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# **Effects of Nutritional Supplements on the Immune Function of Athletes**

By

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A Doctoral Thesis

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## GENERAL ABSTRACT

Prolonged exercise has been associated with depressed immune function, and hence an increased risk of infection. However, several nutritional supplements may reduce or overcome this problem. Thus, the aims of this thesis were to investigate the effects of some nutritional supplements on athletes' immune function. In study 1 (Chapter 3), effects of several vaccine stimulant dose on whole blood culture cytokine production was carried out to determine effective vaccine stimulant dose; which was found to be between a dilution of 4000 (dose 4) and 1000 (dose 6) of the original vaccine. This finding was used for the other studies (Chapter 4 and 5). In addition, the relationship between data obtained from Evidence Investigator analyser and enzyme linked-immuno-sorbent assay (ELISA) for IL-10 was analysed and the results show a positive strong correlation between them. In study 2 (Chapter 4), *in vitro* effects of various immunomodulatory nutritional compounds on antigen-stimulated whole blood culture cytokine production was investigated and it was found that caffeine and quercetin showed tendency towards decrease cytokine production as the doses were increased. On the other hand, an upward trend was evident with kaloba, where high dose of kaloba seemed to increase the cytokine production. Since kaloba appeared to act as an immunostimulant *in vitro*, its effects on the immune response to prolonged exercise were examined in study 3 (Chapter 5). However, 7 days kaloba supplementation (20 mg of the root extract) did not alter athletes' immune response although prolonged moderate intensity exercise significantly decreased S-IgA secretion rate and concentration post-exercise with the values returning to baseline by 1 h post-exercise. A 14-strain probiotic supplement effects on salivary antimicrobial proteins at rest and in response to an acute bout of prolonged exercise was investigated in study 4 (Chapter 6). Unfortunately, 30 days supplementation of the 14-strain probiotic appeared not enough to induce any significant effects on salivary antimicrobial proteins. Lastly, in study 5 (Chapter 7), the effects of a *Lactobacillus* probiotic

on healthy people, who tend to have a higher than normal incidence of infection due to exercise stress-induced immune impairment was studied. In summary, this 16-week intervention study on 267 athletes found that regular ingestion of the probiotic reduced the extent to which training was negatively affected in endurance athletes when infection was present, and increased both S-IgA concentration and secretion rate over time. But it did not appear to reduce URTI incidence or the duration and severity of URTI episodes. Two major confounding factors, namely the unexpectedly low incidence of URTI during the winter period and the lower baseline S-IgA in the probiotic group may have prevented potential beneficial effects of probiotic supplementation from being identified.

**Key words:** nutritional supplements, cytokine, S-IgA, kaloba, probiotic, URTI, immune function, exercise.

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## CONTENTS

<b>General Abstracts</b>	i
<b>Acknowledgements</b>	iii
<b>Publications</b>	v
<b>Contents</b>	vi
<b>List of Tables</b>	xii
<b>List of Figures</b>	xiv
<b>List of Abbreviations</b>	xvi
<b>Chapter 1 – Literature review</b>	
<b>Abstract</b>	1
1.1 The immune system	1
1.1.1 Factors that affect immune function	2
1.2 Effects of exercise on immune function	6
1.2.1 Prolonged exercise and immune function	6
1.3 Exercise and infection risk	8
1.3.1 Prolonged exercise and infection risk	12
1.4 Nutrition and immune function	16
1.4.1 Probiotics	18
1.4.1.1 <i>Probiotics and athletes' immune function</i>	21
1.4.2 Kaloba ( <i>Pelargonium sidoides</i> / EPs7630)	24
1.4.3 Echinacea	26
1.4.4 Quercetin	27
1.4.5 Curcumin	29
1.4.6 Caffeine	30
1.5 Summary	31
<b>Chapter 2 – General methods</b>	
2.1 Ethics approval	32
2.2 Maximal oxygen uptake ( $\dot{V}O_2\text{max}$ ) determination	32
2.3 Familiarisation trials	33



2.4 Analytical methods	33
2.4.1 Saliva sample collection	33
2.4.1.1 <i>Saliva volume/weight, flow rate, and saliva antimicrobial proteins secretion rate calculation</i>	34
2.4.2 Saliva analysis	34
2.4.2.1 <i>Saliva secretory immunoglobulin A (S-IgA) analysis</i>	34
2.4.2.2 <i>Lysozyme analysis</i>	35
2.4.2.3 <i>Lactoferrin analysis</i>	36
2.4.2.4 <i>Alpha (<math>\alpha</math>)-amylase analysis</i>	36
2.4.3 Blood sample collections	37
2.4.4 Haematological analysis	37
2.4.5 Cytokine array	37
2.4.5.1 <i>One hundred times diluted vaccine dose preparation</i>	37
2.4.5.2 <i>Whole blood culture incubation and supernatant storage</i>	38
2.4.5.3 <i>Evidence investigator cytokine array</i>	40

### **Chapter 3 - The effects of stimulant dose on whole blood culture cytokine production**

<b>Abstract</b>	42
3.1 Introduction	44
3.2 Methods	48
3.2.1 Participants	48
3.2.2 Procedures	48
3.2.3 Blood analysis	48
3.2.3.1 <i>Haematological analysis</i>	48
3.2.3.2 <i>One hundred times diluted vaccine dose preparation</i>	48
3.2.3.3 <i>Whole blood culture incubation and supernatant storage</i>	49
3.2.3.4 <i>Evidence investigator cytokine array</i>	49
3.2.3.5 <i>Enzyme linked-immuno-sorbent assay (ELISA) for IL-10</i>	49
3.2.4 Data analysis	51
3.3 Results	51
3.4 Discussion and Conclusion	58

### **Chapter 4 – The *in vitro* effects of various immunomodulatory nutritional compounds on antigen-stimulated whole blood culture cytokine production**

<b>Abstract</b>	59
4.1 Introduction	60
4.2 Methods	61

4.2.1	Participants	61
4.2.2	Procedures	61
4.2.3	Blood analysis	61
4.2.3.1	<i>Haematological analysis</i>	61
4.2.3.2	<i>One hundred times diluted vaccine dose preparation</i>	61
4.2.3.3	<i>Immunomodulatory nutritional compounds preparation</i>	62
4.2.3.4	<i>Whole blood culture incubation and supernatant storage</i>	65
4.2.3.5	<i>Evidence Investigator cytokine array</i>	66
4.2.4	Data analysis	66
4.3	Results	66
4.4	Discussion	70
4.5	Conclusion	73
<b>Chapter 5 – The effects of Kaloba supplementation on the immune response to prolonged exercise</b>		
	<b>Abstract</b>	75
5.1	Introduction	77
5.2	Methods	78
5.2.1	Participants	78
5.2.2	Procedures	79
5.2.2.1	Preliminary tests	79
5.2.2.2	Experimental trials	79
5.2.3	Blood analysis	80
5.2.3.1	<i>Haematological analysis</i>	80
5.2.3.2	<i>One hundred times diluted vaccine dose preparation</i>	80
5.2.3.3	<i>Whole blood culture incubation and supernatant storage</i>	80
5.2.3.4	<i>Evidence investigator cytokine array</i>	81
5.2.4	Saliva analysis	81
5.2.5	Data analysis	81
5.3	Results	81
5.3.1	Physiological parameters	81
5.3.2	Haematological parameters	82
5.3.3	Saliva secretory immunoglobulin A (S-IgA)	84
5.3.4	Cytokines production	86
5.3.5	Questionnaire	86
5.4	Discussion	87
5.5	Conclusion	89

## **Chapter 6 – Effects of a 14-strain probiotic supplement on salivary antimicrobial proteins at rest and in response to an acute bout of prolonged exercise**

<b>Abstract</b>	90
6.1 Introduction	92
6.2 Methods	93
6.2.1 Participants	93
6.2.2 Procedures	93
6.2.2.1 Preliminary test	94
6.2.2.2 Experimental trials	94
6.2.3 Saliva analysis	95
6.2.3.1 <i>Saliva secretory immunoglobulin A (S-IgA) analysis</i>	95
6.2.3.2 <i>Alpha (<math>\alpha</math>)-amylase analysis</i>	95
6.2.3.3 <i>Lysozyme analysis</i>	95
6.2.3.4 <i>Lactoferrin analysis</i>	95
6.2.3.5 <i>Nitrite and nitrate analyses</i>	95
6.2.4 Data analysis	97
6.3 Results	97
6.3.1 Physiological parameters	97
6.3.2 Saliva flow rate, S-IgA concentration, and secretion rate	99
6.3.3 Lactoferrin	101
6.3.4 Lysozyme	102
6.3.5 Alpha ( $\alpha$ )-amylase	103
6.3.6 Nitrite and nitrate	104
6.4 Discussion	104
6.5 Conclusion	108

## **Chapter 7 – Effects of *Lactobacillus* probiotic on common cold infection and mucosal immunity in endurance athletes**

<b>Abstract</b>	109
7.1 Introduction	111
7.2 Methods	113
7.2.1 Participants	113
7.2.2 First visit	114
7.2.3 Study intervention	115
7.2.4 Study protocol	115
7.2.5 Saliva analysis	116

7.2.6 Data analysis	117
7.3 Results	119
7.3.1 Adherence to the study	119
7.3.2 Patterns in baseline characteristics	120
7.3.3 Physical activity levels	121
7.3.4 URTI incidence	122
7.3.5 Medication and visits to the doctor during URTI episodes	123
7.3.6 Outcome of the dummy stepwise regression analysis for URTI incidence	124
7.3.6.1 Stratification I: time interval	124
7.3.6.2 Stratification II: gender	125
7.3.6.3 Stratification III: type of sport	126
7.3.7 Salivary antimicrobial proteins	126
7.3.7.1 Lactoferrin	126
7.3.7.2 Lysozyme	129
7.3.7.3 Saliva secretory immunoglobulin A (S-IgA)	131
7.3.7.4 Alpha ( $\alpha$ )-amylase	133
7.3.8 Outcome of the dummy stepwise regression analysis for salivary antimicrobial proteins	135
7.3.8.1 Lactoferrin	135
7.3.8.2 Lysozyme	137
7.3.8.3 Saliva secretory immunoglobulin A (S-IgA)	139
7.3.8.4 Alpha ( $\alpha$ )-amylase	141
7.3.9 Blood leukocyte counts	141
7.3.10 Blinding	141
7.4 Discussion	143
7.5 Conclusion	147
<b>Chapter 8 – General discussion</b>	
8.1 Effective stimulant (100 x dilution Pediacel vaccine) dose and Evidence Investigator	148
8.2 Kaloba ( <i>Pelargonium sidoides</i> ) supplementation and immune response	149
8.3 A 14-strain probiotic supplementation and salivary antimicrobial proteins (AMPs)	151
8.4 Single strain <i>Lactobacillus</i> probiotic supplementation, common cold infection, and mucosal immunity in endurance athletes	152
8.5 Prolonged exercise and immune response	153
8.6 Summary and future direction	155

