection methods. The analysis of cell counts, vitamin D, EBV status and DNA expression, and SIgA remained as reported in Chapter 5. In addition, whole blood stimulated cytokine release, neutrophil function, lysozyme, lactoferrin, salivary cortisol, and α-amylase were also analysed.

6.2.3 Vitamin D

Vitamin D was analysed using methods detailed in chapter 2 with responses between CHL and PLA groups analysed, and responses of low (< 50 nmol.L^{-1}) and adequate starters (> 50 nmol.L^{-1}).

6.2.4 Cytokines

Immediately following sample collection, 800 µl whole blood (collected into a heparin vaccutainer) was incubated at 37 °C for 60 minutes in a block heater with either 40 µl PBS (Sigma-Aldrich, St. Louis, Missouri, U.S.A.), or 40 µl Stimulant (84015-1VL, Sigma-Aldrich, St. Louis, Missouri, U.S.A.) which had been reconstituted in dH_2O as recommended by the manufacturer, aliquoted, and stored frozen at -80 °C until required. Samples were gently mixed by inversion at the beginning, and half way through the incubation period. As soon as the incubation period was over, samples were centrifuged at 4 °C and 17,000 × g in a microcentrifuge (Fisher Scientific accuSpin Micro 17R Microcentrifuge; Loughborough, U.K.) for 2 minutes. Plasma was aliquoted into 1.5 mL Eppendorf tubes and stored frozen at -80 °C until analysis.

IL-4 and IL-10 were measured by ELISA (Human IL-4 ELISA Max Deluxe; catalogue number: 430305; Human IL-10 ELISA Max Deluxe; catalogue number: 430605; both BioLegend, San Diego, California, U.S.A.). Samples were thoroughly defrosted before use. Stimulated samples were diluted 1:1 prior to use, and the test procedure recommended by the manufacturer was followed. Un-stimulated samples were analysed in singular; stimulated samples were analysed in duplicate (CV for IL-4 and IL-10 = 9.8% and 8.7% respectively). Standard curves and assay results were prepared in Microsoft Excel. Unstimulated values were subtracted from corresponding stimulated samples before statistical analysis. Any samples which fell above or below the standard curve were re-analysed with the dilution factor adjusted accordingly. The concentration was calculated using the following equation:

$$Concentration\ (pg.mL^{-1}) = (\ stim.cytokine\ conc\ (pg.mL^{-1}) - unstim.\ cytokine\ conc\ (pg.mL^{-1})) \times (1 - haematocrit/mL)$$

For statistical analysis, responses to exercise trials and supplementation were analysed but, in addition, subjects were stratified by those who got ill, and those who didn't (Gleeson et al., 2012). IL-4 and IL-10 responses were then compared between these two groups.

6.2.5 Blood contamination

Saliva samples being analysed for alpha-amylase, cortisol, lysozyme, or lactoferrin were screened for blood contamination prior to investigation. Saliva samples were analysed in duplicate (and the mean calculated for each sample) using an ELISA (Salivary Blood Contamination Enzyme Immunoassay Kit, 1-1302; Salimetrics, Pennsylvania, U.S.A.) assay, following the manufacturer instructions. The intraassay CV = 2.8%. Samples with more than 2 mg/dL transferrin were excluded from analysis for lysozyme, lactoferrin, and salivary cortisol.

6.2.6 Lysozyme

Lysozyme was measured by sandwich ELISA (AssayMax™ Human Lysozyme ELISA Kit, EL3010-1; Assaypro LLC, St. Charles, Missouri, U.S.A.) according to the manufacturer instructions. Saliva samples were analysed in duplicate, and the mean calculated for each sample. The intraassay CV = 12.8%. The acute lysozyme responses to exercise pre, post, and 1 hour post 90 minutes steady state cycling are reported, using pre $\dot{V}O_2$max values as a baseline.

6.2.7 Lactoferrin

Lactoferrin was measured by sandwich ELISA (AssayMax™ Human Lactoferrin ELISA Kit, EL2011-1; Assaypro LLC, St. Charles, Missouri, U.S.A.) according to the manufacturer instructions. Saliva samples were analysed in duplicate, and the mean calculated for each sample. The intraassay CV = 4.5% The acute lactoferrin responses to exercise pre, post, and 1 hour post 90 minutes steady state cycling are reported, using pre $\dot{V}O_2$max values as a baseline.

6.2.8 Salivary cortisol

Salivary cortisol was analysed by sandwich ELISA (Expanded Range, High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit, 1-3002; Salimetrics, Pennsylvania, U.S.A.) according to the manufacturer instructions. Saliva samples were analysed in duplicate, and the mean calculated for each sample. The intraassay CV = 2.0%. The acute salivary cortisol responses to exercise pre, post, and 1 hour post 90 minutes steady state cycling are reported, using pre $\dot{V}O_2$max values as a baseline.

6.2.9 α-amylase

α-amylase activity was measured using a kinetic enzyme assay (Salivary α-amylase Kinetic Enzyme Assay Kit, 1-1902; Salimetrics, Pennsylvania, U.S.A.) according to the manufacturer instructions. Saliva samples were analysed in duplicate, and the mean calculated for each sample. The intraassay CV = 6.4%. The acute α-amylase responses to exercise pre, post, and 1 hour post 90 minutes steady state cycling are reported, using pre $\dot{V}O_2$max values as a baseline.

6.2.10 Neutrophil function

Immediately following sample collection, 800 µl whole blood (collected into a heparin vaccutainer) was incubated at 37 °C for 60 minutes in a block heater with either 40 µl PBS (Sigma-Aldrich, St.Louis, Missouri, U.S.A.), or 40 µl Stimulant (84015-1VL, Sigma-Aldrich, St.Louis, Missouri, U.S.A.) which had been reconstituted in dH_2O as recommended by the manufacturer, aliquoted, and stored frozen at -80 °C until required. Samples were gently mixed by inversion at the beginning, and half way through the incubation period. As soon as the incubation period was over, samples were centrifuged at 5 °C and 17,000 × *g* in a microcentrifuge (Fisher Scientific accuSpin Micro 17R Microcentrifuge; Loughborough, U.K.) for 3 minutes. Plasma was aliquoted into 1.5 mL Eppendorf tubes and stored frozen at -80 °C until analysis.

Elastase concentrations were determined by ELISA (AssayMax™ Human Neutrophil Elastase ELISA Kit; catalogue number: EE1001-1; AssayPro, St. Charles, Missouri, U.S.A.). Samples were thoroughly defrosted before use. Un-stimulated and stimulated

samples were diluted 1:50 and 1:1000 respectively prior to use, and the test procedure recommended by the manufacturer was followed. Un-stimulated samples were analysed in singular; stimulated samples were analysed in duplicate. The intraassay CV = 1.5%. Standard curves and assay results were prepared in Microsoft Excel. Any samples which fell above or below the standard curve were re-analysed with the dilution factor adjusted accordingly. Elastase release was calculated using the following equation:

$$Elastase\ release\ (ng.mL^{-1}) = (\ stim.elastase\ conc\ (ng.mL^{-1}) - unstim.\ elastase\ conc\ (ng.mL^{-1})) \times (1 - haematocrit)$$

Elastase release per neutrophil was calculated using the following equation:

$$Elastase\ release/neurophil\ (fg.mL^{-1}) = \frac{Elastase\ release\ (ng.mL^{-1})}{Neutrophil\ count}$$

The acute neutrophil responses to exercise pre, post, and 1 hour post 90 minutes steady state cycling are reported, using pre supplementation values as a baseline.

6.2.11 Statistical analysis

All results are presented as mean (SD). A significance level of 0.05 was pre-set for all statistical analyses. Normal distribution within the data was analysed using the Shapiro-Wilk test. For normally distributed variables, means of subject characteristics, and exercise trial performance were compared using Paired Samples T-Tests. Illness episodes were compared between arms using the Chi Squared Test with the McNemar Test statistic reported (for within group comparison). The log transformed and square-roots of data for which normal distribution could not be assumed were first tested using the Shapiro-Wilk test before non-parametric tests were undertaken. Wilcoxon tests and Friedman tests were undertaken for monocyte, granulocyte, haemoglobin, haematocrit, and neutrophil-lymphocyte ratios, IL-10 and IL-4 responses. All other means and changes over time were compared using two-way ANOVAs. For vitamin D status analysis, pre-supplementation vitamin D status was also included as a covariate in order to determine the effects of pre vitamin D status on the effectiveness of the supplementation induced changes of vitamin D

status. Significant differences were identified using the Holm-Bonferonni Test. All tests were carried out using SPSS Version 22.0 (IBM Corp, Armonk, NY, USA

6.3 Results

6.3.1 Subject characteristics

There were no significant differences in subjects' physical and performance characteristics between trials (tables 6.1 and 6.2).

6.3.2 Exercise trial physiological responses

There were no significant differences in subjects' absolute (P = 0.113) or relative $\dot{V}O_2$max (P = 0.117); lower gas exchange threshold relative to body mass ($ml.kg^{-1}.min^{-1}$) (P = 0.052); W_{max} (P = 0.539); or time to exhaustion (P = 0.954) between $\dot{V}O_2$max tests. Subjects' lower ventilatory exchange threshold ($L.min^{-1}$), and subjects' lower gas exchange threshold when expressed as a percent of $\dot{V}O_2$max ($L.min^{-1}$) were significantly lower in subjects' CHL trials (P = 0.009 and 0.013 respectively) (table 6.2).

Table 6.2 Physiological Responses to the $\dot{V}O_2$max Test on Day 1 of the Training Intervention, n = 14

	Chlorella		**Placebo**		***P*-Value**
	Mean (SD) or Median (IQR)	**Range**	**Mean (SD) or Median (IQR)**	**Range**	
VO_2max ($L.min^{-1}$)	3.9 (0.7)	3.0 – 5.1	3.7 (0.7)	2.9 – 4.8	0.113
$\dot{V}O_2$max ($ml.kg^{-1}.min^{-1}$)	55.0 (13.2)	35.1 – 79.7	53.6 (13.2)	34.2 – 78.7	0.117
VT1 ($L.min^{-1}$)	1.6 (0.3)	1.3 – 2.3	**1.8 (0.4)***	1.1 – 2.3	**0.009**
VT1 ($ml.kg^{-1}.min^{-1}$)	23.7 (6.3)	14.2 – 36.4	25.8 (7.0)	15.4 – 37.7	0.052
VT1 (% max)	42.3 (4.5)	34.2 – 52.4	**47.9 (5.4)***	39.3 – 61.1	**0.013**
W_{max} (watts)	326 (52)	254 - 425	327 (47)	264 - 403	0.539
Time to exhaustion (min)	13.9 (1.7)	11.5 – 17.2	13.9 (1.6)	11.8 – 16.4	0.954

* ($P < 0.05$) indicate significantly higher than chlorella

There were no significant differences in power output (P = 0.524); target VO_2 (P = 0.961); mean RPE (P = 0.216); meanVO_2 (P = 0.289); or mean RER (P = 0.685) between trials during the 90 minute trials (table 6.3). There was, however, a significant difference in the relative work-rate when expressed as a percent of $\dot{V}O_2$max ($L.min^{-1}$) (P = 0.047) with subjects working at a higher relative work-rate in the placebo trial. There was not a main

effect of trial for NBM pre to post exercise between trials, or a main effect of time, or a trial × time interaction (table 6.4).