<b>References</b>	156
<b>Appendix A</b>	192
<b>Appendix B</b>	193

## LIST OF TABLES

Table 1.1	Main elements of the immune system	2
Table 2.1	Advantages and disadvantages of whole blood culture and freshly isolated PBMC methods	39
Table 3.1	Cytokine families	45
Table 3.2	Cytokines and their principal sources, and functions	46
Table 3.3	Volume of 100 x diluted vaccine and RPMI added to each well for each blood sample. To these volumes, 0.25 ml heparinised blood was added to give the respective dilution factor	49
Table 3.4	Mean and SD values of haematological parameters	52
Table 4.1	Volume of RPMI, 100 x diluted vaccine, and immunostimulants solution added to each well for each blood sample: to these volumes, 0.25 ml heparinised blood was added	65
Table 4.2	Mean and SD values of haematological parameters	67
Table 4.3	Antigen-stimulated (vaccine dose 5) whole blood culture cytokine production (pg/ml) in response to alcohol, caffeine, curcumin, echinacea, kaloba, and quercetin at low and high doses	68
Table 5.1	Physiological parameters (n = 9)	82
Table 5.2	Mean and SD values of haematological parameters in both non-Kaloba and Kaloba trials	83
Table 5.3	Wisconsin Upper Respiratory Symptom Survey-21 (WURSS-21) results	86
Table 6.1	Physiological parameters (n = 9)	98
Table 6.2	Nitrite and nitrate concentration in both trials	104
Table 7.1	Flow diagram showing numbers of subjects in week 1 and 16	120
Table 7.2	Baseline characteristics of the subjects who completed the study	120

Table 7.3	Number of URTI episodes, number of days with URTI symptom score $\geq 5$ , and mean total symptom score and duration for URTI episodes in the 16-week period	123
Table 7.4	Total and differential blood leukocyte counts before and after 16 weeks of the intervention period	142

## LIST OF FIGURES

Figure 1.1	‘J’-shaped model of the relationship between varying amounts of exercise and risk of upper respiratory tract infection	10
Figure 1.2	‘S’-shaped model of the relationship between exercise load and infection rate	10
Figure 3.1	IL-2 responses to different stimulant dose	53
Figure 3.2	IL-4 responses to different stimulant dose	53
Figure 3.3	IL-6 responses to different stimulant dose	54
Figure 3.4	IL-8 responses to different stimulant dose	54
Figure 3.5	IL-10 responses to different stimulant dose	55
Figure 3.6	Interferon-gamma (IFN- $\gamma$ ) responses to different stimulant dose	55
Figure 3.7	Tumor Necrosis Factor-alpha (TNF- $\alpha$ ) responses to different stimulant dose	56
Figure 3.8	IL-1 $\alpha$ responses to different stimulant dose	56
Figure 3.9	IL- $\beta$ 1 responses to different stimulant dose	57
Figure 3.10	Correlation graph between Evidence Investigator and ELISA data for IL-10	57
Figure 5.1	S-IgA flow rate ( $\mu\text{g}/\text{min}$ ) in non-Kaloba and Kaloba trials	84
Figure 5.2	S-IgA concentration ( $\mu\text{g}/\text{ml}$ ) in non-Kaloba and Kaloba trials	85
Figure 5.3	S-IgA secretion rate ( $\mu\text{g}/\text{min}$ ) in non-Kaloba and Kaloba trials	85
Figure 6.1	Saliva flow rate in both trials	99
Figure 6.2	S-IgA concentration in both trials	100
Figure 6.3	S-IgA secretion rate in both trials	100
Figure 6.4	Lactoferrin concentration in both trials	101
Figure 6.5	Lysozyme concentration in both trials	102
Figure 6.6	$\alpha$ -amylase concentration in both trials	103



Figure 7.1	Training loads in MET-h/week over the 16-week study period for subjects who completed the study (mean $\pm$ SEM)	121
Figure 7.2	Percentage of the cohort reporting a URTI episode for each week of the study period	123
Figure 7.3	Number of common cold (URTI) episodes during the study period	124
Figure 7.4	Number of common cold (URTI) episodes in males during the study period	125
Figure 7.5	Number of common cold (URTI) episodes in females during the study period	126
Figure 7.6	Lactoferrin concentration (A) and secretion rate (B) before and after 4, 8, 12 and 16 weeks of the study	128
Figure 7.7	Lysozyme concentration (A) and secretion rate (B) before and after 4, 8, 12 and 16 weeks of the study	130
Figure 7.8	S-IgA concentration (A) and secretion rate (B) before and after 4, 8, 12 and 16 weeks of the study	132
Figure 7.9	$\alpha$ -amylase activity (A) and secretion rate (B) before and after 4, 8, 12 and 16 weeks of the study	134
Figure 7.10	Change in lactoferrin concentration (A) and secretion rate (B) over the study period	136
Figure 7.11	Change in lysozyme concentration (A) and secretion rate (B) over the study period	138
Figure 7.12	Change in s-IgA concentration (A) and secretion rate (B) over the study period	140
Figure 7.13	UK Health Protection Agency Influenza Surveillance data (2012) demonstrating incidence of influenza-like illness in current and recent seasons (A) and proportion of calls for cold/flu symptoms in current and recent seasons (B)	146

## LIST OF ABBREVIATIONS

ANOVA	analysis of variance
beats.min <sup>-1</sup>	beats per minute
BMI	body mass index
CD	clusters of differentiation
CHO	carbohydrate
CI	confidence interval
CO <sub>2</sub>	carbon dioxide
CV	coefficient of variation
DC	dendritic cells
ELISA	enzyme linked-immuno-sorbent assay
g	grams
h	hour/s
Hb	haemoglobin
Hct	haematocrit
HR	heart rate
IFN	interferon
Ig	immunoglobulin
IL-	interleukin-
K <sub>3</sub> EDTA	tripotassium ethylene-tetra acetic acid
kg	kilogram
km	kilometre
L	litre
m	metre
mg	milligram
min	minute
ml	millilitre

mM	millimole
nm	nanometre
NK	natural killer cells
O <sub>2</sub>	oxygen
PBMC	peripheral blood mononuclear cell
RBC	red blood cells
RPE	rating of perceived exertion
rpm	rounds per minute
s	second
S-Ig	salivary secretory immunoglobulin
SD	standard deviation
URTI	upper respiratory tract infection
$\dot{V}O_2$	oxygen uptake/expired gas
$\dot{V}O_{2max}$	maximum rate of oxygen uptake
W	watt
WBC	white blood cells
°C	degrees Celsius
~	approximately
$\alpha$	alpha
$\beta$	beta
$\gamma$	gamma
$\mu$	micro
%	percent

# CHAPTER 1

## LITERATURE REVIEW

### 1.1 The immune system.

The human body has a complex, self-identification defence system called the immune system which protects the host from infection by pathogens that may lead to various diseases. Since the types of pathogens are diverse, this defence system produces different types of immune response which can be schematically divided into two types called innate and acquired immunity (Pinchuk, 2002; Male, 2003; Sompayrac, 2003). Innate immunity is a natural protective mechanism that we are born with and it comprises physical barriers (skin, mucus, enzymes, commensal flora, etc.), and physiological barriers (Decker, 1999). It is mainly represented by monocytes, natural killer (NK), macrophages, and dendritic cells (DC) (Sanson et al., 2008). Innate immunity is a non-specific cellular and humoral response, which serves as the first line of defence system. It produces an immediate response and fights against many but not all pathogens.

On the other hand, acquired (adaptive) immunity is highly specific in response, inducible, and discriminatory (Clancy, 1998). It is activated when the innate immunity is unsuccessful eliminating the pathogens. B and T lymphocytes play a major role at this stage (Sanson et al., 2008). However, acquired immunity takes several days or weeks after the first exposure to pathogen to produce a response. Interestingly, it produces an immune memory, so that when the same pathogens invade the body for the next time, it will produce a faster and greater response (Decker, 1999). Basically, the immune response has two phases: recognition and

effector phases. In the recognition phase, the immune system tries to identify the pathogen while in the effector phase, the pathogen will be eliminated or at least restrained sufficiently to control the damage created by the pathogen (Male, 2003). The various immune system components (both innate and adaptive) are shown in Table 1.1.

*Table 1.1: Main elements of the immune system (Gleeson, 2006).*

<b>Innate components</b>	<b>Adaptive components</b>
<i>Cellular:</i>	<i>Cellular:</i>
Natural killer cells (CD16 <sup>+</sup> , CD56 <sup>+</sup> )	T-cells (CD3 <sup>+</sup> , CD4 <sup>+</sup> , CD8 <sup>+</sup> )
Phagocytes (neutrophils, eosinophils, basophils, monocytes (CD14 <sup>+</sup> ), macrophages)	B-cells (CD19 <sup>+</sup> , CD20 <sup>+</sup> , CD22 <sup>+</sup> )
<i>Soluble:</i>	<i>Soluble:</i>
Acute-phase proteins	Immunoglobulins (Ig)- IgA, IgD, IgE, IgG, IgM
Complement	
Lysozymes	
Cytokines (interleukins (IL), interferons (IFN), colony-stimulating factor (CSF), tumour necrosis factor (TNF))	

CD = Clusters of Differentiation or Cluster Designators

### 1.1.1 Factors that affect immune function.

It is clear that defence mechanisms of human body largely depend on the effectiveness of the immune system. There are several factors that have been demonstrated to have a profound influence on the immune system, including age, inflammatory or autoimmune disease, exercise, psychological stress, and diet

(Gleeson, 2006). This explains why each individual's immunity is different from each other. It is generally assumed that the aging process progressively deteriorates and subsequently shows significant adverse changes on the structure and function of the immune system (Makinodan and Kay, 1980; Herbert and Cohen, 1993; Castle, 2000). However, the evidence suggests that innate immunity is better preserved, while adaptive immunity is deteriorates (Hirokawa et al., 1994; Franceschi et al., 2000). Immunosenescence is established to be associated with increased risk of infectious disease, malignancy, and autoimmune disorders (Miller, 1991; Graham et al., 2006). It has been reported that, there are losses of phagocytic capacity, increases of early pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF), reduced dendritic cell (DC) traffic, decreased number of naïve T cells, higher numbers of memory cell types, decreased T and B cell proliferation, and reduced expression of important surface molecules for intercellular interaction in the elderly (Miller, 1996; Pawelec and Solana, 1997; Ginaldi et al., 1999). Hirokawa et al. (1994) proposed that physiological thymic involution, which includes changes in thymic hormone production and overall function of thymus ability is responsible for those age-related changes in T-cell-dependent immune function. Thymic involution is believed to start at the age of puberty and is almost complete at the end of the sixth decade of life (Sansoni et al., 2008). Yet, age-related changes in immune function are not unavoidable and irrevocable since there is a complex relationship between this factor and other factors (psychological stress, diet, exercise, etc.) (Fiatarone et al., 1989).

Psychological stress is another factor that can affect immune function where even a fairly, mild stress can result in immune dysregulation (Glaser and Kiecolt-Glaser, 2005; Graham et al., 2006). Stress is defined as what one feels when life's demands exceed one's ability to meet those demands (Kelly, 1999). School

examinations or tests were found to diminish NK cell cytotoxic activity (NKCA) (Kang et al., 1997), increase neutrophil and platelet numbers and decrease eosinophil, monocyte, basophil, and lymphocyte numbers in the circulation (Qureshi et al., 2002), and delay the wound healing process (Kiecolt-Glaser et al., 1998; Marucha et al., 1998). Chronic stress leads to greater impact on immune function compared with acute stress since acute stress causes only a temporary decrease in immune function while chronic stress will result in continued impairment in immune function manifest as decreased NKCA and decreased salivary secretory IgA (S-IgA) secretion (Kelly, 1999). It was found that stress and age are interactive and that psychological stress can imitate, worsen (Graham et al., 2006), and speed up (Kiecolt-Glaser and Glaser, 2001) the effects of aging on immune function. The same decline in immune functions appears to occur in psychologically stressed individuals and aged individuals (Burns and Goodwin, 1997; Kiecolt-Glaser et al., 1998; Epel et al., 2004; Hawkey and Cacioppo, 2004).

Gender also affects both humoral and cell-mediated immune responses (Chao et al., 1995). Statistics shows that incidence of autoimmune disease, i.e., systemic lupus erythematosus, Hashimoto's thyroiditis, and rheumatoid arthritis, is higher in females than males (Olsen and Kovacs, 1996; Gaillard and Spinedi, 1998; Butts and Sternbrg, 2004). Kiecolt-Glaser and Newton (2001) reported that following conflicts in married people, women show greater immune dysregulation than men. The suggested explanation was that female possesses higher levels of Immunoglobulin (Ig) than males (Gaillard and Spinedi, 1998; Butts and Sternbrg, 2004). It is also believed that differences in sex steroids are responsible for this gender differences in immune response (Schuurs and Verheul, 1990; Olsen and Kovacs, 1996). A study conducted in 2001 revealed that a predisposition to acute stress by women during the

luteal phase of their menstrual cycle affects immune function by suppressing the immune response, decreasing the CD4/CD8 ratio, and decreasing NK cells as a percentage of total lymphocytes (Pehlivanoglu et al., 2001). In relation to exercise, females had greater macrophage antiviral resistance than males in both control-sedentary and moderate exercise groups (Brown et al., 2006). In a separate study, researchers discovered that exercise (cycling for 60 min at 70% maximum oxygen uptake,  $\dot{V}O_2\text{max}$ ) induced increases in total leukocytes, lymphocytes, and NK counts that were 35% greater or more in girls compared with boys of similar pubertal status (Timmons et al., 2006).

It is known that engaging in regular moderate physical activity can enhance immune function and protect against upper respiratory tract infection (URTI) (Nieman et al., 1990; Nehlsen-Canarella et al., 1991). However, immune function can be detrimentally affected during and after prolonged, strenuous exercise, thus increasing the infection risk (Mackinnon, 2000; Pedersen and Toft, 2000). Thus, it was suggested that exercise-induced changes in immune function depend on the exercise intensity and duration (Gleeson and Bishop, 1999) and the training status of the individual (Keast et al., 1988; MacKinnon, 1989). The effects of different types of exercise on immune function and infection risk will be further discussed in the following sections.

Certain nutritional strategies have been shown to limit exercise-induced immune dysfunction and are thus recommended to athletes engaged in heavy training and competition. These nutritional strategies include ensuring adequate intake of macronutrients (carbohydrate, protein, and fat), avoidance deficiencies of micronutrients (e.g. the minerals Iron, Zinc, Copper, and the Vitamins A, B<sub>12</sub>, C, D, E and Folic acid), and taking supplements such as antioxidants, probiotics, herbs, and



bovine colostrum. However, questions remain about their efficacy in reducing infection risk or ameliorating symptoms and duration of infections. There are numerous studies in this field to help athletes to cope with the exercise-induced immune function dysregulation and thus to reduce the risk of infection. The details of how these nutritional supplements work in this area will be explained later in this chapter.

## **1.2 Effects of exercise on immune function.**

Cumulating evidence has shown that impaired immune function may increase infection risk, which in turn is associated with health impairment and performance decrement in athletes. Thus, athletes and fitness enthusiasts are very concerned about the effects of exercise on immune function. It is well established that exercise-induced immunodepression depends on the intensity and duration of exercise (Keast et al., 1988; MacKinnon, 1989; Gleeson and Bishop, 1999; Gleeson and Walsh, 2012). Numerous studies have been conducted regarding the effects of exercise on immune function where it is generally indicated that regular moderate intensity exercise enhances immune function above those having sedentary lifestyle. In contrast, prolonged exercise and periods of intensive training and competition may impair immune function.

### **1.2.1 Prolonged exercise and immune function.**

According to Gleeson (2006a), post-exercise depression of immune function is most pronounced when the exercise is continuous, prolonged (> 1.5 h), of moderate to high intensity (55 – 75% of aerobic capacity), and performed without food intake. Prolonged exercise may impair both innate and adaptive immune function (Gleeson

and Walsh, 2012). Several studies reported that suppression of type 1 cytokines were found immediately after a prolonged intensified exercise, and persisted for 24 h (Starkie et al., 2001; Lancaster et al., 2005). Furthermore, Libicz and colleagues (2006) reported that there was negative cumulative effect of an intense exercise repeated daily on basal levels of S-IgA on elite triathletes. In a separate study, it was also reported that S-IgA secretion was decreased after prolonged bouts of exercise (Bishop and Gleeson, 2009).

Other evidence demonstrated that during and for several hours after an exercise trial to exhaustion before, immediately after, and following 2 weeks of recovery from a 6-day intensified training period, production of IL-2 and IFN- $\gamma$  by T lymphocytes were inhibited, while a 6-day period of intensified training decreased the percentage of type 1 T cells in circulation in the resting state (Lancaster et al., 2004). It was suggested that bouts of intense prolonged exercise induce decreased number and function of leukocytes as a result of increased stress hormone secretion, thus resulting in the entry of less mature leukocytes into circulation, particularly NK cells (Gleeson, 2005).

In 2001, analysis of blood samples collected after a competitive marathon run revealed decreased number of cells producing IL-1 $\alpha$  and IL-6, increased numbers of monocytes with less cytokine production, and marked increases in the plasma concentrations of IL-6, TNF- $\alpha$ , adrenaline (epinephrine), and noradrenaline (norepinephrine) (Starkie et al., 2001). Subsequently, Gomez-merino and colleagues (2003) conducted a study on 26 male soldiers, where they found that S-IgA activity was lower and circulating IL-6 was higher in the soldiers after a 5-day military course following 3 weeks of combat training. Studies conducted on rats indicated that 8

weeks of exercise on running wheels caused decreases in T cell activity, increases in B cell proliferation (Lin et al., 1993), and induced an immune response that mimicked some aspects of fever (Rowsey et al., 2009). Production of IL-2 was found to be lower in an animal study in which rats exercised by running at 60 – 70%  $\dot{V}O_2\text{max}$  for 30 min which was then extended up to 60 min per day for 5 days per week for 10 weeks (Lin et al., 1993). Another study found that cycling at an intensity of 10% above the ventilatory threshold level in sedentary males for 8 weeks, 3 days per week, 20 min per day, did not significantly increase the total circulating leukocyte number, but significantly decreased the CD3<sup>+</sup> and CD4<sup>+</sup> counts as much as 13 and 17%, respectively (Unal et al., 2005). Thus, it is believed that prolonged strenuous exercise have a temporary negative impact on immune function.

### **1.3 Exercise and infection risk.**

Based on numerous studies conducted regarding the relationship between exercise and infection risk up to 1994, it has been proposed that the relationship between exercise intensity or training volume and risk of upper respiratory tract infection (URTI) may be best explained in the form of ‘J’-shaped model (Nieman, 1994). As shown in Figure 1.1, the ‘J’-shaped model suggests that regular moderate intensity exercise decreases the relative risk of URTI compared with a sedentary lifestyle while prolonged high intensity exercise or periods of strenuous exercise training increase the relative risk of URTI above that of a physically inactive individual. According to Malm (2006), the ‘J’-shaped model was proposed based on the findings of a small number of studies before 1994, where most of the studies investigated the relationship between prolonged, high intensity exercise (e.g. marathon and ultramarathon running) and the URTI risk in the weeks that followed.

Furthermore, this hypothesis was built based from observational and case series surveys (Moreira et al., 2009).

There are several factors that may influence infection risk other than exercise and these include: vaccine history and time since inoculation, previous infections, nutrition status, and pathogen exposure. This suggests that infection is a risk for anybody, and anywhere. On average, children experience between six and eight colds per year, while adults may have between two and four colds per year (Lissiman et al., 2009). However, elite athletes, compared to subelite athletes, tend to maintain their health status even after they have completed heavy exercise training. Hence, in 2006, based on a re-evaluation of previous studies, Malm proposed an 'S'-shaped relationship (Figure 1.2) between exercise load and risk of infection. However, he recommended that this 'S'-shaped model be verified using data from a large number of subjects since it was limited to one runners' training log over 16 years (Malm, 2006). This finding may be attributed to physiological fitness of that elite athlete, thereby healthy enough to compete even after a strenuous training period. In addition, it may be due to genetic factors.