Table 6.3 Physiological Responses to 90 Minute Cycling on Day 2 of the Training Intervention (n = 14)

	Chlorella (n = 14)		**Placebo (n = 14)**		***P*-Value**
	Mean (SD) or Median (IQR)	**Range**	**Mean (SD) or Median (IQR)**	**Range**	
Work rate (W)	148 (34)	101 - 201	146 (30)	106 - 190	0.524
Target VO_2 ($L.min^{-1}$)	2.2 (0.4)	1.7 - 2.8	2.2 (0.4)	1.6 - 2.8	0.961
Mean RPE	12 (1)	11 - 15	13 (1)	11 - 14	0.216
Mean VO_2 ($L.min^{-1}$)	2.1 (0.4)	1.4 - 2.9	2.2 (0.4)	1.6 - 2.9	0.289
Work rate (% max)	56.1 (7.7)	38.0 - 66.6	**59.9 (6.0)***	52.8 - 72.3	**0.047***
Mean RER	0.94 (0.10)	0.85 - 1.11	0.95 (0.08)	0.88 - 1.08	0.685

Data are presented mean (SD), with the exception of mean RER, whereby median (IQR) is reported
* ($P < 0.05$) indicate significantly higher than chlorella

Table 6.4 Change in NBM (kg) 90 Minute Cycling on Day 2 of the Training Intervention

	Chlorella (n = 14)		**Placebo (n = 14)**		***P*-Value**		
	Median (IQR)	**Range**	**Median (IQR)**	**Range**	**Trial**	**Time**	**Trial × Time**
NBM Pre (kg)	68.1 (16.4)	56.4 - 91.0	68.5 (18.5)	58.0 - 89.8	0.093	0.077	0.051
NBM Post (kg)	67.1 (10.0)	55.8 - 90.4	69.1 (18.7)	58.0 - 93.1			

6.3.3 Illness incidence

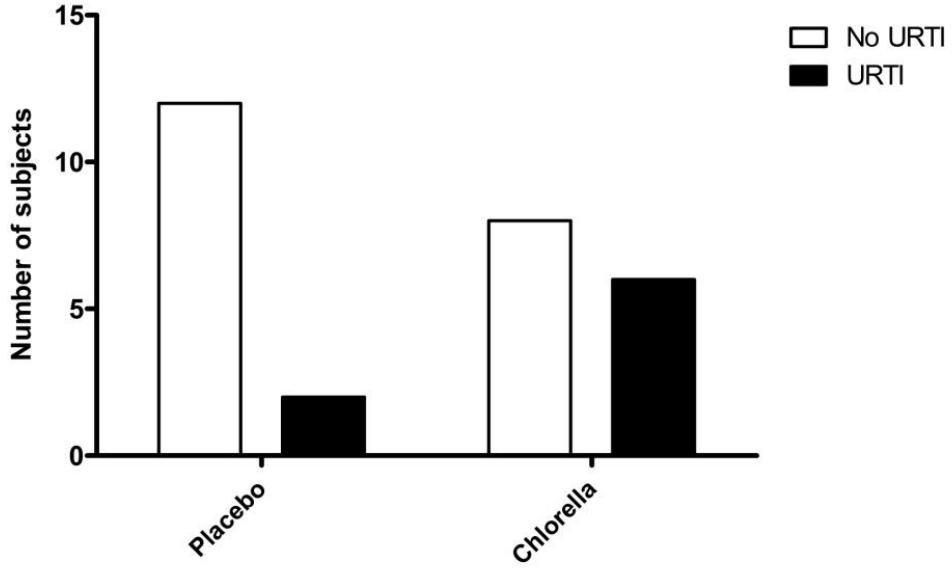
Two subjects in the PLA trial (14%), and 6 subjects in the CHL trial (43%) suffered with a URTI in the 2 weeks following the exercise intervention which was not a significant difference (P = 0.094) (figure 6.1). Subjects in the PLA trial reported that symptoms lasted an average of 7 ± 3 days, had a peak severity of 2 ± 1, and a symptom score of 15 ± 9. In the CHL trial, subjects reported that symptoms lasted an average of 7 ± 4 days, had a peak severity of 2 ± 1, and a symptom score of 15 ± 11.

6.3.4 Cell counts

There were no significant main effects of trial for total WBC count (P = 0.530), total circulating lymphocyte count (P = 0.137), total circulating monocyte count (P = 0.132), or

total circulating granulocyte count (P = 0.321). There were significant effects of time for total WBC count (P <0.001), total circulating lymphocyte count (P <0.001), total circulating monocyte count (P <0.001), and total circulating granulocyte count (P <0.001). There a not a significant time × trial interaction for total WBC count (P = 0.330), but there was for total lymphocyte count (P = 0.031). Post-hoc tests showing a significant increase in total WBC count from baseline immediately post-exercise (90 min bout) (P <0.001), and 1 hour post-exercise (P <0.001) (table 6.5); a significant increase in lymphocyte count immediately post-exercise (90 min bout) (P = 0.024) in the placebo trial; a significant increase in monocyte count from baseline immediately post-exercise (90 min bout) (P <0.001); and a significant increase in granulocyte count from baseline immediately post-exercise and 1 hour post-exercise (90 min bout) (P <0.001 and <0.001 respectively). There was no significant main effect of trial for the granulocyte to lymphocyte ratio (P = 0.201), but there was a significant effect of time (P <0.001) with post-hoc tests showing a significant increase in the granulocyte to lymphocyte ratio at 1 hour post-exercise (90 min bout) (P <0.001). There were no significant main effects of trial for haemoglobin concentration (P = 0.968) or haematocrit (P = 0.625). There were significant effects of time for both haemoglobin (P = 0.006) and haematocrit (P <0.001) but post-hoc tests did not identify any specific time differences (table 6.5).

Figure 6.1 URTI Incidence between trials



6.3.5 Vitamin D status

There was no significant main effect of trial (P = 0.620) or time (P = 0.454), nor was there a significant time × trial interaction (P = 0.133) for total vitamin D concentration. When baseline values for each condition were included as a covariate, there was not a significant difference between adjusted means for placebo (mean ± SEM: 65.43 ± 1.40 nmol/L) and chlorella (mean ± SEM: 62.75 ± 1.14 nmol/L) with a mean difference of 2.682 nmol/L (P = 0.174). There was not a significant change between adjusted means for pre (mean ± SEM: 63.14 ± 0.00 nmol/L) and post (mean ± SEM: 65.04 ± 1.77 nmol/L) with a mean difference of -1.90 nmol/L (P = 0.307), and differences between trials were not dependent on changes over time as there was not a significant trial by time interaction (p = 0.172). There was no significant main effect of trial (P = 0.778), time (P = 0.554), or a trial × time interaction (P = 0.569) for subjects who started with adequate vitamin D levels (>50 $nmol.L^{-1}$). There was not a significant main effect of trial (P = 0.478) or a main effect of time (P = 0.121), but there was there a significant time × trial interaction (P = 0.042) for subject who started the trial with insufficient vitamin D levels (<50 $nmol.L^{-1}$). Post-hoc analysis showed no significant effects of chlorella (P = 0.056) or placebo supplementation (P = 0.758) when subjects started with low vitamin D levels (table 6.6).

6.3.6 SIgA responses

There were no significant main effects of trial (P = 0.145 and 0.348) or time (P = 0.112 and 0.139) for SIgA concentration or secretion rate, respectively, between resting time-points throughout the trial. There was a significant time × trial interaction for SIgA concentration (P = 0.024), but not secretion rate (P = 0.329). Post-hoc analysis identified a significant difference in SIgA concentration between trials 1-week-post intervention (P = 0.040) with SIgA concentration higher in the CHL trial. There was not an effect of time on SIgA concentration in the PLA group (P = 0.748) but there was an effect of time in the CHL trial (P = 0.011) but post-hoc tests did not detect any changes over time (table 6.7).

Table 6.5 Acute Blood Responses Compared to Baseline during 90 min Steady State Endurance Ride on Day 2

			Bout 3 (Day 2 morning): 90 min SS		
	Baseline	**Pre- VO_2max**	**Pre-Ex**	**Post-Ex**	**1h-Post-Ex**
Total WBC (×10^9 cells.L^{-1})				**	**
PLA (*n* = 13)	6.02 (1.80)	6.41 (2.04)	5.50 (1.44)	8.94 (3.37)	9.80 (3.56)
SC (*n* = 13)	5.60 (1.94)	5.82 (1.46)	5.53 (1.89)	8.56 (3.20)	10.23 (3.45)
Lymphocytes (×10^9 cells.L^{-1})					
PLA (*n* = 13)	1.54 (0.71)	1.84 (0.82)	1.37 (0.49)	**2.72 (1.35)** †	1.16 (0.47)
SC (*n* = 13)	1.48 (0.53)	1.53 (0.60)	1.42 (0.61)	1.71 (0.52)	1.15 (0.46)
Monocytes (×10^9 cells.L^{-1})				**	
PLA (*n* = 13)	0.64 (0.42)	0.79 (0.65)	0.69 (0.49)	0.96 (0.53)	0.77 (0.42)
SC (*n* = 13)	0.55 (0.43)	0.63 (0.32)	0.62 (0.46)	0.76 (0.43)	0.82 (0.27)
Granulocytes (×10^9 cells.L^{-1})				**	**
PLA (*n* = 13)	3.93 (1.99)	3.41 (1.46)	3.61 (1.97)	4.20 (2.71)	7.62 (7.82)
SC (*n* = 13)	3.70 (2.03)	3.69 (2.35)	3.67 (2.09)	5.31 (2.92)	7.51 (4.06)
Haemoglobin (g.L^{-1})					
PLA (*n* = 13)	152.1 (15.0)	159.0 (14.5)	152.0 (15.0)	159.1 (12.9)	160.0 (19.7)
SC (*n* = 13)	156.0 (10.5)	157.0 (13.5)	155.0 (19.0)	161.0 (16.5)	160.3 (17.0)
Haematocrit (%)					
PLA (*n* = 13)	41.2 (3.89)	41.4 (2.56)	39.9 (3.2)	41.3 (4.2)	41.4 (5.1)
SC (*n* = 13)	40.7 (2.5)	40.1 (2.6)	41.0 (4.4)	42.7 (3.4)	42.0 (4.32)
Granulocyte:Lymphocyte					**
PLA (*n* = 13)	2.94 (1.76)	2.12 (2.01)	2.53 (1.45)	1.91 (2.25)	6.62 (4.57)
SC (*n* = 13)	2.35 (1.20)	2.45 (1.15)	2.24 (1.12)	3.06 (1.63)	8.09 (7.49)

90 min SS: steady state endurance ride. Values are median (IQR) with the exception of total WBC and lymphocytes whereby mean (SD) are presented

* ($P < 0.05$) and ** ($P < 0.01$) indicates a significant change from baseline (post hoc follow-up for time: both groups pooled due to no group × time-point interaction).

† ($P < 0.05$) indicates a significant change from baseline (post post hoc follow-up for time: each group analysed separately due to significant group × time-point interaction).