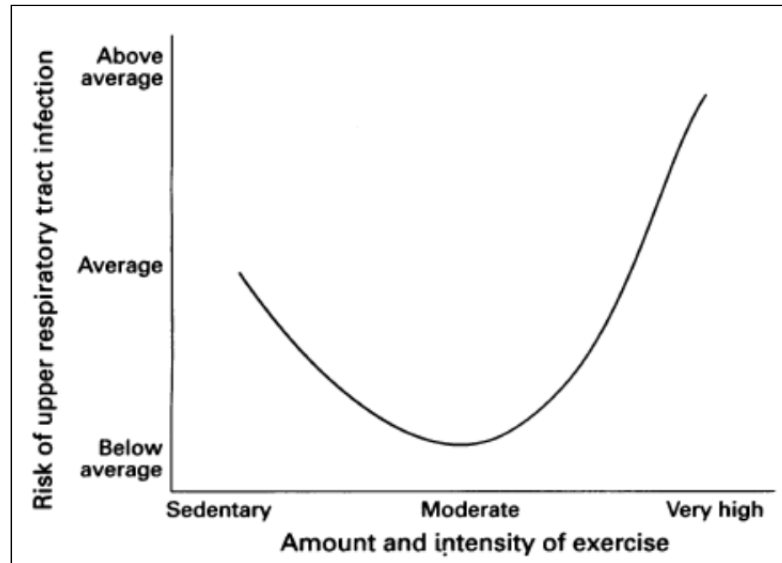


Figure 1.1: 'J'-shaped model of the relationship between varying amounts of exercise and risk of upper respiratory tract infection (Nieman, 1994).

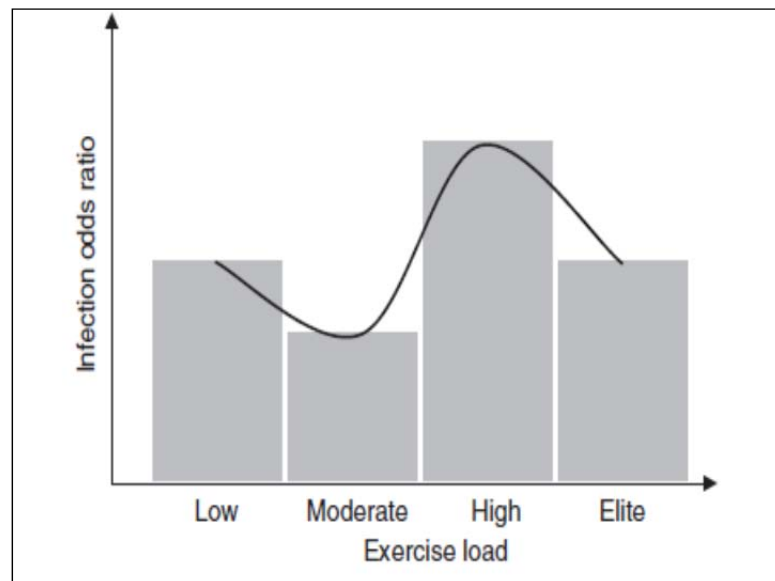


Figure 1.2: 'S'-shaped model of the relationship between exercise load and infection rate (Malm, 2006).

It is generally assumed that illness will decrease performance and the ability to train hard or compete. Even small changes in several immune parameters may alter resistance to URTI. Thus, information of how to decrease risk of infection is valuable especially to athletes and fitness enthusiasts. It has been suggested that individuals should avoid overtraining, have adequate rest and recovery during the training cycle and after competition, limit exposure to potential sources of infection, ensure adequate nutrition, and take selected supplements (vitamin C, probiotics, herbs, antioxidants, etc.) to prevent URTI (Mackinnon, 2000). It is thought that periods of prolonged and intense training are associated with high risk of infection because during this period, also known as open window period, virus and bacteria can gain a foothold (high susceptibility for pathogen entrance) (Nieman, 2000). Linde (1987) suggested that athletes tend to develop more frequent but not necessarily more severe URTI than the general public, and changes in URTI incidence are related to training volume and intensity.

An interesting article published in 2009 reviewed 30 published studies that had investigated the effect of exercise, physical activity, sport, and training on susceptibility to URTI and included 4 descriptive, 18 observational, and 8 interventional studies (involving 8595 athletes and 1798 non-athletes) (Moreira et al., 2009). This systematic assessment covered areas of agreement, areas of controversy, and areas timely for developing research (growing points). Among the 30 reviewed studies, areas of agreement included: moderate activity may enhance immune function, whereas prolonged, high-intensity exercise temporarily impairs immune competence; athletes experience a higher rate of URTI after training and competition compared with less active individuals; increasing physical activity in previously sedentary people is associated with a decreased risk of URTI. On the other hand, areas

of controversy among these 30 studies are: elite athletes may have a decreased susceptibility to URTI; poorly known individual determinants (e.g. genetic factors, fitness, nutritional status or disposition towards developing allergic hypersensitivity reactions) might affect the relationship between exercise and URTI.

### 1.3.1 Prolonged exercise and infection risk.

There is growing evidence to indicate that regular moderate exercise decreases the risk of acquiring URTI, while too much or too little exercise intensity may raise the infection risk. In a large scale study over 12 weeks involving over 1000 participants, it was recently reported that regular moderate intensity exercise reduces URTI incidence by ~ 20 – 45% compared with a sedentary lifestyle (Nieman et al., 2011). In addition, among athletes, it was suggested that relatively short duration or less competitive events were not associated with elevated risk of URTI (Nieman et al., 1989). In response to this, recreational or non-competitive athletes believe that they are more protected against URTI compared with others.

Therefore, there is a perception among top athletes, coaches, and sport physicians that athletes are at a higher risk of getting infections during period of intense exercise training and after major competition (Peters, 1997; Mackinnon, 1989), because during these periods there is a chronic mild depression of some immune parameters (Mackinnon and Hooper, 1996). It was suggested that prolonged bouts of strenuous exercise have a temporary negative effect on immune function (Gleeson and Walsh, 2012). Furthermore, the depression of immune function is most pronounced when the exercise is continuous, prolonged (> 1.5 h), of moderate to high intensity (55 – 75 %  $\text{VO}_2\text{max}$ ), and performed without food intake (Gleeson, 2006a).

It was previously observed that URTI incidence was elevated as much as 6-fold in runners compared with matched non-participants in the first 1 – 2 weeks after an endurance event (Peters and Bateman, 1983; Nieman et al., 1990). An observational, case-control study in 1983 revealed that 1 week after the two Oceans Marathon in 1982, there were significantly more self-reported symptoms of URTI in runners compared with individually matched controls who did not run. In addition, this self-reported URTI incidence was higher in the fastest runners (likely to be both the fittest and the ones who had the highest training loads), with no differences between the slower runners and controls (Peters and Bateman, 1983). Nieman and colleagues (1989) conducted a prospective study on 273 marathon runners with a 2-month training period prior to a 5-km, 10-km, or half-marathon race. The outcome was that self-reported URTI symptoms were more common in runners who trained more than 15 miles per week. Furthermore, they found that there was no increase in infectious episodes during the week following the run compared with the week prior to the race. In one study, Gleeson and colleagues (2011b) reported that high levels of physical activity are associated with a higher risk of URTI, where they found that those who exercised  $\geq 11$  h/week (HIGH) had significantly more URTI episodes than those who exercised 3 – 6 h/week (LOW). They also found an elevated level of anti-inflammatory cytokines IL-4 and IL-10 in response to antigen challenge in the HIGH group, and suggested that this may explain the higher incidence of infection in the HIGH group.

The mucosal immune system provides resistance to respiratory infection, with the major secretory immunoglobulin being IgA. Thus, IgA play a role in the first line of defence against infectious agents on mucosal surfaces (Gleeson and Pyne, 2000). Numerous studies have observed a decline in S-IgA levels after prolonged intense



exercise in endurance athletes, which in turn is sometimes associated with an increased incidence of URTI symptoms. A review article reported that low levels of S-IgA and IgM, particularly IgA1 subclass are associated with an increased risk of respiratory illness (Gleeson, 2000). Nevertheless, this association remains to be confirmed in well-controlled large scale studies. In 2005, a study was conducted on 21 male cadets from a military school who took part in a commando training (3 weeks of commando training followed by a 5-day combat course) (Tiollier et al., 2005). It was found that after the 3 weeks of training, S-IgA concentration was unchanged, but it was reduced after the 5-day combat course with a return to baseline values within 1 week of recovery. In addition, they found that the incidence of URTI was increased during the trial but was unrelated to S-IgA levels. Hence, they concluded that a stressful event results in an adverse effect on mucosal immunity and incidence of URTI, yet the relationship between S-IgA levels and illness remains uncertain.

In a separate study, researchers investigated the association between tennis training and competition, the incidence of URTI and S-IgA in 17 elite female tennis players (Novas et al., 2003). Results from this 12-week observational study showed that increased training duration and load, and competition level was positively associated with incidence of URTI. Salivary IgA was reduced markedly after a 1 h tennis training session (whole unstimulated saliva samples were collected before and after selected 1 h training sessions at 2-weekly intervals). Besides, this study reported that over the 12-week period, pre-exercise S-IgA concentration and secretion rate were directly associated with the amount of training undertaken during the previous day and week. The decline in S-IgA after 1 h of intense tennis play was also positively associated to the amount of training undertaken during the previous day and week. It was concluded that although suppression of S-IgA due to exercise may be a

risk factor; the occurrence of URTI in this study could not be accurately predicted (Novas et al., 2003).

A year longitudinal study on American college football players reveals that there was a significant reduction in the concentration and secretion rate of S-IgA (Fahlman and Engels, 2005). As a result of this, it was found that the incidence of URTI in these football players was increased. Another study on elite professional athletes over 50 weeks of training found that there was a significant decrease in S-IgA concentration 3 weeks before the URTI episodes, and return to baseline value after 2 weeks of infection (Neville et al., 2008). It was indicated that there is a one in two chance of contracting an URTI within 3 weeks if the S-IgA value is lower than 40% of an individual's mean healthy value. This suggests that a decline of S-IgA concentration over 3 weeks is associated with elevated URTI incidence, where the magnitude of relative decrease in S-IgA was related to the URTI risk but independent of the absolute S-IgA concentration. These two studies somewhat support the theory that reduction in S-IgA level associated with increase susceptibility to URTI.