There were no significant main effects of trial (P = 0.833 and 0.225) or a trial × time interaction (P = 0.947 and 0.588) for SIgA concentration or secretion rate, respectively, in response to the $\dot{V}O_2$max test. There was a significant main effect of time (P = 0.004) for SIgA concentration, but not secretion rate (P = 0.251). However, post-hoc analysis could not identify any changes in SIgA concentration over time. When pre-supplementation values were excluded and exercise responses analysed in isolation, there was not a main effect of trial (P = 0.685 and 0.271) or time (P = 0.181 and 0.444) for SIgA concentration or secretion rate, respectively. There was a significant time × trial interaction (P = 0.011) for SIgA concentration, but not secretion rate (P = 0.320). Post-hoc analysis of SIgA concentration identified a time effect in both the placebo and chlorella trials (P = 0.004 and 0.032 respectively). In both the placebo and chlorella trials, there were no significant changes in SIgA concentration from pre-$\dot{V}O_2$max test to immediately post (P = 0.152 and 0.626, respectively), or 1 hour post (P = 0.071 and 0.060, respectively) (table 6.8).

There was no significant main effect of trial for SIgA concentration or secretion rate in response to HIIE1 (P = 0.434 and 0.853, respectively). There was no main effect of time (P = 0.240) or a time × trial interaction (P = 0.156) for SIgA secretion rate, however there was a significant main effect of time (P <0.001), and a time × trial interaction (P = 0.045) for SIgA concentration. Post-hoc analysis of SIgA concentration in response to HIIE1 identified a significant effect of time in the placebo condition with a significant decrease in SIgA concentration from baseline pre (P = 0.006) and 1 hour post (P <0.001). The same effects were not observed in the chlorella trial (P = 0.371). When pre-supplementation values were excluded and exercise responses analysed in isolation, there was a significant main effect of trial (P <0.001) for SIgA concentration, but not SIgA secretion rate (P = 0.695). No significant main effects of time (P = 0.243 and 0.444), or time × trial interactions (P = 0.051 and 0.066) were observed for SIgA concentration or secretion rate, respectively. Post-hoc analysis of SIgA concentration in response to HIIE1 identified a significant main effect of time in the placebo trial (P <0.001) with a significant decrease in SIgA concentration pre-HIIE1 (P = 0.004) and 1 hour post HIIE1 (P <0.001). There was no significant main effect of time in the chlorella trial (P = 0.139) (table 6.8).

There was no significant main effects of trial (P = 0.632 and 0.926) for SIgA concentration or secretion rate, respectively, in response to 90 minutes steady state cycling. There was a

Table 6.6 Vitamin D Responses to Supplementation

	Baseline (ALL) (*n* = 14)	**Post Supplementation (ALL)** (*n* = 14)	**Baseline (low starters)** (*n* = 5 PLA, 4 CHL)	**Post Supplementation (low starters)** (*n* = 5 PLA, 4 CHL)	**Baseline (adequate starters)** (*n* = 9 PLA, 10 CHL)	**Post Supplementation (adequate starters)** (*n* = 9 PLA, 10 CHL)
Total Vitamin D ($nmol.L^{-1}$)						
PLA	66.9 (31.9)	63.9 (24.6)	38.3 (5.9)	43.3 (18.0)	35.4 (3.0)	34.0 (9.9)
SC	59.4 (23.7)	66.1 (16.9)	29.6 (6.8)	51.2 (12.6)	71.3 (15.6)	72.1 (14.3)
Vitamin D_2 ($nmol.L^{-1}$)						
PLA	5.8 (3.1)	4.6 (2.1)	4.4 (2.0)	3.4 (1.4)	6.5 (3.5)	5.2 (2.3)
SC	3.8 (1.8)	27.7 (12.0)	5.5 (2.2)	32.5 (17.5)	3.1 (1.1)	25.8 (9.6)
Vitamin D_3 ($nmol.L^{-1}$)						
PLA	61.2 (32.0)	59.4 (23.8)	34.0 (4.4)	39.8 (17.8)	76.3 (30.6)	70.2 (19.8)
SC	55.6 (24.3)	38.5 (17.1)	24.2 (7.3)	18.7 (6.3)	68.1 (14.9)	46.4 (12.8)

Values are mean (SD)
Low starters are subjects with a starting Total Vitamin D of <50.00 $nmol.L^{-1}$; Adequate starters are subjects with a starting Total Vitamin D of >50.00 $nmol.L^{-1}$

Table 6.7 Resting SIgA Responses compared to baseline, pre supplementation

	Baseline	Pre Exercise Day 1	Pre Exercise Day 2	1 Week Post	2 Weeks Post
SIgA concentration ($mg.L^{-1}$)					
PLA (*n* = 14)	185 (71)	190 (80)	195 (88)	168 (71)	190 (52)
SC (*n* = 14)	209 (163)	201 (96)	187 (95)	**267 (123)***	355 (268)
SIgA secretion rate ($\mu g .min^{-1}$)					
PLA (*n* = 14)	79.5 (51.6)	79.6 (55.2)	84.5 (67.3)	104.8 (92.0)	90.3 (70.1)
SC (*n* = 14)	59.9 (54.6)	70.3 (52.5)	69.1 (41.2)	79.0 (39.7)	64.4 (47.4)

Values are mean (SD)
* ($P < 0.05$) indicate significantly higher than placebo.

Table 6.8 Acute SIgA responses to each exercise bout during the 2-day intensified training period (*n* = 14)

		Bout 1 (Day 1 morning): $\dot{V}O_2$max test			Bout 2 (Day 1 afternoon): HIIE			Bout 3 (Day 2 morning): 90 min SS			Bout 4 (Day 2 afternoon):HIIE		
	Baseline	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**
SIgA concentration ($mg.L^{-1}$)								*		*			
PLA	185 (71)	190 (80)	229 (107)	150 (66)	**127 (66)** †	210 (111)	**101 (48)** ‡	195 (88)	156 (40)	133 (65)	142 (70)	221 (110)	159 (75)
SC	209 (163)	201 (96)	238 (163)	145 (67)	153 (63)	175 (109)	157 (105)	187 (95)	192 (157)	149 (56)	135 (68)	199 (103)	134 (82)
SIgA secretion rate ($\mu g.min^{-1}$)													
PLA	79.5 (51.6)	79.6 (55.2)	63.3 (37.7)	69.4 (35.5)	58.3 (25.9)	50.8 (31.7)	71.1 (56.4)	84.5 (67.3)	64.0 (48.0)	58.0 (36.3)	61.9 (39.0)	62.3 (49.6)	107.5 (94.3)
SC	59.9 (54.6)	70.3 (52.5)	67.6 (64.1)	67.0 (57.5)	63.6 (27.9)	59.0 (33.7)	59.1 (46.0)	69.1 (41.2)	59.6 (27.8)	75.3 (33.9)	57.3 (27.6)	43.3 (21.7)	68.1 (41.1)

HIIE: High-intensity interval exercise session; 90 min SS: steady state endurance ride. Values are mean (SD)
* ($P < 0.05$) indicates significant change from Pre-Ex, Bout 1 ($\dot{V}O_2$max test) (post hoc follow-up for time: both groups pooled due to no group × time-point interaction).
† ($P < 0.05$) and ‡ ($P < 0.01$) indicate significant change from baseline (post hoc follow-up for time: each group analysed separately due to significant group × time-point interaction).

significant effect of time for SIgA concentration (P = 0.044) but not SIgA secretion rate (P = 0.753). There was no time × trial interaction (P = 0.452) for SIgA concentration but there was for SIgA secretion rate (P = 0.032). Post-hoc analysis showed a significant decrease in SIgA secretion rate 1 hour post compared to baseline (P = 0.009), however there was no significant effect of time in either the placebo trial (P = 0.054) or the chlorella trial (P = 0.185). No significant differences were identified in SIgA concentration. When pre-supplementation values were excluded and exercise responses analysed in isolation, there was a significant main effect of trial (P = 0.048) and a significant main effect of time (P = 0.006) for SIgA concentration, but not SIgA secretion rate (P = 0.343 and 0.369, respectively). There were no significant time × trial interactions for either SIgA concentration (P = 0.529) or secretion rate (P = 0.384). Post-hoc analysis identified a significant decrease in SIgA concentration pre 90 minutes (P = 0.024) and 1 hour post 90 minutes (P = 0.020) compared to pre $\dot{V}O_2$max test (table 6.8).

There were no significant main effects of trial for SIgA concentration or secretion rate in response to HIIE2 (P = 0.082 and 0.161 respectively), nor were there time × trial interactions (P = 0.427 and 0.258). There was a significant main effect of time (P = 0.016) for SIgA concentration, but post-hoc analysis could not identify a difference. No significant main effects of time were identified for SIgA secretion rate (P = 0.069). When pre-supplementation values were excluded and exercise responses analysed in isolation, there was a significant main effect of trial for SIgA concentration and secretion rate (P = 0.001 and 0.031 respectively) but not time (P = 0.253 and 0.220 respectively). There was no time × trial interaction for SIgA concentration (P = 0.748), but there was for SIgA secretion rate (P <0.001). Post-hoc analysis of SIgA secretion rate in response to HIIE2 identified a significant main effect of time in the placebo trial (P = 0.013) but no specific changes across time points could be identified. There was no main effect of time in the chlorella condition (P = 0.075) (table 6.8).

There was no significant main effect of trial (P = 0.060), time (P = 0.084), or a time × trial interaction (P = 0.099) for saliva flow rate at resting time points (table 6.9). There was no main effect of trial (P = 0.249) or a time × trial interaction (P = 0.600) on saliva flow rate in response to the $\dot{V}O_2$max test, but there was a main effect of time (P = 0.004) but post-hoc tests failed to find any specific differences between time points. When pre-supplementation values were excluded and exercise responses analysed in isolation, there was no main effect of trial (P = 0.393), or a time × trial interaction (P = 0.673) on saliva

flow rate in response to the $\dot{V}O_2$max test, but there was a main effect of time ($P < 0.001$) with post-hoc tests identifying a significant decrease in saliva flow rate immediately post $\dot{V}O_2$max test compared to pre $\dot{V}O_2$max test ($P = 0.027$). There was no main effect of trial on saliva flow rate in response to the HIIE1 ($P = 0.307$), however there was a significant main effect of time ($P = 0.017$) and a significant time × trial interaction ($P = 0.003$). Post-hoc tests identified a significant decrease in saliva flow rate from baseline in the placebo group immediately post HIIE1 ($P = 0.049$), and a significant increase in saliva flow rate from baseline 1 hour post the HIIE1 ($P = 0.036$). When pre-supplementation values were excluded and exercise responses analysed in isolation, there was no main effect of trial ($P = 0.527$), however there was a main effect of time ($P < 0.001$), and a time × trial interaction ($P = 0.004$) for saliva flow rate in response to HIIE1. Post-hoc tests identified a significantly higher saliva flow rate in the CHL condition compared to PLA immediately post HIIE1 ($P < 0.001$) and significantly lower in the CHL trial compared to PLA 1 hour post HIIE1 ($P = 0.037$). There was no main effect of trial ($P = 0.753$), time ($P = 0.061$), or a time × trial interaction ($P = 0.054$) for saliva flow rate in response to 90 minutes steady state cycling. When pre-supplementation values were excluded and exercise responses analysed in isolation, there was no main effect of trial ($P = 0.893$), time ($P = 0.102$), or a time × trial interaction ($P = 0.209$) for saliva flow rate in response to 90 minutes steady state cycling. There was no main effect of trial ($P = 0.778$), or a time × trial interaction ($P = 0.577$) for saliva flow rate in response to HIIE2, however there was a significant main effect of time ($P = 0.004$). Post-hoc tests did not identify any significant changes in saliva flow rate at any time point from baseline in response to HIIE2. When pre-supplementation values were excluded and exercise responses analysed in isolation, there was no main effect of trial ($P = 0.999$), nor a time × trial interaction ($P = 0.690$), however there was a main effect of time ($P = 0.002$) for saliva flow rate in response to HIIE2. Post-hoc tests identified a significant decrease in saliva flow rate immediately post HIIE2 compared to pre ($P < 0.001$) (table 6.10).

6.3.7 Salivary lysozyme, salivary lactoferrin, salivary cortisol, and α-amylase

There was no significant main effect of trial ($P = 0.477$ and 0.549), nor was there a significant trial × time interaction ($P = 0.198$ and 0.451) for salivary lysozyme concentration or secretion rate, respectively. There was a significant main effect of time ($P <0.001$ and 0.010) for both salivary lysozyme concentration and secretion rate, with post-

Table 6.9 Resting saliva flow rate (ml.min^{-1}) responses compared to baseline, pre supplementation

	Baseline	Pre Exercise Day 1	Pre Exercise Day 2	1 Week Post	2 Weeks Post
PLA (*n* = 14)	0.39 (0.18)	0.44 (0.23)	0.45 (0.41)	0.38 (0.38)	0.25 (0.26)
SC (*n* = 14)	0.35 (0.32)	0.38 (0.36)	0.42 (0.42)	0.45 (0.66)	0.42 (0.44)

Values are median (IQR)

Table 6.10 Acute saliva flow rate (ml.min^{-1}) responses to each exercise bout during the 2-day intensified training period (*n* = 14)

		Bout 1 (Day 1 morning): $\dot{V}O_2$max test			Bout 2 (Day 1 afternoon): HIIE			Bout 3 (Day 2 morning): 90 min SS			Bout 4 (Day 2 afternoon):HIIE		
	Baseline	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**	**Pre-Ex**	**Post-Ex**	**1h Post-Ex**
			††			#†	#					††	
PLA	0.39 (0.18)	0.44 (0.23)	0.30 (0.44)	0.47 (0.47)	0.47 (0.42)	**0.24 (0.49) ***	**0.87 (0.77) ***	0.45 (0.41)	0.36 (0.31)	0.47 (0.36)	0.48 (0.54)	0.26 (0.30)	0.59 (0.62)
SC	0.35 (0.32)	0.38 (0.36)	0.30 (0.29)	0.41 (0.49)	0.46 (0.43)	**0.40 (0.35)** †	0.52 (0.59)	0.42 (0.42)	0.34 (0.42)	0.51 (0.36)	0.48 (0.42)	0.26 (0.32)	0.43 (0.67)

HIIE: High-intensity interval exercise session; 90 min SS: steady state endurance ride. Values are median (IQR)
* ($P < 0.05$) indicates significant change from baseline
† ($P < 0.05$) and †† ($P < 0.001$) indicate significant change from pre exercise values (post hoc follow-up for time: each group analysed separately due to significant group × time-point interaction).
# ($P < 0.05$) indicates significant difference between conditions

hoc tests showing a significant decrease in salivary lysozyme concentration one hour post-exercise (90 min bout) (P = 0.015), with no significant changes in salivary lysozyme secretion rate at any specific time points. There was no significant main effect of trial (P = 0.909 and 0.908), nor was there a significant trial × time interaction (P = 0.686 0.495) for salivary lactoferrin concentration or secretion rate, respectively. There was no significant main effect of time for salivary lactoferrin secretion rate (P = 0.383), but there was a significant main effect of time for salivary lactoferrin concentration (P = 0.010). Post-hoc tests did not identify any changes at specific time points for salivary lactoferrin concentration. There were no significant main effects of trial (P = 0.830 and 0.657), nor was there a significant trial × time interaction (P = 0.209 and 0.309) for salivary cortisol concentration or secretion rate, respectively. There was no main effect of time for salivary cortisol concentration (P = 0.065), but there was for salivary cortisol secretion rate (P = 0.047) with post-hoc tests showing a significant decrease in salivary cortisol secretion rate one hour post-exercise (90 min bout) (P = 0.045). There were no significant main effects of trial (P = 0.415 and 0.906) or time (P = 0.111 and 0.051), nor were there significant trial × time interactions (P = 0.415 and 0.691) for α-amylase concentration or secretion rate (table 6.11).

6.3.8 EBV expression

Twelve out of fifteen subjects (80%) were seropositive for EBV. For saliva EBV DNA concentration, there was a main effect of trial (P = 0.035), and a trial × time interaction (P = 0.012), but no main effect of time (P = 0.088). Post-hoc analysis identified a greater concentration of EBV DNA at baseline, and the start of day 2 of the exercise intervention in the CHL trial, but not the start of day 1 of the exercise intervention (P = 0.004, 0.032, and 0.986 respectively). During the placebo trial, there was a significant increase in EBV DNA concentration from baseline at the start of first day of exercise (P = 0.026), but not at the start of the second day of exercise (P = 0.252). In the chlorella trial, there was no significant change in EBV DNA concentration from baseline at the start of the first (P = 1.000), or the second day of exercise (P = 1.000). EBV DNA concentration was compared between subjects who fell ill, and those who didn't. There was no main effect of group (P = 0.195), time (P = 0.091), or a group × time interaction (P = 0.986) in the PLA trial. There was no main effect of group (P = 0.803), or time (P = 0.428), but there was a group × time interaction (P = 0.047) in the CHL group. Post-hoc tests did not identify any

Table 6.11 Salivary Markers Compared to Baseline during 90 min Steady State Endurance Ride on Day 2

			Bout 3 (Day 2 morning): 90 min SS	
	Pre- $\dot{V}O_2max$	**Pre-Ex**	**Post-Ex**	**1h-Post-Ex**
Lysozyme				
Concentration(mg.L^{-1})				*
PLA (*n* = 10)	2.3 (1.7)	2.7 (2.5)	4.7 (2.6)	1.3 (0.9)
SC (*n* = 10)	4.0 (3.2)	23. (2.0)	5.8 (5.3)	1.7 (1.6)
Secretion rate (mg.min^{-1})				
PLA (*n* = 9)	1.2 (1.0)	1.6 (1.5)	1.9 (1.4)	0.8 (0.6)
SC (*n* = 9)	1.6 (1.2)	1.3 (1.2)	2.1 (1.9)	1.1 (1.1)
Lactoferrin				
Concentration (mg.L^{-1})				
PLA (*n* = 10)	5.7 (11.1)	7.1 (7.7)	7.3 (18.3)	6.8 (6.0)
SC (*n* = 10)	6.0 (6.0)	6.1 (8.7)	6.7 (15.9)	6.0 (9.1)
Secretion rate (mg.min^{-1})				
PLA (*n* = 9)	4.9 (4.4)	4.8 (4.1)	6.0 (4.6)	3.8 (2.3)
SC (*n* = 9)	3.7 (3.1)	4.8 (2.7)	5.2 (4.1)	4.6 (2.8)
Salivary Cortisol				
Concentration (nmol.L^{-1})				
PLA (*n* = 10)	11.8 (5.4)	9.1 (5.4)	9.0 (4.2)	6.4 (4.8)
SC (*n* = 10)	10.0 (3.6)	8.8 (3.7)	10.9 (4.3)	5.7 (3.3)
Secretion rate (nmol.min^{-1})				*****
PLA (*n* = 9)	6.2 (3.2)	5.2 (4.0)	3.8 (2.0)	3.3 (2.3)
SC (*n* = 9)	5.1 (3.2)	5.6 (3.8)	3.9 (1.5)	2.8 (1.7)
α-amylase				
Concentration (U.mL^{-1})				
PLA (*n* = 10)	74 (93)	141 (192)	102 (69)	86 (61)
SC (*n* = 10)	48 (36)	182 (210)	55 (27)	89 (48)
Secretion rate (U.min^{-1})				
PLA (*n* = 9)	22.1 (31.3)	41.0 (38.9)	57.2 (52.7)	45.2 (61.9)
SC (*n* = 9)	22.7 (23.2)	34.6 (81.9)	34.4 (33.1)	53.0 (41.1)

90 min SS: steady state endurance ride. Values are mean (SD) with the exception of Lactoferrin Concentration and α-amylase secretion rate whereby the values are the median (IQR)
* ($P < 0.05$) indicates a significant decrease from baseline (post hoc follow-up for time: both groups pooled due to no group × time-point interaction).

Table 6.12 Change in EBV concentration compared to baseline, pre supplementation

	Baseline	Pre Exercise Day 1	Pre Exercise Day 2
EBV Concentration (ng.µl^{-1} x10^{-6})	*		*
PLA (*n* = 12)	0.07 (0.21)	**0.3 (0.16)†**	0.08 (0.19)
SC (*n* = 12)	0.91 (1.51)	1.07 (1.48)	1.13 (4.66)
EBV Concentration (ng.µl^{-1} x10^{-6})			
No URTI (*n* = 16)	0.16 (0.60)	0.11 (0.87)	0.15 (4.29)
URTI (*n* = 8)	0.83 (1.76)	1.16 (2.26)	1.02 (2.17)

Values are median (IQR)

* ($P < 0.05$) indicate significant difference between groups at time point (post-hoc follow up: group comparisons made due to significant trial interaction)

† ($P < 0.05$) indicate significant increase from baseline (post-hoc follow up: each group analysed separately due to significant group × time point interaction).

significant differences between subjects who developed a URTI and those who didn't in the chlorella group (table 6.12).

6.3.9 Neutrophil degranulation (Elastase release)

For stimulated elastase release per neutrophil, there was no significant main effect of trial ($P = 0.622$) nor was there a significant trial × time interaction ($P = 0.747$). There was a significant main effect of time ($P < 0.001$) with post hoc tests identifying significant increases in neutrophil degranulation immediately post ($P = 0.030$) and 1 hour post exercise ($P < 0.001$) (table 6.13).

6.3.10 Cytokine responses

There were no significant effects of trial for stimulated IL-4 or IL-10 responses ($P = 0.091$ and 0.945 respectively). There was no significant effect of time for stimulated IL-4 responses ($P = 0.052$), but there was a significant effect of time for stimulated IL-10 responses ($P = 0.010$). Post-hoc tests could not identify any specific time effects for IL-10 ($P = 0.120$) (table 6.13). When subjects were stratified into those who got ill and those who didn't, there were no significant effects of time ($P = 0.388$ and 0.622), group ($P = 0.526$ and 0.882), or a group × time interaction ($P = 0.745$ and 0.676) for stimulated IL-4 or IL-10, respectively (table 6.13).