In contrast, a 4-month winter training period in 20 highly trained international level male distance runners found no substantial relationship between intensity or training load and number of illness episodes reported (Fricker et al., 2005). In 2006, Ekblom and colleagues observed no relation between training volume 6 months before the race, finishing time and socioeconomic and demographic factors and 3 weeks before or 3 weeks after the Stockholm Marathon 2000. Furthermore, the incidence of infectious episodes 3 weeks after the race (post-IE) in runners, who had not experienced any infectious episodes in the 3 weeks before the race was 16%; whereas post-IE in runners who had experienced an infectious episode 3 weeks before

the race was 33%. They also found that younger runners were more likely to experience infectious episodes both before and after the race. Hence, this study does not support the common perception of intense training causing high infection risk, but does suggest that increased infection risk is somewhat related to engaging in strenuous exercise too soon after a recent infection (Ekblom et al., 2006).

In general, the evidence indicates that moderate exercise training in sedentary or less active individuals reduces their infection risk but endurance athletes experience higher infection incidence after intense training and competition compared with sedentary individuals. There is limited evidence for a reduction of risk of infection in elite athletes. For some athlete group, there appears to be a dose-response relationship between infection risk and exercise load during training and competition. There are many other factors other than exercise load, that may increase the susceptibility to infection and S-IgA levels may play some role in resistance to respiratory illness.

#### **1.4 Nutrition and immune function.**

Early in the chapter, it was briefly discussed that diet may affect immune function. It was proposed that inadequate diets lead to immunodepression which in turn can increase susceptibility to infection (Gleeson and Bishop, 2000). Two available mechanisms (direct and indirect) may clearly explain this effect. Inadequate diets may induce a direct negative effect on immune function by altering the availability of nutrients, coenzymes, or cofactors involved in immune cell energy metabolism and protein synthesis. Otherwise, inadequate nutrition may cause an indirect negative effect on immune function through immunoregulatory effects of stress hormones and altered hormonal responses to stress (e.g. exercise), thereby

decreasing immune function. This may include decreased cell mediated immunity, cell proliferation, complement formation, phagocyte function, and antibody production or affinity (Walsh, 2006). However, there is insufficient evidence to associate this inadequate nutrition-induced immunodepression to increased risk of URTI in athletes.

Numerous studies have been published regarding the effects of various types of diet and nutrition supplements on immune function, particularly in athletes. This growing field of study aims to provide sufficient evidence of the benefit of certain foods or supplements that may boost an athlete's immune function, thereby reducing the infection risk. Macronutrients (carbohydrate, protein and fat), micronutrients, vitamins, herbs, and probiotics are among those dietary components that have been studied.

As exercise results in increased energy expenditure, there is need for an increased intake of macronutrients. Deficiency in macronutrients may lead to compromised performances, premature fatigue, and impact on muscle building, maintaining and repairing. Some researchers have investigated the effects of combination of diets (e.g. CHO and protein, CHO and vitamin) in order to reveal significant positive effects of these combinations on athletes' immune function. Endurance athletes typically consume a low-fat-high-CHO diet (15% fat, 65% CHO, 20% protein of total calories) (Venkatraman et al., 2000).

The need for trace minerals may rise with exercise training since they are secreted through sweat produced during exercise, and are also lost through urine. Their concentration in blood and tissue is decreased after training and competition, where the magnitude of losses depend on mode and intensity of exercise, exercise

duration, environmental temperature, and mostly related to nutrition status of the individual (Konig et al., 1998). Zinc and iron are among these important trace minerals with roles in immune function besides copper, selenium, manganese, and magnesium. Zinc is a component of antioxidant enzymes and is thought to protect against increases in free reactive oxygen species, while iron is important in energy metabolism and synthesis of haemoglobin, myoglobin, and cytochromes (Speich et al., 2001). Hence, supplementation of these minerals may be beneficial to enhance athletes' immune function. However, too much of them may lead to increase susceptibility to infection since their excess is somewhat associated with immune function impairment (Gleeson and Bishop, 2000).

Many vitamins are precursors of coenzymes involved in energy metabolism and protein or nucleic acid synthesis. Several vitamins are essential for normal immune function: vitamin A, B-complex, C, D and E. Deficiency in vitamin B-complex may lead to fatigue and muscle soreness. However, vitamin deficiency in athletes is less reported since vitamin loss via sweat and urine during exercise is negligible.

The following sections describe some nutritional supplements that were examined in the studies reported in this thesis: probiotics, Kaloba (*Pelargonium sidoides*/ EPs7630), echinacea, quercetin, curcumin and caffeine.

#### 1.4.1 Probiotics.

Intestinal epithelia are constantly exposed to varied, dynamic enteric bacteria which comprise more than 500 species of anaerobic bacteria (Ohland and MacNaughton, 2010). Microbiota within the gastrointestinal (GI) tract give benefit to

their host whereby they maintain normal mucosal immune function, epithelial barrier integrity, motility, and nutrient absorption. Whenever this relationship between microbiota and host is disrupted, GI function and disease susceptibility may be altered (Collins and Bercik, 2009). Its defensive barrier functions to protect against an uncontrolled inflammatory response and includes a mucous layer, antimicrobial proteins (AMPs), secretory IgA, and epithelial junction adhesion complex (McGuckin et al., 2009). Although adult bacterial colonic flora are generally stable, it appears that age, nutritional requirement, immune status, use of antibiotic, stress, alcohol use, pH, transit time, and the presence of materials in the gut, may influence them (Collin and Gibson, 1999). Numerous studies have proposed that consumption of non-pathogenic bacteria, especially probiotics, may decrease paracellular permeability, provide innate defence, and enhance physical impediment of the mucous layer (Boirivant and Strober, 2007).

Probiotics, also known as ‘friendly bacteria’, is a Greek word which means ‘for life’ (Salminen et al., 1998). Officially, it is defined by World Health Organisation (WHO) as ‘live microorganisms which when administrated in adequate amounts confer a health benefit on the host’ (FAO, 2001). These non-pathogenic microorganisms can be found in fermented food products such as yoghurt, tempeh, kefir, sauerkraut, cabbage kimchee, soybean based miso, and natto (Nichols, 2007). A nationwide survey reported that approximately 2 million adults in the United States use probiotics for health reasons with middle aged females being the highest consumers (French, 2006). *Lactobacillus* species, *Bifidobacterium* species, *Streptococcus* species, and *Escherichia coli* are the commonly used bacterial probiotics. *Lactococcus lactis* and some *Enterococcus* species are also used. Both the *Lactobacillus* (L.) and *Bifidobacterium* (B.) species are lactic acid bacteria which

convert carbohydrate to lactic acid, which gives the sour taste to yoghurt and other similar products. Lactic acid reduces the pH, thus reducing the growth of spoilage organisms.

To ensure its safety for human consumption, most of these microorganisms were isolated from healthy humans (Boirivant and Strober, 2007). To date, probiotics consumption in humans has a good safety record (Gueimonde et al., 2006). However, special precautions are needed when it is to be consumed by ill patients. Previous studies showed that passage of probiotics through the GI tract is typically between 3 and 8 days (Klijn et al., 1995). To be classified as a genuine probiotic, these bacteria should have viability during processing, transport, and storage, ability to survive gastric transport, ability to adhere and colonise the GI tract, ability to antagonize pathogenic bacteria, and demonstrated clinical health outcomes (West et al., 2009). Yet, there is no optimum dosage established for probiotic ingestion to induce beneficial effects. The current dosage is between  $10^8$  and  $10^{11}$  colony forming units (CFU) per day (Takashi et al., 2009; Kudsk, 2002).

While probiotics are live microbial food ingredients, prebiotics are indigestible food ingredients that selectively promote the growth or activity of beneficial enteric bacteria, thereby benefiting the host (Gibson and Roberfroid, 1995). Combinations of probiotics and prebiotics are called synbiotics and are designed to improve the survival of ingested microorganisms and their colonization in the GI tract (Boirivant and Strober, 2007). Studies show that probiotics exert their effects either with or without colonization. Those with colonization (e.g. *Bifidobacterium longum* and *Bifidobacterium thetaiotamicron*) become part of human intestinal microflora (Sonnenburg et al., 2006; Pochart et al., 1992), while those without colonization (e.g.

*Lactobacillus casei* and *Bifidobacterium animalis*) exert their effects indirectly either in a transient manner as they pass through or more likely by remodelling or influencing the existing microbial community (Preidis and Versalovic, 2009; Pochart et al., 1992) with potential effects on mucosal and systemic immunity (Calder and Kew, 2002).

The proposed mechanisms of action of probiotics include altering the expression levels of host cell-derived anti-microbial peptides and directly inhibiting growth or promoting killing of pathogens by producing anti-microbial molecules including short-chain fatty acids (SCFA) and bacteriocins or microcins (Collins and Bercik, 2009). Probiotic bacteria may also compete against invading pathogens for binding sites to epithelial cells and the overlying mucous layer, and augment levels of total and pathogen-specific secretory IgA upon infection, while typically not inducing production of probiotic-specific secretory IgA (Galdeano and Perdigon, 2006). Furthermore, probiotic bacteria may promote tolerogenic dendritic cell and regulatory T cell phenotypes, inhibit inflammatory cytokine production, and enhance NKCA (Ng et al., 2009). In addition, French (2006) reported that probiotics may speed the breakdown of organic waste fragments, cleaning the intestinal tract, increasing the production of important enzymes, assisting the regulation of digestion, relieving constipation, and strengthening the immune system.

#### *1.4.1.2 Probiotics and athletes' immune function.*

Evidence of an ergogenic effect of probiotics on athlete performance is lacking but this is not surprising given that there is no rationale for such effect. However, probiotics may confer secondary health benefit to athletes by enhancing recovery from fatigue, improving the immune function, and maintaining healthy of GI



tract function (Nichols, 2007). Yet, the available published studies regarding probiotics' benefit on athletes is limited.