6.4 Discussion

The aim of the current study was to assess the effects of chlorella supplementation on immune responses (leucocyte responses, salivary immune and stress response markers, SIgA, EBV DNA, and illness incidence) in response to a two day intensified training period using a double blind, cross-over design. The main findings of this study were that a daily dose of 6 g CHL for 4 weeks before (during, and for the 2 weeks following the training intervention) increased resting SIgA concentration by week 5, but did not influence the frequency, severity, or duration of self-reported URS. Neither SIgA secretion rate or concentration were acutely affected by any one individual exercise bout, but SIgA concentration in the CHL group appears to increase in response to some of the exercise bouts. The same response was not observed in the PLA group. WBCs,

Table 6.13 Elastase and Cytokine responses to 90 min Steady State Endurance Ride on Day 2, Compared to Baseline

			Bout 3 (Day 2 morning): 90 min SS		
	Baseline	**Pre- $\dot{V}O_2$max**	**Pre-Ex**	**Post-Ex**	**1h-Post-Ex**
Elastase (fg.mL^{-1})					*
PLA (*n* = 9)	705 (197)	712 (472)	678 (198)	1781 (2012)	1625 (977)
SC (*n* = 9)	689 (431)	615 (392)	805 (361)	1303 (490)	1332 (458)
IL-4 production (pg.mL^{-1})					
PLA (*n* = 14)	0.06 (0.21)	0.07 (0.15)	0.04 (0.06)	0.07 (0.08)	0.02 (0.03)
SC (*n* = 14)	0.04 (0.02)	0.11 (0.27)	0.05 (0.05)	0.06 (0.10)	0.09 (0.09)
IL-10 production (pg.mL^{-1})					
PLA (*n* = 14)	0.21 (0.47)	0.25 (0.49)	0.00 (0.40)	0.66 (2.01)	0.13 (0.45)
SC (*n* = 14)	0.40 (0.89)	0.18 (0.99)	0.13 (0.38)	0.33 (0.70)	0.22 (0.72)
IL-4 production (pg.mL^{-1})					
No URTI (*n* = 16)	0.05 (0.04)	0.12 (0.28)	0.04 (0.07)	0.07 (0.09)	0.004 (0.12)
URTI (*n* = 8)	0.02 (0.18)	0.07 (0.05)	0.05 (0.03)	0.06 (0.04)	0.10 (0.05)
IL-10 production (pg.mL^{-1})					
No URTI (*n* = 16)	0.41 (0.69)	0.26 (0.50)	0.22 (1.31)	0.74 (1.01)	0.28 (0.49)
URTI (*n* = 8)	0.29 (0.72)	0.73 (0.85)	0.20 (0.66)	0.87 (1.7)	0.57 (1.29)

90 min SS: steady state endurance ride. Values are median (IQR) with the exception of elastase whereby mean (SD) are presented

* ($P < 0.05$) indicates a significant change from baseline (post hoc follow-up for time: both groups pooled due to no group × time-point interaction).

lymphocytes, monocytes, and granulocytes increased from baseline immediately post the 90 minute cycle, with WBCs and granulocytes remaining elevated 1 hour post exercise, but there were no differences between the CHL and PLA conditions. The GLR increased from baseline 1 hour after the 90 minute cycle on day 2 of the training intervention, but there were no effects of CHL supplementation. Supplementation with CHL did not appear to affect the GLR; haemoglobin; haematocrit; vitamin D (even in those whose levels were inadequate to start with (>50 nmol.L^{-1}); lysozyme; lactoferrin; α-amylase; salivary cortisol; IL-4; or IL-10.

The previous study (Chapter 5) reported that a daily dose of 6 g CHL for 4 weeks before (and for the 2 weeks following) the training intervention increased resting SIgA secretion rate by week 4, accompanied by a non-significant trend for resting SIgA concentration to increase by week 5 which could not be explained by changes in saliva flow rate. However, in the present study, there were no changes in responses of SIgA secretion rate in either the PLA or CHL group. There was, however, an increase in resting SIgA concentration by week 5 which is in line with the previous study. As discussed in the previous chapter, there is very little research that has tracked salivary SIgA responses to multiple exercise sessions over such a short period of time. The findings of the current study neither concur, nor differ categorically with previous research (Davison., 2011; Hall et al., 2007; Otsuki et al., 2012; Papacosta et al., 2013). Because a decrease in SIgA has been shown to be related to an increased risk of developing URTI and the development of associated URS, it was hypothesised that any increase (or the avoidance of a decrease) in SIgA would result in a reduced risk of URS (i.e. subjects would report fewer symptoms). As discussed in the previous chapter, a decrease in the number of URS reported would be in line with previous research studies on intensified training periods/training camps and long-term monitoring of athletes (Fahlman & Engels, 2005; Gleeson et al., 2012; Gleeson et al., 2011; and Neville et al., 2008). Typically, an acute bout of moderate intensity exercise results in a drop of SIgA below baseline immediately post-exercise, with levels returning to normal within the following hour. For more intensive bouts of exercise, or in periods of intensified or over training, SIgA levels can take much longer to return to baseline. This can leave athletes at an increased risk of developing URTI in the hours that follow exercise (Kakanis et al., 2010). In the present study, however, there were no differences in URTI incidence between the CHL and PLA conditions. This finding, taken in combination with the fact that CHL supplementation did not increase SIgA secretion rate or concentration following the majority of exercise sessions, suggests that CHL supplementation, or at least the

supplementation schedule employed by the present study, does not provide protection against URTI via the SIgA pathway, or otherwise. It is not only SIgA which forms the first line of defence for pathogens entering via the oral cavity, however. AMPs and enzymes such as lysozyme, lactoferrin, and α-amylase also help in the prevention of illness. There are fewer studies investigating the role of these AMPs on URTI risk compared to SIgA but, owing to the fact these proteins and enzymes possess bacteriocidal properties, low levels in saliva should, in theory, result in an increase in URTI. However, owing to the fact the research pool is limited, this is merely speculation. The general consensus is that intense and exhaustive exercise results in increased levels of lysozyme (Allgrove et al., 2008; West et al., 2010; West et al., 2006), lactoferrin (West et al., 2010) and α-amylase (Allgrove et al., 2008; Bishop et al., 2000; Li & Gleeson, 2004; West et al., 2006) but in all cases, it is very much dependent on the exercise intensity. No changes in lysozyme, lactoferrin, or α-amylase were observed in the present study, however, which is not in line with previous research. Li & Gleeson (2004) reported that 2 hours cycling at 60% $\dot{V}O_2$max increased α-amylase activity. The increases in α-amylase activity were observed 1 hour into the exercise trial and remained elevated for the remainder of the trial period (a further 1 hour). These findings are similar to previous research in the area (Blannin et al., 2000; Walsh et al., 1999). It is the increases in plasma catecholamines and β-sympathetic activity associated with exercise that are believed to be responsible for the increases in α-amylase activity during, and immediately following exercise (Dawes, 1981; Walsh et al., 1999). The present study did not investigate plasma catecholamines, but it did investigate salivary cortisol which is an indicator of sympathetic activity. The fact that no increases in salivary cortisol were observed in response to exercise, makes it unsurprising that no changes to AMPs were observed either.

The increase in the concentration of EBV DNA detected in saliva in the chlorella trial is interesting insomuch as it appears CHL supplementation may decrease *in vivo* immunity which goes against our hypothesis. However, subjects at the start of the CHL condition had an increased expression of EBV DNA in their saliva compared to when they were supplemented with PLA. This may have had an impact on how they responded to the exercise intervention, albeit 4 weeks later, resulting in the increased EBV DNA concentrations observed at the start of the second exercise intervention day. In addition, although no significant differences in URTI incidence were identified by statistical analysis, a greater number of subjects reported a URTI following exercise when supplemented with CHL, compared to PLA (6 vs. 2 subjects respectively). This may be

indicative of a link between EBV concentration and the incidence of URTI, as demonstrated in the study by Yamauchi et al., 2011. It also appears in the present study, that CHL supplementation may actually protect against changes in EBV DNA expression, as significantly increased levels of EBV DNA were observed in the PLA trial, but not the CHL trial, but further research with a larger sample size may be required to investigate this further.

It is unlikely that there was a seasonal effect as half the subjects were supplemented with CHL first, and vice versa, minimising any seasonal variation between conditions (Matthews et al., 2002). SIgA only rises in the CHL condition five weeks after supplementation, and a week after the intensified training period. The immune permutations that would occur up-to 72 hours after the intensified training period (Kakanis et al., 2010) would have passed, and therefore the likelihood of contacting an illness in either group during week 5 or week 6, would be decreased somewhat. The fact that the beneficial effects of CHL do not appear to transpire until 5 weeks after the start of supplementation indicates it is possible that a longer supplementation period may be required to yield any beneficial effects on immune function and it would be feasible to suggest that, had the training period commenced after 5 or 6 weeks of supplementation, rather than 4, that this may have translated into greater effects on URS reports. However, this will require further study.

The most likely mechanisms for the increase in salivary SIgA concentration observed after 5 weeks of supplementation with CHL are via the immunostimulating properties of compounds found in CHL, discussed in chapter 5. One nutrient CHL is abundant in which has been shown to have a strong influence on immunity, is vitamin D. The primary outcome analysis for subjects' vitamin D levels in the present study showed that there was no difference in vitamin D status between the CHL and PLA groups. Furthermore, when subjects were stratified by starting vitamin D status (either low <50 $nmol.L^{-1}$, or normal >50 $nmol.l^{-1}$), no differences were observed between conditions. This finding is not in-line with the previous study (Chapter 5), in which CHL supplementation for subjects starting with a low vitamin status increased total vitamin D however this was due to an increase in vitamin D_2 and an decrease in vitamin D_3. There was a trend for subjects total vitamin D to decrease over the course of this study however, and the data show a substantial, although insignificant, increase in vitamin D_2 and a slight decrease in vitamin D_3 following supplementation with CHL (table 6.6). Further study with a larger sample size may be

required to explore this further. This may go some way to explaining why there was not a decrease in the number of URTI/URS reported in the CHL group as these subjects would have been more susceptible to infection, however this can only be speculated at this stage as the statistics do not support this interpretation..

The leucocytosis observed in response to the training intervention and, specifically, the 90 minute prolonged cycle at 25% Δ, is in line with the findings of previous research (Robson et al., 1999), and the previous study (chapter 5). Typically, prolonged endurance exercise results in a delayed leucocytosis which usually sees a doubling of leucocytes in circulation but, in some instances, a four-fold increase has been observed (Eskola et al., 1978). The significant increase in circulating lymphocytes following exercise is due to the redistribution of white blood cells already present. Typically, following intensive exercise lasting less than one hour, leucocytosis is caused by a significant increase in circulating neutrophils and lymphocytes. Following prolonged exercise, leucocytosis is predominately caused by neutrophilia. In this study, we were unable to differentiate granulocytes into neutrophils, eosinophils, basophils, and mast cells. Increases in eosinophils, basophils, and mast cells in response to exercise within a healthy population are unlikely, however, that we can be confident in our assumption that the significant increases in granulocytes observed in this study, are in fact neutrophils. Therefore, the cellular blood responses in the present study, where total WBC, lymphocytes, monocytes, and granulocytes (neutrophils) all significantly increased from baseline immediately following the 90 minute cycling on day 2 of the intervention, with granulocytes remaining elevated 1 hour post, are not atypical of the research currently in circulation (Blannin, 2006; Nieman et al., 1994, Pyne, 1994; Robson et al., 1999). Despite the fact a doubling of total WBCs from baseline was not observed (which would be typical of exercise sessions lasting in excess of one hour), the significant increases in WBCs do suggest that our training intervention increased cardiac output/blood flow and catecholamine output, resulting in the mechanical shear stress of WBCs.

One measure of physiological stress is salivary cortisol. In the present study, there was a significant decrease in cortisol secretion rate 1 hour post the 90 minute cycle, however, this is not in line with previous research (Allgrove et al., 2008; Jacks et al.. 2002). Typically, an increase in cortisol would be observed following a prolonged, acute bout of exercise as seen in the present study (Allgrove et al., 2008; Jacks et al., 2002; Walsh et al., 2011*a*). The responses observed in this study may be due to the diurnal responses of salivary

cortisol which typically start to decline 3 hours after waking. In addition, subjects who awake early (as was required for this study) have a tendency to secrete more cortisol in the hour or so after waking, followed by a steeper decline (Edwards et al., 2001). Neutrophil count, and GLR, as measured in the present study, can also be used as a measure of immunological stress (Chen et al., 2017). It is believed that an increase in IL-6 (not measured in the present study) increases the rate and number of neutrophils released from the bone marrow resulting in a neutrophilia and, as a result, an increased GLR ratio (Wang et al., 2011). In the present study, there was a significant increase in granulocytes immediately, and one hour post the 90 minute cycle. This was accompanied by an significant increase in the GLR 1 hour post. This is in line with present research that uses GLR, amongst other techniques, to measure stress responses to a stimuli, or levels of inflammation (i.e. the greater the stress response/inflammation, the greater the GLR) (Chen et al., 2017; Lopes et al., 2016; Wang et al., 2011). The level of stress the body experiences as a result of an exercise bout is important as the "*stress responses*" (i.e. cell counts, neutrophilia, increases in cytokines, etc) are intricately linked to immune function. The greater the stress response to any given exercise bout, the greater the impact on the host's ability to recover from said exercise bout, and effectively fight infection. Therefore, in the present study, we hypothesised that an increase in the "*stress response*" to the intensified training period would result in an increase of URTI and self reported URS. In the context of the present study, however, it does not appear that CHL supplementation blunts the "*stress response*" to exercise as there were no significant differences between conditions observed for salivary cortisol, granulocytes, or the GLR, and this may go some way towards explaining why no differences were observed in URTI between conditions, when taken into consideration alongside the arguments outlined above.

In the present study, there were no significant changes in IL-4 or IL-10 production in response to exercise. Increases in anti-inflammatory cytokines tend to increase with the exercise intensity (Lancaster, 2006*a*). The increases in IL-10 and IL-4, observed following exercise tend to be relatively mild compared to IL-6, for example. Studies which have monitored the acute changes in IL-4 and IL-10 in response to exercise, have found little, or no change in these specific cytokines (Lancaster, 2006*a*; Lasisi & Adeniyi, 2016). Gleeson et al. (2012) reported that illness prone athletes had impaired pro-inflammatory, and elevated anti-inflammatory states most notably, with a 2.5 fold increase in IL-4 and IL-10 production by antigen stimulated whole blood culture. In the present study, when athletes were grouped into those who got ill (i.e. experienced at least one episode of self-reported

URTI when scored using the Fricker method), or those who didn't (i.e. did not experience any episodes of self-reported URTI when scored using the Fricker method), there were no differences in either IL-4 or IL-10 at any of the time-points analysed. Although these findings are not in line with Gleeson et al. (2012) and others (Handzlik et al., 2013), our study does not track the chronic effects of exercise on immune responses, and therefore the acute effects of exercise may not reflect what is observed longitudinally (i.e. over a 4 month period as in the abovementioned study). The fact that the present study did not report any changes in IL-4 or IL-10 is most likely due to the nature of the monitoring period (i.e. acute response to exercise) and the intensity of the exercise session (i.e. moderate, 25% Δ for 90 minutes).

It should be noted that, although there were no differences in any biological markers of physiological stress, there was a difference in the physiological marker of VT1 and $VT1_{\%max}$ in response to the $\dot{V}O_2max$ test, with VT1 and $VT1_{\%max}$ lower in the chlorella condition. This resulted in an increased relative work-rate for the 90 minute cycle in the placebo condition, as the calculation of 25% Δ relies upon VT1. Previous research has reported improvements to both cardiovascular fitness and physical performance following supplementation with CHL. Following four weeks of supplementation with Chlorella pyrenoidosa, Umemoto & Otsuki (2014) reported a significant, 9% increase in peak oxygen uptake during a maximal cycling test to exhaustion, attributed by the study's authors to be caused by branch chain amino acids (although the levels contained within the administered dose would be small compared to the amounts used in branch chain amino acid performance studies), the bioactive effects of some of the vitamins and nutrients, or to do with the wide spectrum of nutrients available (as opposed to the bioactive effects). This built on the findings of a murine study 3 years previously which reported a two-fold increase in time to exhaustion when undertaking a swimming challenge in mice that were supplemented with CHL, attributed by the authors to be due to a greater preservation of glycogen stores, and an increase in the amount of energy being produced by fatty acid degradation in mice supplemented with CHL (Mizoguchi et al., 2011). The findings of the current study, therefore, are not in line with previous research. VT1 is an important threshold as it's considered the threshold at which exercise intensity changes from easy/steady (below VT1) to moderate (above VT1) (Lansley et al., 2011). Essentially, the closer VT1 is to $\dot{V}O_2max$, the greater the intensity an athlete can exercise while remaining in an easy, or steady state. This is advantageous, particularly for endurance athletes. In the present study, 25% Δ (i.e. the difference between VT1 and $\dot{V}O_2max$) would be lower when

expressed as $\dot{V}O_2$, in subjects whose VT1 was further away from their $\dot{V}O_2$max, as seen in the CHL condition. This would go some way to explaining the higher relative work rate during the 90 minute cycle as the VT1 in the PLA condition was higher. The present study was not designed to measure cardiovascular responses to supplementation, however, and future research would be required to substantiate these findings.

6.5 Limitations

One limitation of this study is that we did not measure $\dot{V}O_2$max pre-supplementation, so it is not possible to see the effect of CHL supplementation on performance parameters, such as $\dot{V}O_2$max, VT1, and W_{max}. However, this study was not designed to measure cardiovascular or performance responses to supplementation, but rather the immunological responses to a controlled period of intensified training (i.e. with all subjects exposed to the same relative training demand). We also did not record physiological responses during HIIE sessions to ensure that exercise intensity was comparable between PLA and CHL groups. However, we are confident that subjects produced a maximal effort (and hence the same relative demand) for these sessions based on the maximal RPE values expressed following all HIIE sprints. One further limitation is that we did not collect and incubate a full complement of whole blood samples for analysis of cytokines as the equipment was not available when data collection commenced. It was also the intention to investigate the responses of an array of cytokines (IL-1RA, IL-2, IL-6, IL-8, INF-γ, and TNF-α in addition to IL-4 and IL-10 in response to 24 hour incubations) however, there were too many missing timepoints to make the analysis worthwhile and the decision was made to investigate IL-4 and IL-10 only, using the samples originally intended for the sole investigation of elastase. Future studies should look to use a multiplex cytokine array, or incubate a greater quantity of whole blood to increase the yield of incubated sample for analysis.

6.6 Conclusion

The aim of the current study was to assess the effects of chlorella supplementation on acute and delayed immune responses to a two day intensified training period. In conclusion, daily supplementation with CHL was able to increase salivary SIgA concentration at rest, but only following 5 weeks of supplementation. Supplementation did not reduce the acute stress responses to exercise, as observed by leucocyte and cortisol responses.

Together with previous research, there is now substantial evidence to show that CHL can enhance salivary SIgA; however, in the present study, it appears that a longer supplementation period may be required to translate to protection against URTI and reduced URS reports. The evidence in the present study does not show as much promise as the results presented in the previous chapter. However, the nature of a crossover design means that the results are more powerful owing to the elimination of inter-person variability, compared to those subjected to a between groups comparison. Future studies should seek to extend the supplementation period, and increase the number of subjects.

Chapter 7

General Discussion and Conclusion: The use of salivary EBV DNA as a marker of *in vivo* immunity

7.1 Main findings of the thesis

The main findings from this thesis are that the concentration of EBV DNA detected in saliva following prolonged exercise does not increase; carbohydrate, nor *Chlorella pyrenoidosa* alter the amount of viral DNA shed by EBV into saliva; and a rise in salivary EBV DNA concentration is not associated with an increased incidence of URTI or changes in SIgA (e.g. decreases in SIgA secretion rate). EBV, within the context of these studies, does not appear to be a useful marker of *in vivo* immune function. Studies within this thesis observed no correlations between EBV and DTH responses (chapter 4), SIgA (chapters 4, 5, 6, and 7), or URTI (chapters 4, 5, 6 and 7). Exercise does not appear to change the expression of EBV within an acute context (up to 24 hours post-exercise) (chapters 4, 5, 6, summarised in chapter 7), and neither carbohydrate or *Chlorella pyrenoidosa* (CHL) supplementation appears to affect EBV expression (this is with the exception of chapter 6, whereby EBV expression appeared to be higher overall in the CHL condition, but there were greater, and significant changes in EBV concentration within the placebo group only).

In vivo markers of immune function are highly suitable for immunomodulation studies as they measure the integrated immune response, and are biologically relevant (Albers et al., 2005 & 2013). Measures of *in vivo* immune function, such as vaccine responses, DTH responses, and the response to attenuated pathogens all have a sound, comprehensive evidence base (Albers et al., 2005 and 2013; Bermon et al., 2017; Davison et al., 2016; Diment et al., 2015; Harper-Smith et al., 2011; Walsh et al., 2011*a*; Weidner et al., 1998). However, relatively few studies to date have investigated the use of EBV as a marker of *in vivo* immune function in athletes (Cox et al., 2004; Gleeson et al., 2002; Yamauchi et al., 2011). The aim of this thesis was to investigate the use of salivary EBV DNA as a marker of *in vivo* immunity in response to training and nutritional intervention. In addition, an array of established *in vivo* and *in vitro* markers of immune function were measured to help inform conclusions.

Throughout this thesis, the response of EBV, and its relationship to other immune markers following physical and/or nutritional intervention has been reported and discussed in a general nature, in amongst a wide range of physiological, and immunological markers. Within this, the final chapter of the thesis, all available EBV data collected throughout this PhD were consolidated, and the relationship between EBV and URTI incidence, and the

relationship between EBV and SIgA investigated. The change in EBV concentration within an acute context (i.e. pre to post an isolated exercise event) were compared to delayed changes 24 hours after exercise.

To date, only a handful of studies have investigated the role EBV may play in the URTI incidence rates in athletes. He et al. (2013*b*) monitored 236 student athletes over a 4 month winter training period and found that positive EBV serostatus did not influence the number, severity, or duration of URTI infections that athletes suffered. However, latent viral EBV shedding has been linked to URS in elite swimmers, with low levels of SIgA and increases in EBV DNA being detected in saliva before the occurrence of a URTI (Gleeson et al., 2002). A more detailed review of the literature can be found in section 1.3.3 of chapter 1.

The data from chapters 4, 5 and 6 were pooled to provide a larger data set than any of the single studies provided. It is acknowledged that the study design varied between chapters, but the aim of this chapter is to consider the relationship between salivary SIgA and EBV DNA, which was assessed in all studies. However, all studies within this thesis measured salivary SIgA and EBV DNA, and monitored URTI incidence for the 2 weeks that followed the intervention study. It is these data that have been analysed in order to provide a better understanding on the utility of salivary EBV DNA as a measure of *in vivo* immune function. Based on research published to date, we hypothesised that the concentration of EBV DNA detected in saliva following prolonged exercise would increase, owing to the immune-modulation that occurs following exercise, however we also hypothesised that the nutritional interventions built into the studies contained in chapters 4, 5, and 6 would alter the amount of EBV DNA in saliva either by reducing the amount of EBV DNA shed into saliva, or by depressing any exercise-induced increases in the concentration of EBV DNA. We further hypothesised that a rise in salivary EBV DNA concentration would be associated with an increased incidence of URTI and be linked to changes in SIgA (e.g. decreases in SIgA secretion rate). Subjects were included when EBV serology had been determined, a baseline saliva sample which had been analysed for both SIgA and EBV was available, and where 2 weeks of post-exercise URTI questionnaires had been collected. In the case of the Chlorella pyrenoidosa studies (chapters 5 and 6), only subjects in the placebo conditions were included in this analysis. This provided data for 28 individual subjects (table 7.1). As in previous chapters, and as detailed in Chapter 2, subjects were stratified as seropositive, or seronegative for EBV. EBV concentration pre, and post

exercise were used to calculate the absolute change in EBV concentration over time, and the percent change in EBV concentration. In addition, subjects were split into groups based on whether they had suffered with a URTI in the 2 weeks following exercise. SIgA pre, and post exercise were used to calculate the absolute change in SIgA over time, and the percent change in SIgA. Data from chapter 4 were included to compare the acute reponses immediately pre and post exercise of EBV to SIgA and URTI (Acute). Data from chapters 5 and 6 were included to compare the delayed responses (24 hours) in EBV to SIgA and URTI (Delayed).