A recent published study reported that regular ingestion of *L. salivarius* probiotic in 66 highly active individuals during 16 weeks of spring training does not affect blood leukocyte counts, levels of salivary AMPs, and also not appears to be beneficial in reducing URTI frequency (Gleeson et al., 2012). However, previously it was found that daily probiotic (*L. casei* Shirota) supplementation in 84 highly active individuals for 16 weeks during winter period was beneficial in reducing URTI frequency, which might be related to better maintenance of S-IgA levels (Gleeson et al., 2011a).

In a separate study, 20 healthy elite male distance runners received 3 capsules, twice per day for 28 days of *L. fermentum* VRI-003 (PCC) ( $1.2 \times 10^{10}$ ) or placebo (Cox et al., 2010). Twenty-eight days after completing the first supplementation period, elite runners received the second supplement (PCC or placebo), followed by follow-up. The authors reported that there was less than half the number of days of respiratory symptoms in subjects with PCC compared with placebo. Furthermore, illness severity was lower, and unstimulated *in vitro* whole blood culture production of IFN- $\gamma$  was 2-fold greater with PCC supplementation. There were no significant differences in mean change in S-IgA and plasma Ig-A levels or in IL-4 and IL-12 serum levels between treatments. Another study which using the same strain of probiotic (minimum  $1 \times 10^9$  *L. fermentum* (PCC) per day for 11 weeks) found that the probiotic lowered the respiratory illness symptoms in male competitive cyclists compared to those taking placebo (West et al., 2011). However, its effect on female cyclists was uncertain.

Clancy et al. (2006) conducted a study on 9 athletes with fatigue and impaired performance and symptoms consistent with re-activation of Epstein Barr virus (EBV) infection and 18 healthy athletes (control group), where athletes were given daily capsules of  $2 \times 10^{10}$  *L. acidophilus* LAFTI® L10 for 4 weeks. It was found that stimulated IFN- $\gamma$  production by T cells which was low in the fatigued athletes was significantly increased in the fatigued athletes, to the same level as the healthy controls after the probiotic treatment.

In a separate study, 5 male cyclists were given probiotics drink contains  $1.3 \times 10^{10}$  *L. casei* Shirota or placebo daily for 2 weeks during training. Then, they completed a cycle ergometer exercise trial (Gleeson, 2008). This study found that the circulating CD4+ increased and the CD4+/CD8+ ratio improved at rest and post exercise. Another study found a smaller decrease in circulating NK cells after an exercise stress (60 minutes at 75%  $\dot{V}O_2\text{max}$ ) after a month of daily supplementation with 500 ml probiotic (DN-114 001) drink containing  $3.2 \times 10^8$  CFU/ml of *L. casei* (Pujol et al., 2000). These findings suggest that some probiotics may enhance athletes' immune function.

During 3 months of training after the Helsinki City Marathon, 141 marathon runners were given a milk-based fruit drink (130 ml per day) which contained  $4 \times 10^{10}$  CFU/ml of *L. rhamnosus* GG or placebo (Kekkonen et al., 2007). Then, they were followed-up for 2 weeks after the event. Results revealed that GI illness episodes showed a trend to be of shorter duration during training, significantly shorter duration after the marathon, but with no difference in GI illness incidence.

#### 1.4.2 Kaloba (*Pelargonium sidoides* / EPs7630).

Kaloba is an extract derived from the root of plants that are indigenous to South Africa. It comes from the family Geraniaceae and genus *Pelargonium* and was traditionally used to treat dysentery, diarrhoea, colds, wounds, fatigue, fevers, hepatic complaints, generalized malaise, and respiratory tract infections such as tuberculosis, bronchitis, and sinusitis (Kolodziej, 2000; Lewu et al., 2006). Furthermore, studies reported that it is successfully employed for the treatment of ear, nose, and throat disorders as well as respiratory tract infections (Kolodziej and Kiderlen, 2007; Matthys and Funk 2008; Thale et al., 2008; Matthys et al., 2010). Besides, it was found that its usage is well tolerated, efficacious, safe, and no adverse effects. African geranium, umckaloaba, kalwerbossie, rabassamin, and zucol are among its common names. Kaloba composed of 6 main groups of constituents which include unsubstituted and substituted oligomeric prodelphinidins, monomeric and oligomeric carbohydrates, minerals, peptides, purine derivatives and highly substituted benzopyranones.

To date, there is one study investigating the effects of this plant extract on athletes' immune function available in literature (PubMed search). Luna and colleagues (2010) had investigated immune response induced by *Pelargonium sidoides* (Kaloba) extract in serum and nasal mucosa of athletes after exhaustive exercise. In that study, participants performed a high-intensity running session (~ 85% of VO<sub>2</sub>max) after 28 days of supplementation (3 x 30 drops/day of a solution of 80 g of extract/100 ml in solution). They found that saliva secretory IgA concentration was increased suggesting that *Pelargonium sidoides* able to modulate the immune response of athletes during intense physical activity. However, the post-exercise

sample was collected 48 h after exercise completed. The authors did not justify the selection of this sampling time point and it would be of interest to know if the IgA response to exercise was altered in the immediate post-exercise recovery period.

A study was conducted to determine its efficacy in the treatment of patients (1 – 18 years old) with acute bronchitis, outside the strict indication for antibiotics (Kamin et al., 2012). Patients were given 7 days supplementation of either EPs7630 (1 - 6 years: 3 x 10 drops per day; > 6 - 12 years: 3 x 20 drops per day; > 12 - 18 years: 3 x 30 drops per day) or placebo. The mean total score of bronchitis-specific symptoms (BSS) was significantly more improved in the EPs7630 group throughout the supplementation period. Improvements were most pronounced for ‘coughing’ and ‘rales at auscultation’. There were no serious adverse effects observed from either supplement. Thus, this study supports the traditional belief that this plant extract is effective in treating bronchitis. In a separate study, 400 patients (6 – 18 years old) were given 30 mg, 60 mg, or 90 mg of EPs7630 or placebo for 7 days (Kamin et al., 2010b). They found that BSS total score (especially ‘coughing’, ‘sputum’ and ‘lung sounds’ (small clicking, bubbling, or rattling sounds) heard during auscultation) was significantly better with a dose of 60 and 90 mg of EPs7630.

Another study investigated the efficacy of EPs7630 in treating the common cold (Lizogub et al., 2007). Patients (103 males and females adult patients with at least 2 major and 1 minor or with 1 major and 3 minor cold symptom) received either 30 drops (1.5 ml) of the liquid herbal drug preparation EPs7630 or placebo, 3 times per day for 10 days. It was found that the sum of symptom intensity differences improved, the decrement of the mean of cold intensity score was higher, percentage of patients cured after 10 days supplementation was higher, and the mean duration of

inability to work was lower in the EPs7630 group compared with placebo. As the adverse effects observed were not serious, it was concluded that EPs7630 is effective and tolerable in treating the common cold.

#### 1.4.3 Echinacea.

Immunomodulatory properties of Echinacea, the most widely consumed botanical product in the United States (Bardia et al., 2007), are unclear because of different methods of its preparation, different plant parts used, and heterogeneity of species. Three widely available species which include *purpurea*, *angustifolia*, and *pallida*, appear to have different immunomodulatory potential based on cytokine stimulatory properties (Barnes et al., 2005; Senchina et al., 2009).

Interestingly, a recent published study found that Echinacea supplementation (8000 mg/day, 4 times per day, for 28 days) significantly improved running economy (as indicated by a decrease in submaximal  $\dot{V}O_{2\max}$  during the first 2 stages of maximal graded exercise tests) (Whitehead et al., 2012). In a study involving two different exercises; a  $\dot{V}O_{2\max}$  test and a 90 min cycling at 85% of ventilatory threshold, blood samples pre- and post-exercise were stimulated in vitro with extracts from bloodroot (*Sanguinaria canadensis*) or coneflower (*Echinacea tenesseeensis*) (Senchina et al., 2009). It was found that in the  $\dot{V}O_{2\max}$  test and the 90-min bout, bloodroot extracts significantly increased cytokine production compared with controls. In a separate study, the effects of 4 weeks Echinacea supplementation on mucosal immunity and URTI incidence and duration before an exercise was examined compared with control group (without Echinacea supplementation) (Hall et al., 2007). This exercise involved 3 consecutive, 30 sec all-out efforts Wingate cycling tests with 0.075 kg per kg body mass frictional resistance. Tests were separated by 3 min

recovery. It was found that, in the control group, S-IgA level and secretion rate decreased following the intervention. Furthermore, URTI duration was significantly different between groups where it was found to be longer in the control group. This suggests that Echinacea may blunt the intense exercise-induced mucosal immune suppression and reduce the URTI duration.

A double-blind, placebo-controlled, cross-over study on horses with 42 days supplementation of either Echinacea or placebo reported increased phagocytic ability of isolated neutrophils, boosted peripheral lymphocyte counts, and to some extent stimulate neutrophil migration from peripheral circulation in to the tissues (O'Neill et al., 2002). In addition, it was also found that the size and concentration of peripheral red blood cells and the concentration of haemoglobin and packed cell volume were increased. Researchers concluded that Echinacea supplementation act as a haematinic agent (an agent that stimulates the production of red blood cells or increases the amount of haemoglobin in the blood) and stimulate equine immunocompetence.

#### 1.4.4 Quercetin.

Quercetin is a flavonol that can be found in fruits and vegetables which is believed to reduce infection risk during intense exercise since it has anti-inflammatory and antioxidant properties, beside its psychostimulant actions and its ability to stimulate mitochondrial biogenesis (Davis et al., 2009). A recent study found that quercetin supplementation (500 mg supplemental quercetin capsule) daily for 8 weeks in active male students had significantly increased  $VO_2\text{max}$ , lean body mass, total body water, basal metabolic rate, and total energy expenditure (Askari et al., 2013). Similarly, another study also claimed that quercetin increased  $VO_2\text{max}$  in untrained subjects (Davis et al., 2010). However, one study reported that quercetin (250 mg)

supplementation for 3 weeks on runners did not affect the ratings of perceived exertion (RPE) during a 160-km endurance run (Utter et al., 2009). An animal (mice) study also found no effects of 6 weeks quercetin supplementation on  $\dot{V}O_2$  peak, speed at  $\dot{V}O_2$  peak, or endurance time to exhaustion (Casuso et al., 2013).

A study conducted in 2007 revealed that 2 weeks ingestion of quercetin (1000 mg per day) did not affect exercise-induced immune function depression (NKCA, PHA-stimulated lymphocyte proliferation, polymorphonuclear oxidative-burst activity, and S-IgA output) in trained male cyclists who completed 3 h of cycling per day for 3 days (57% PPO). However, it was found that quercetin ingestion significantly reduced the URTI incidence in the cyclists (Nieman et al., 2007b). In a separate study, Nieman and colleagues reported that 3 weeks quercetin ingestion (1000 mg per day) by ultra-marathon runners before a competition (160 km) did not blunt the muscle damage, inflammation, increase in plasma cytokine and hormone concentrations, and alterations in leukocyte cytokine mRNA expression (Nieman et al., 2007a).

In an animal study, mice were randomly distributed into four different groups: exercise-placebo, exercise-quercetin, control-placebo, and control-quercetin (Davis et al., 2008). Mice were fed with quercetin (12.5 mg/kg) via gavage for 7 days before they were exposed to the influenza virus A/Puerto Rico/8/34 (H1N1). The animals were intranasally inoculated at 30 min after the last bout of exercise (run to fatigue (approximately 140 min) on a treadmill for 3 consecutive days) or rest. The exercise-induced increase in susceptibility to infection (morbidity, mortality, and symptom severity on days 5 – 7 after influenza virus inoculation) was blunted with quercetin supplementation. Morbidity in mice was identified by the presence of ruffled fur,

redness around the eyes, nose, or mouth and altered respiration which occurred around day 4 to day 5, hunched-back pose, which occurred around day 5 to day 6, and unresponsiveness, which occurred after 6 days.

#### 1.4.5 Curcumin.

Curcumin which is contained in the rhizome of the plant is the major bioactive compound in turmeric (*Curcuma longa*). It has multiple biological activities such as anti-oxidants, anti-inflammatory, and anti-carcinogenic (Huang et al., 1991; Rao et al., 1995; Cai et al., 2013), thus used in folk medicine for the therapy of inflammatory and infectious diseases. In addition, a recent study suggested that curcumin reduces glomerulosclerosis (hardening of the glomerulus in the kidney), improves kidney function and could serve as a therapeutic agent during serum sickness (Jacob et al., 2013). However, it has poor bioavailability and lack of solubility in aqueous solvents (Yadav et al., 2010), thus its optimal potential is difficult to discover. Studies reported that curcumin causes reduction in production of TNF- $\alpha$ , IL-1, and IL-8 (Chan, 1995; Hidaka et al., 2002).

Furthermore, Yun et al. (2011) found that curcumin suppressed cytokine release in human monocytes cells. These findings suggest that curcumin is an immunosuppressant. Bharti and colleagues (2010) reported that curcumin dose-dependently decreased IFN- $\alpha$  induction in cultures (peripheral blood lymphocytes), but has marginal effect on IL-4 expression. They also reported that curcumin effectively suppresses massive Th1 cytokines induction after renal transplantation.

It was also suggested that curcumin ingestion and aerobic exercise training can increase flow-mediated dilation in post-menopausal women, hence can potentially



improve the age-related decline in endothelial function (Akazawa et al., 2012). In a separate study, curcumin supplementation in rats shows an inhibitory role, where it attenuated lead-induced neurotoxicity (Hosseinzadeh et al., 2013).

#### 1.4.6 Caffeine.

Immunomodulatory effects of caffeine, a well-known psychostimulant substance, have been extensively studied and it appears that caffeine modulates both innate and adaptive immune responses. Caffeine is present in coffee and tea and is a member of the methylxanthine family of drugs; it is the most widely consumed psychoactive substance by humans (Fredholm et al., 1999). Numerous studies suggest caffeine has anti-inflammatory effects. For instance, one study found that caffeine supplementation 1 h before exercise increased IL-6 and IL-10 plasma levels (anti-inflammatory cytokines) following a 15-km running competition in athletes (Tauler et al., 2013). Besides, studies have also reported that caffeine decreased lymphocyte proliferation (Kantamala et al., 1990; Rosenthal et al., 1992; Horrigan et al., 2005). It also appears that caffeine supplementation suppresses antibody production (Laux and Klesius, 1973; Saxena et al., 1984; Rosenthal et al., 1992). Furthermore, previous studies also showed that the production (whole blood culture) of pro-inflammatory cytokines such as TNF- $\alpha$  (Van Furth et al., 1995; Horrigan et al., 2004) and IFN- $\gamma$  (Horrigan et al., 2005) were decreased with caffeine.

It was suggested that many of caffeine's immunomodulatory effects occur at concentrations that are relevant to normal human consumption (Horrigan et al., 2006). However, one study found that one large dose (6 mg/kg body mass) of caffeine did affect (attenuated) the exercise-induced increase in antigen-stimulated NK cell CD69 expression 1 h following strenuous intermittent exercise (Fletcher and Bishop, 2011).

But, this effect was not evident when the caffeine was ingested in smaller amounts (2 mg/kg body mass) on 3 separate occasions (this model is typical of habitual caffeine consumption) during the day. Overall, people in Ireland, UK, and US consume caffeine in amounts of approximately 200 mg/person/day, whilst over 400 mg/person/day are consumed in the Netherlands, Sweden, and Norway.

### **1.5 Summary.**

Throughout this literature review, it is clearly shown that exercise-induced immunodepression depends on the duration and intensity of exercise, where prolonged and high intensity exercise is the most detrimental to human immune function. The relationship between the amount and intensity of exercise with the risk of upper respiratory tract infection was described in the J-shaped model as discussed previously. High-level athletes are at a higher risk to suffer from infections compared with recreational athletes because high-level athletes engage in high intensity training for longer periods especially when they are about to take part in competition. Various diet and nutritional supplement interventions have been studied and recommended by researchers to be taken to enhance immune function and subsequently reduce infection risk. However, the effects of some of these nutritional supplements on immune responses to exercise and/or URTI incidence are scarce in literature.

## CHAPTER 2

### GENERAL METHODS

#### 2.1 Ethics approval.

Each study obtained ethics approval from the local Research Ethics Committee. Before each study was conducted, participants were informed about the study background and procedures before they signed the written informed consent form (Appendix A). Participants recruited were all physically active and healthy. To confirm their overall health status, they were asked to complete a health screening questionnaire (Appendix B) before participating in each study.

#### 2.2 Maximal oxygen uptake ( $\dot{V}O_2\text{max}$ ) determination.

During each first visit, participants completed the health screen questionnaire form and consent form. Then, their body mass and height were measured. After that, participants sat on an electrically braked cycle ergometer (Lode Excalibur, Holland) for a  $\dot{V}O_2\text{max}$  test. They began cycling at 95 watt (W) for 3 min (warm-up), and then the power output was increased to 165 W for the following 3 min. After that, the power output was increased by 35 W at every 3 min, until volitional exhaustion. Expired gas was collected in a Douglas bag during the final minute at each 3-min stage. In addition, heart rate (HR) and rating of perceived exertion (RPE) were measured during this period. Heart rate was measured using a telemetric device (Polar Electro, Kempele, Finland) and the Borg 6 – 20 point scale was used to estimate RPE (Borg, 1982). Participants were asked to give signal when they thought they could continue for only one more minute (i.e., as they approached volitional exhaustion). During this period, RPE and HR were measured and expired gas was collected in a

new Douglas bag. Verbal encouragement was also given at this point. After that, participants were allowed to cool down before leaving.

Expired gas samples collected in the Douglas bags were then analysed by an oxygen (O<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>) analyser (Servomex series 1400, Crowborough, UK) and the volume of expired gas at each work rate was measured using a dry gas meter (Harvard apparatus, Cambridge, UK). From these results,  $\dot{V}O_{2\max}$  was determined. The relationship between oxygen uptake and work rate was used to determine the work rate that was equivalent to 60%  $\dot{V}O_{2\max}$ . This work rate was then used during the experimental trials.

### **2.3 Familiarisation trial.**

Approximately 1 week after  $\dot{V}O_{2\max}$  test, participants completed a familiarisation trial which was cycling at the work rate which would be required during the experimental trial (equivalent to  $\sim 60\% \dot{V}O_{2\max}$ ) for 90 min on a cycle ergometer to familiarise them with the protocol of the study. This also ensured that the exercise intensity given did elicit a relative intensity of  $\sim 60\% \dot{V}O_{2\max}$  and participants were able to maintain the exercise for 90 min after an overnight fast. Expired gas was measured during the trial at minute 10, 30, 60 and 90.

### **2.4 Analytical methods.**

#### **2.4.1 Saliva sample collection.**

Participants came in the morning after an overnight fast. Before saliva samples were collected, participants were seated for at least 10 min. Saliva samples were obtained by 2 min unstimulated dribbling into a pre-weighed sterile bijoux tube. They

were asked to sit on a chair, lean the head forward and let the saliva passively dribble into the tube; without using their tongue or any mouth movement. Whenever the saliva volume collected in 2 min was insufficient, the collection was continued further for another 1 or 2 min. The bijou tube (with saliva sample) was then weighed.

#### *2.4.1.1 Saliva volume/weight, flow rate, and saliva antimicrobial proteins secretion rate calculation.*

Following are how the saliva volume/weight, flow rate, and saliva antimicrobial protein secretion rate were calculated in this study:

Saliva volume (ml) = Difference in weight (g) of bijou tube after collection of saliva assuming a saliva density of 1.0 g/ml

Saliva flow rate (ml/min) =  $\frac{\text{Saliva volume (ml)}}{\text{Collection time (min)}}$

Saliva antimicrobial protein secretion rate ( $\mu\text{g}/\text{min}$ ) = Saliva flow rate (ml/min) X Saliva antimicrobial protein concentration ( $\mu\text{g}/\text{ml}$ )

#### 2.4.2 Saliva analysis.

##### *2.4.2.1 Saliva secretory immunoglobulin A (S-IgA) analysis.*

Saliva samples were analysed for S-IgA level by using a Salimetric S-IgA ELISA kit. Before analysis, saliva samples were thawed at room temperature and then spun for 2 min at 12,000 rpm in microcentrifuge. Then, a dilutor dispenser Hamilton 400X (with 5 ml and 100  $\mu\text{l}$  syringes) was used to deliver 10  $\mu\text{l}$  of standard, control or sample to 4 ml of working conjugate solution into each labelled polypropylene tube. Each sample was done in duplicate. They were then incubated at room temperature

for 90 min. After 90 min incubation, 50  $\mu$ l from each tube was pipetted into the microwell plate in duplicate. The plate was covered with an adhesive plate sealer. After that, it was incubated at room temperature for 90 min on a plate shaker set to 250 rpm. Next, the plate was washed by using an automated washer before 50  $\mu$ l of TMB was added to all wells. It was then incubated for 20 min in the dark without shaking. After that, 50  $\mu$ l of stop solution was added to all wells. Then, the absorbance was read on Dynex Opsys plate reader at 450 nm wavelength.