Table 7.1 Subject characteristics

	Acute	**Delayed**
Gender (Male/Female)	13 / 0	10 / 5
Age (years)	30 (9)	31 (10)
Height (m)	1.79 (0.06)	1.72 (0.06)
Weight (kg)	78.31 (10.75)	69.88 (11.50)

Data are reported as mean (SD), except for height whereby median (IQR) is reported

All results are presented as median ± IQR unless otherwise stated. A significance level of 0.05 was pre-set for all statistical analyses. Normal distribution within the data was analysed using the Shapiro-Wilk test. For normally distributed variables, independent samples T-tests were undertaken, and ANOVA for SIgA concentration and secretion rates between Acute and Delayed groups. Illness episodes were compared using the Chi Squared Test with the Pearson Chi Square test statistic reported. The log transformed and square-roots of data for which normal distribution could not be assumed were first tested using the Shapiro-Wilk test before non-parametric tests were undertaken. Mann-Whitney U tests were undertaken for height; differences in baseline and post exercise EBV concentration between groups; absolute and percent changes in EBV concentration between groups; percent changes in SIgA concentration between groups; and absolute and percent changes in SIgA secretion rate between groups. Wilcoxon tests were undertaken for changes in EBV concentration from baseline to post-exercise in the Acute and Delayed groups. Pearson correlations were used to analyse the correlations between EBV and SIgA and EBV and URTI. Significant differences were identified using Holm-Bonferonni corrected T-Tests. Cohen's *d* is reported for effect size. All tests were carried out using SPSS Version 24.0 (IBM Corp, Armonk, NY, USA).

In the analysis undertaken within this chapter, a total of 21 (75%) subjects were seropositive for EBV (9 (69%) subjects in the Acute, and 12 (80%) subjects in the Delayed groups). This is in line with the findings of chapters 4, 5 and 6 within this thesis whereby 83, 75, and 80% of subjects were seropositive respectively. This also aligns with the findings of other research studies within a similar field whereby rates of 75 - 84% have been reported (Cox et al, 2004; Gleeson et al., 2002; He et al., 2013*b*). Out of 42 samples tested in this chapter, EBV DNA was detectable in 33 samples (79%) which is much higher than rates previously reported by Mehta et al. (2000) and Pierson et al. (2005) who detected EBV DNA in 17 and 23% of samples respectively. Mehta et al. (2000) and Pierson et al. (2005) studied the effects of Antarctic expedition and space flight on EBV expression, and reported the detection of the EBV polymerase accessory gene, BMRF1. The difference in subject demographic, or the gene studied may provide some explanation as to why a greater number of samples tested positive for EBV in the present study. However, when the expression of BALF5 was investigated in response to a 1 month training camp in rugby players, a similar proportion of samples collected (22%) tested positive for EBV (Yamauchi et al., 2011). It is impossible to know why such a high number of samples in the present study had detectable levels of EBV, but it may due to the acute nature of the study design, the design of the qPCR, or the populations studied. Alternatively, the assay developed within this thesis for the detection of BALF5 may have a greater sensitivity or specificity compared to the assays employed by previous research. Without further study, we can only speculate as to the cause and therefore future studies should look to include a monitoring period both before and after the exercise trial, and/or investigate alternative methods for the detection of EBV DNA in saliva. There were no significant changes in EBV DNA concentration pre to post exercise between the Acute and Delayed groups, baseline EBV concentration, post exercise EBV concentration, or the absolute change or percent change in EBV concentration between the Acute and Delayed groups (tables 7.2 and 7.3).

In the present analysis, a total of 4 subject (14%) suffered with a URTI in the 2 weeks following their exercise intervention, of which 75% of these subjects were seropositive, and 25% were seronegative. The average duration of each episode was 6 ± 4 days. He et al. (2013*b*) reported that previous exposure to EBV does not influence the likelihood of developing a URTI. These findings are supported by the findings of the present chapter whereby one out of seven seronegative (14%), and three out of twenty-one seropositive (14%) subjects developed a URTI in the 2 weeks following the exercise intervention.

Subjects' serology to EBV did not affect the likelihood of them developing a URTI in the 2 weeks following exercise (P = 1.000). Baseline exercise EBV DNA concentration was not linked to the occurrence of URTI (No URTI: 0.76 (3.63), URTI: 1.44 (-) ng.µl^{-1} ×10^{-6}; P = 0.616; Cohen's d = 0.52), nor was post exercise EBV DNA concentration (No URTI: 0.48 (1.97), URTI: 0.67 (-) ng.µl^{-1} ×10^{-6}; P = 0.800; Cohen's d = 0.14). There was not a significant change in EBV DNA concentration pre to post exercise in subjects who didn't (Pre: 0.76 (3.63), Post: 0.48 (1.97) ng.µl^{-1} ×10^{-6}; P = 0.647) or those who did suffer with a URTI in the 2 weeks post exercise (Pre: 1.44 (-), Post: 0.67 (-) ng.µl^{-6} ×10^{-6}; P = 0.180). The absolute change in EBV concentration was not linked to the occurrence of URTI (No URTI: -0.46 (3.05), URTI: 0.00 (-) ng.µl^{-1} ×10^{-6}; P = 0.336; Cohen's d = 0.00). The percent change in EBV concentration was not linked to the occurrence of URTI (No URTI: -64.03 (179.80), URTI: 0.00 (-)%; P = 0.193; Cohen's d = 0.18). EBV infection has previously been linked to the incidence of URTI in athletes (Gleeson et al., 2002), but the sample size was relatively small, in comparison to He et al. (2013*b*). Gleeson et al. (2002) reported a greater incidence of URTI in athletes seropositive for EBV than the present study, but there were no URTI instances recorded in seronegative subjects, leading to their conclusion that previous exposure to EBV was associated with the increased incidence of URTI. However, studies which investigate the incidence of URTI typically require a large sample size to accumulate enough URTI episodes, and statistical power to make them worthwhile. When you introduce a variable such as serology for EBV of which, we have already discussed, 75 - 84% of subjects will be seropositive, that means that only 16 - 25% of subjects will be seronegative. When the likelihood of developing a URTI naturally (as opposed to being inoculated with a URTI causing pathogen within a controlled study, such as that conducted by Weidner et al., 1998) is considered, the number of URTIs likely to occur in the sample of seronegative subjects is relatively few (in the present study we observed only one incidence of URTI in seronegative subjects). Future studies should seek to recruit as large a sample size as possible in order to increase the statistical power of their subsequent analyses and should, ideally, consider controlled inoculation with a URTI causing pathogen in order to increase the likelihood of developing a URTI.

It is not only the association between EBV concentration and the incidence of URTI in the present study which presented null findings. The association between EBV concentration and SIgA responses also failed to demonstrate a relationship between these variables. The data analysed in the present chapter demonstrated that baseline SIgA concentration was not linked to the occurrence of URTI (No URTI: 238 ± 113, URTI: 204 ± 109 mg.L^{-1}; P =

0.671; Cohen's *d* = 0.31) and neither was SIgA secretion rate (No URTI: 94.6 ± 58.2, URTI: 83.3 ± 58.5 µg.min^{-1}; *P* = 0.976; Cohen's *d* = 0.19). Neither post exercise SIgA concentration (No URTI: 218 ± 98, URTI: 226 ± 120 mg.L^{-1}; *P* = 0.703; Cohen's *d* = -0.07) or post-exercise SIgA secretion rate (No URTI: 98.9 ± 63.5 µg.min^{-1}, URTI: 69.7 ± 47.5; *P* = 0.196; Cohen's *d* = 0.52) were linked to the incidence of URTI. There was no significant change in SIgA concentration pre to post exercise in subjects who didn't (Pre: 238 ± 112, Post: 218 ± 96 mg.L^{-1}; *P* = 0.203) or those who did suffer with a URTI in the 2 weeks following exercise (Pre: 201 ± 121, Post: 228 ± 137 mg.L^{-1}; *P* = 0.733) with the same being true for SIgA secretion rate pre to post exercise in subjects who didn't (Pre: 93.2 ± 58.6, Post: 94.0 ± 103 µg. min^{-1}; *P* = 0.939) or those who did suffer with a URTI in the 2 weeks following exercise (Pre: 92.6 ± 55.9, Post: 102.7 ± 66.2 µg.min^{-1}; *P* = 0.437).

Table 7.2 Baseline and post-exercise EBV and SIgA between groups

	Acute	**Delayed**	***P*-value**	**Effect Size**
Baseline EBV (ng.µl^{-1} ×10^{-6})	0.93 (3.11)	0.76 (2.91)	0.943	0.20
Post Exercise EBV (ng.µl^{-1} ×10^{-6})	0.46 (1.24)	0.82 (7.08)	0.332	0.48
Baseline SIgA (mg.L^{-1})	310 (105)	187 (74)	**0.002**	1.35
Post Exercise SIgA (mg.L^{-1})	253 (89)	198 (99)	0.142	0.59
Baseline SIgA (µg.min^{-1})	118.7 (56.5)	74.0 (52.2)	**0.043**	0.82
Post Exercise SIgA (µg.min^{-1})	126.5 (60.7)	71.3 (58.3)	**0.024**	0.93

Data are presented as median (IQR), with the exception of baseline and post exercise SIgA concentration and secretion rate whereby mean (SD) is reported.

When absolute changes in SIgA were analysed in response to the occurrence of URTI, no link was found between the absolute change in SIgA concentration and the occurrence of URTI (No URTI: -19.84 ± 80.13, URTI: 21.92 ± 157.26 mg.L^{-1}; *P* = 0.129; Cohen's *d* = -0.46), or the absolute change in SIgA secretion rate and the incidence of URTI (No URTI: -2.49 (50.76), URTI: 6.22 (64.68) µg.min^{-1}; *P* = 0.768; Cohen's *d* = 0.38). Furthermore, when the percent changes in SIgA were analysed in response to the occurrence of URTI, the percent change in SIgA concentration was not linked to the occurrence of URTI (No URTI: -10.75 (45.31), URTI: -4.39 (147.34)%; *P* = 0.680; Cohen's *d* = -0.36) and neither was the percent change in SIgA secretion rate (No URTI: -6.75 (79.54), URTI: 7.42 (94.43)%; *P* = 0.953; Cohen's *d* = 0.18). There were no significant correlations between EBV DNA concentration and SIgA concentration post-exercise (*r* = 0.022, *P* = 0.919); EBV DNA concentration and SIgA secretion rate post-exercise (*r* = -0.143, *P* = 0.506) (table 7.2); the absolute changes in EBV DNA and SIgA concentrations post-exercise (*r* = -0.096, *P* = 0.654); the absolute changes in EBV DNA concentration and SIgA secretion

rate post-exercise (r = -0.194, P = 0.364); the percent changes in EBV DNA and SIgA concentrations (r = 0.065, P = 0.763); or between the percent changes in EBV DNA concentration and SIgA secretion rate (r = 0.056, P = 0.795) (table 7.3). SIgA has been identified as a predictor of URTI (Fahlman & Engels, 2005; Gleeson et al., 1999*a*) but the present study also failed to identify such an association for either SIgA concentration or secretion rate and URTI risk. Gleeson et al. (2002) reported that SIgA levels were lower immediately before the first signs of a URTI. The present study did not find an association between SIgA levels post-exercise and URTI risk, but the study design differed from that of Gleeson et al.'s (2002), whereby the incidence of URTI was monitored for 2 weeks following exercise as opposed to longitudinally over a longer study period. It is therefore difficult for direct comparisons to be made. The association of SIgA and URTI was not the main focus of the present study, however, although it is noteworthy that there is not a significant correlation of SIgA, a predictor of URTI, and EBV as this supports the finding of the present study, discussed above, that there is also not a significant correlation between EBV concentration and URTI.

Table 7.3 Changes in EBV and SIgA between groups

	Acute	Delayed	*P*-value	Effect Size
Absolute change EBV ($ng.\mu l^{-1} \times 10^{-6}$)	-0.44 (3.63)	-0.20 (2.26)	0.546	0.44
Percent change EBV (%)	-46.98 (172.44)	-34.10 (213.93)	0.858	0.44
Absolute change SIgA ($mg.L^{-1}$)	-56.24 (76.66)	11.36 (93.22)	0.054	0.80
Percent change SIgA conc. (%)	-18.39 (35.82)	1.30 (49.44)	0.205	0.63
Absolute change SIgA ($\mu g.min^{-1}$)	10.10 (117.60)	-2.49 (34.10)	0.591	0.21
Percent change SIgA SR (%)	18.46 (94.70)	-6.75 (73.30)	0.495	0.41

Data are presented as median (IQR), with the exception of absolute change in SIgA concentration whereby mean (SD) is reported.

However, the majority of studies which have investigated the role of established *in vivo* immune markers in response to acute bouts of exercise have, to date, only demonstrated differences in *in vivo* markers between rested controls and experimental subjects (Davison et al., 2016; Harper-Smith et al., 2011), or exercise intensities (Diment et al., 2015). The fact that no discernible differences in EBV concentration could be determined from the studies contained within this thesis may not, therefore, be indicative of its lack of usefulness within the context of immunomodulation research but, instead, reflective of the manner in which it has been employed. However, Jones et al. (2017), who, it should be noted, also reported a lack of significant differences to the overall *in vivo* reactivity to DPCP following supplementation with bovine colostrum, analysed the DPCP responses

further by sensitivity analysis. They found that the minimum dose required to elicit a response at 48 hours was 1.8 fold greater in the placebo group, indicating that the sensitivity to DPCP was relevant to host defence, and that it is possible to identify a discernible difference between exercising groups without the need for a rested control. That said, the majority of research contained within this thesis has also failed to show any significant changes in established, *ex vivo* and *in vitro* markers of immune function, such as SIgA, blood counts, cytokine responses, and the incidence of URTI and the potential reasons for this are discussed below. The present findings cannot rule out any associations between EBV, immune markers and URTI risk, but just there are no associations within the context of the type of exercise/training employed in the present study. It is possible that relationships may exist under conditions of greater stress (e.g. more prolonged exercise and/or longer periods of intensified training), that cause greater perturbations in immunity but this will require further studies being conducted.

7.2 Strengths and limitations of the thesis

The research conducted within this thesis was well controlled which would allow for replicability, and integrated the use of established markers of immune function, such as SIgA, alongside the novel method of salivary EBV DNA detection. The research contained within this thesis is not without limitation, however. The relatively small samples sizes of each of the projects limited statistical power. Subject recruitment transpired to be one of the biggest hurdles to overcome. The frequency with which spontaneous URTI infections were recorded was also limited to just a handful of subjects. The frequency with which URTI episodes were observed were not too dissimilar to rates reported in previous literature but this, in combination with low subject numbers, resulted in an extremely limited data pool for subsequent analyses. Finally, as supported by the findings of chapters 5 and 6, and the summary presented above, it appears that the magnitude of difference in markers of *in vivo* immunity are most pronounced between exercising, and non-exercising controls. The studies contained within this thesis failed to include a non-exercising control group and therefore there is a limitation of the data presented as, in retrospect, the inclusion of a non-exercising control could, potentially, have been extremely insightful. The inclusion of a resting control trial or group would have allowed further comparisons of the overall affects of exercise, which are generally greater in magnitude than the effects between exercise trials with vs. without nutritional intervention.

7.3 Future research

The utility of EBV as a marker of *in vivo* immune function warrants further investigation, despite the null findings within this thesis. First and foremost, it appears, based on the studies contained within this thesis and previous research in the field, that the use of *in vivo* markers of immune function may be better suited to assess differences of greater magnitude (e.g. exercise vs. rest) than those typically observed in nutritional intervention studies. A study should, therefore, be conducted to investigate if what has been observed for DTH responses (Davison et al., 2016 and Harper-Smith et al., 2011) is also true for EBV. Secondly, the lack of spontaneous URTIs that arose within the studies contained within this thesis certainly limited the statistical power when comparing these data. One model that could be employed in future EBV research is the inoculation of subjects with a pathogen known to cause URTI (e.g. Weidner et al., 1998). Once studies following these models have been undertaken, it is hoped that a better understanding of the role of EBV in acute exercise immunology studies may be better understood. The research conducted within this thesis are reflective of what athletes do as part of their training, however, and therefore the evidence presented within the context of this thesis, suggests that there are no benefits of the nutritional interventions investigated (with the exception that supplementation with *Chlorella pyrenoidosa* may potentially be beneficial in athletes, especially those with low SIgA, as it appears to increase SIgA concentration at rest). However, the evidence from previous research is strong for the use of salivary EBV DNA as a marker within longitudinal monitoring studies, and therefore it may be that EBV's utility is limited to this research context, at least until further research on the effects of acute exercise can be conducted.

7.4 Conclusion

In line with the findings of chapters 4, 5 and 6, and the subsequent analysis undertaken in this chapter, it does not appear that salivary EBV DNA is a useful marker of immune function within an acute setting (up to 24 hours after exercise within the context of the type of exercise/training employed in the thesis). It should also be acknowledged that the small sample sizes recruited, and the limited incidences of URTI observed may have contributed to the null findings of this thesis. Future research is needed to further investigate the use of

EBV as a marker of *in vivo* immune function before conclusive decisions can be made regarding its utility.

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Appendix A

Comparison of AmpliSens EBV-EPh PCR kit, and the DreamTaq reaction with Yamauchi et al. (2011)

An initial experiment used samples that had previously been shown to contain EBV DNA using the AmpliSens EBV-EPh PCR kit (AmpliSens, Bratislava, Slovakia). The AmpliSens EBV-EPh PCR kit was run concurrently with the DreamTaq reaction and the Yamauchi primers. Both were run using their individual cycling recommendations (table A1.1)

Table A1.1 Thermal Cycling Conditions AmpliSens and DreamTaq

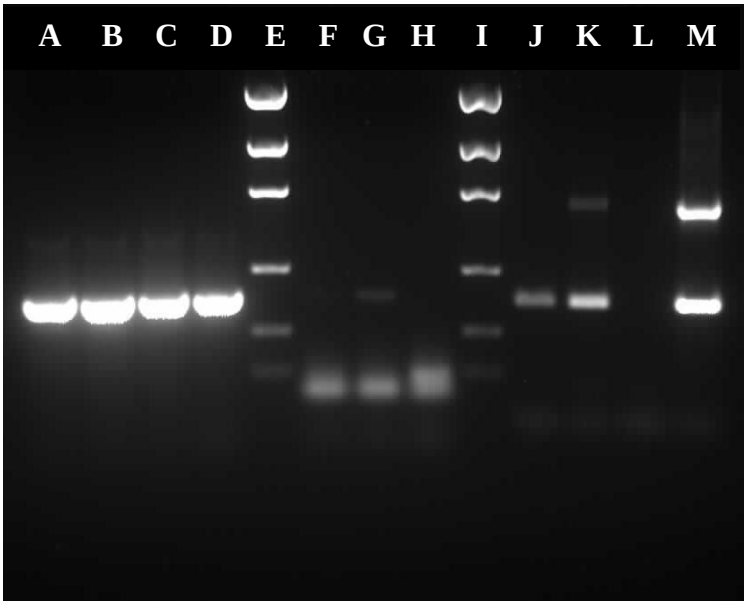
	AmpliSens EBV-EPh PCR Kit			**DreamTaq**		
Step	**Temp °C**	**Time**	**Cycles**	**Temp °C**	**Time**	**Cycles**
Initial denaturation	95	5 min	1	95	5 min	1
Denaturation	95	15s		95	15s	
Annealing	65	25s	42	55	25s	30
Extension	72	25s		72	25s	
Final extension	72	1 min	1	72	1 min	1

The amplified PCR products were analysed using gel electrophoresis but the DreamTaq reaction was unsuccessful. The low annealing temperature was possibly responsible for the failure of amplification, or the relatively low number of cycles. The experiment, therefore, was repeated using a gradient for 42 cycles (table 3.7). DreamTaq reactions were placed in the block at 55.00, 58.13, 61.25, and 65.00 °C (figure A1.1).

The DreamTaq reactions with plasmid amplified successfully across the temperature gradient, however there was a failure of amplification of an extracted saliva sample (Lane F) that had previously provided a positive result for EBV using the AmpliSens EBV-EPh PCR kit. The sample had been extracted using the AmpliSens DNA-sorb-AM kit, and therefore it is most likely that the kit extracts specific targets from biological samples compatible with its kits for downstream application (e.g. AmpliSens EBV-EPh PCR kit).

The plasmid amplified successfully, however, and therefore the cycling conditions (i.e. an annealing temperature of 65 °C for 42 cycles) and PCR design (table *.*) for EBV detection were accepted as a viable methodology. However, as mentioned above, the primers were run *in-silico* using NCBI's Primer-BLAST, and failed checks at the first stage. As such, alternative experiments were tested using the BALF5 primers which amplified a region of the gene at 452 and 275 bp (chapter 3).

Figure A1.1 Results of 55 - 65 °C gradient PCR testing the AmpliSens EBV-EPh PCR kit (Amp), and the DreamTaq reaction with Yamauchi et al. (2011) primers



Lane A: DreamTaq (DT) and plasmid at 55.00 °C; **Lane B:** DT and plasmid at 58.13 °C; **Lane C:** DT and plasmid at 61.25 °C; **Lane D:** DT and plasmid at 65.00 °C; **Lane E:** Low DNA Mass Ladder; **Lane F:** DT and extracted saliva sample at 65 °C; **Lane G:** DT -ve control at 65 °C; **Lane H:** DT and AMP +ve control at 65 °C; **Lane I:** Low DNA Mass Ladder; **Lane J:** Amp and Plasmid at 65 °C; **Lane K:** Amp and +ve sample at 65 °C; **Lane L:** Amp and -ve control at 65 °C; **Lane M:** Amp and Amp +ve control. Ladder bands are visible, from top to bottom, at 2,000, 1,200, 800, 400, 200, and 100 bp.