#### *2.4.2.2 Lysozyme analysis.*

Before analysis was carried out, saliva samples were diluted 1000 times with Phosphate-Buffered Saline (PBS). Then, 50  $\mu$ l of standards and saliva samples were pipetted into the designated duplicate wells. The plate was covered tightly with a plastic seal, and then incubated at room temperature for 1 h. Next, the plate was washed by using an automated washer. After that, 100  $\mu$ l of the Lysozyme antiserum was added to each well. Once again, the plate was covered tightly with a plastic seal, and then incubated at room temperature for 1 h. After washing, 100  $\mu$ l of the diluted Donkey anti-Goat IgG Peroxidase was added to each well and then incubated for another 1 h at room temperature. One volume of TMB solution (BT-497) was mixed with one volume of Hydrogen Peroxidase solution (BT-498) and then put aside. The plate was washed again, and then 100  $\mu$ l of substrate mix was immediately added to all wells and incubated at room temperature, in the dark, for 15 min. Finally, 100  $\mu$ l of stop solution was added to all wells, mixed by shaking and the absorbance measured at 450 nm on a Dynex Opsys plate reader within 15 min.

#### 2.4.2.3 Lactoferrin analysis.

Saliva samples were diluted 1000 times with PBS as described for lysozyme analysis. Firstly, 100  $\mu$ l of standard/sample was added to each well. Then the plate was covered tightly with a plastic seal and incubated for 1 h at 37°C. Next, the plate was washed by using an automated washer. Then, 100  $\mu$ l of diluted anti-lactoferrin solution was added to each well. The plate was covered tightly with a plastic seal, and then incubated at for 1 h at 37°C. Next, the plate was washed by using an automated washer. After that, 100  $\mu$ l of diluted avidin-HRP (horseradish peroxidase) solution was added to each well. The plate was covered tightly with a plastic seal, and then incubated at for 15 min at 37°C. Next, the plate was washed by using an automated washer. Then, 100  $\mu$ l of OPD (*o*-phenylenediamine) was added to each well. The plate was covered tightly with a plastic seal, and then incubated at for 5 to 10 min at 37°C. Finally, 50  $\mu$ l of stop solution was added, mixed by shaking, and the absorbance was read at 450 nm on a Dynex Opsys plate reader.

#### 2.4.2.4 Alpha ( $\alpha$ )-amylase analysis.

The assay was carried out in a micro-titration plate by using a kit (Amylase-20, No. 577, Sigma). Firstly, saliva was diluted 1 in 100 in 1.0 mM calcium chloride ( $\text{CaCl}_2$ ) solution. Then, 20  $\mu$ l of this diluted saliva was mixed with 180  $\mu$ l of amylase reagent. Purified  $\alpha$ -amylase from human saliva (A1031, Sigma) was used as a standard. The plate was then incubated for 1 min at 20°C, and then the absorbance was read at 450 nm wavelength on a Dynex Opsys plate reader. Absorbance was read again after 2 min. Finally, amylase activity (units of enzyme activity corrected to 37°C) was calculated by using the following formula:

$$\text{Amylase activity (U/L)} = \frac{\Delta A(\text{The difference of absorbance reading})}{2 \text{ min}} \times 2515$$

#### 2.4.3 Blood sample collections.

Blood samples collections were always performed after saliva sample collections. Blood samples were taken from an antecubital vein by venepuncture with a 21 gauge needle into heparin and/or K<sub>3</sub>EDTA collection tube/s using the vacutainer method. To make the vein become prominent, a tourniquet was used on the upper arm and was immediately released as soon as blood started to flow into the collection tube. Blood samples collected in K<sub>3</sub>EDTA tube were used for haematological analysis while blood samples collected in heparin tube were used for determination of antigen-stimulated cytokine production.

#### 2.4.4 Haematological analysis.

On the same day as the blood sample collection, blood from the K<sub>3</sub>EDTA tube was used to measure haematological parameters (haematocrit (Hct), haemoglobin (Hb) concentration, red blood cell (RBC) count, and total and differential leukocyte counts) using an automated haematology analyser (AcT.5 diff, Beckman Coulter, UK). Each sample was measured twice and the average value was recorded.

#### 2.4.5 Cytokine array.

##### 2.4.5.1 *One hundred times diluted vaccine dose preparation.*

Pediacel vaccine (containing purified diphtheria toxoid, purified tetanus toxoid, 5 purified components of the *Bordetella pertussis* bacteria, 3 types of inactivated poliovirus, and purified components *Haemophilus influenzae* type b) was



diluted 100 times by adding 50 µl Pediacel vaccine to 4.95 ml RPMI-1640 medium (Sigma-Aldrich, UK) in a bijoux tube. This solution of diluted vaccine was kept at 4°C and only used for up to 1 week. A new stock dilution was prepared each week when participants came to the laboratory.

#### *2.4.5.2 Whole blood culture incubation and supernatant storage.*

Since the effective dose range was found to be between dose 4 and 6 (1,000-4,000-fold dilution of vaccine) (Chapter 3), we decided to use dose 5 (2,000-fold dilution of vaccine) in the second (Chapter 4) and third (Chapter 5) studies. On the same day as the blood sample collection, blood samples from heparin tube were incubated for 20 h. Firstly, on the culture plate, 50 µl of 100 x diluted vaccine was added to 0.7 ml of RPMI-1640 medium. Then 0.25 ml blood from heparin collection tube was added to it and gently mixed. RPMI-1640 medium contains sodium bicarbonate and L-glutamine which support the growth of the cultured cells. After that, the lid was placed on the culture plate before it was placed in the CO<sub>2</sub> incubator (MiniGalaxy E, UK) (5% CO<sub>2</sub>, 37°C) for 20 h.

After 20 h incubation, by using a 1 ml disposal pastette, blood culture was transferred from each well to separate labelled eppendorf tubes and spun for 4 min at 12,000 rpm in a microcentrifuge (Thermo IEC, UK). After that, the supernatant fluid was aspirated using a 1 ml disposal pastette and placed in a labelled eppendorf tube. These were stored in a cryobox in a -20°C freezer until further analyses were carried out.

Whole blood culture was used in this study instead of freshly isolated peripheral blood mononuclear cells (PBMCs) because whole blood culture system

represents more closely the natural environment with the presence of various immunomodulating and pro- and anti-inflammatory mediators of the blood. Furthermore, a whole blood culture system, which needs no purification procedure as needed in isolating PBMCs reduces the risk of cellular activation due to the purification procedure and avoids the disturbance in the ratios of different cell types.

Table 2.1 shows the advantages and disadvantages of both methods.

*Table 2.1: Advantages and disadvantages of whole blood culture and freshly isolated PBMC methods (Hanekom et al., 2007).*

<b>Assay approaches</b>	<b>Advantages</b>	<b>Disadvantages</b>
Whole blood	-Smaller blood volumes are required, compared with PBMC-based assays.	-Incubation with antigens is on a per-volume basis; T cell depletion may therefore result in loss of sensitivity and may require adjustment of blood volumes.
	-Assessment of a whole immunological compartment is accomplished - The measured T cell response will reflect cellular and soluble components that affect antigen presentation and T cell activation.	-Whole blood may be used undiluted when incubation is $\leq$ 18 hours, but longer term incubation requires dilution with RPMI, and therefore more resources.
	-The stimulation phase of this approach requires relatively few resources; assessment may often be completed in 2 phases: first immediate incubation with antigen, followed by cryopreservation of plasma, fixed white cells or RNA for later analysis, e.g., soluble, cell-associated or mRNA expression analysis of cytokine in the respective samples.	-The measured response reflects not only T cells, but other peripheral blood components also – for example, when cytokines levels are determined in plasma obtained from whole blood, measurements may reflect contributions of neutrophils and monocytes.

			-The need for immediate processing of collected blood limits versatility in what may be measured.
Freshly isolated PBMC		-PBMC-based assays are incubated on a per-cell basis, resulting in increased sensitivity in the face of T cell depletion, such as in HIV infection, compared with whole blood assays.	-PBMC isolation requires more resources, compared with whole blood stimulation.
		-PBMC isolation may allow for a description of T cell responses without the influence of other whole blood components such as neutrophils and plasma.	-PBMC isolation is associated with considerable cellular loss, requiring larger blood volumes.
			-Incubation of PBMC often involves introduction of foreign material, such as foetal calf or unrelated human serum; however, serum-free media may also be used.
			-In short term assays, the cellular activation as a result of PBMC isolation may result in higher backgrounds, compared with similar incubation of whole blood.

#### 2.4.5.3 Evidence Investigator cytokine array.

Supernatant fluid stored in -20°C freezer was removed from the freezer and kept at room temperature to thaw for approximately 1 h before it was assayed for the cytokine concentrations using an Evidence Investigator™ cytokine and growth factors array kit (Randox, County Antrim, UK). Briefly, 200 µl of assay buffer and 100 µl of standard or sample were added to each well of the carrier. Then, for 60 min, it was incubated at 37°C at 370 rpm on the thermoshaker (Randox, UK). This allowed the

cytokines present in the sample to bind to the immobilised antibodies bound to the base of each carriers' well. After that, the liquid from each well was decanted and washed by performing two quick washes and four 2-min soaks, followed by decanting the liquid and tapping the carrier onto lint free tissue paper.

Next, 300  $\mu$ l of conjugate was added to each well and incubated at 37°C at 370 rpm on the thermoshaker for 60 min. The wells were then washed as previously described before 250  $\mu$ l of signal reagent (a mixture of luminol and peroxide) were added to each well followed by 2-min incubation in the dark. Finally, it was analysed by using the Evidence Investigator machine (Randox, UK).

## CHAPTER 3

### **STUDY 1: The effects of stimulant dose on whole blood culture cytokine production.**

#### **Abstract**

One informative measure of immune function is the production of cytokines in response to an antigen challenge. Diseases, infections, psychological stress, and exercise are among those factors that cause immunodepression, and in this situation, cytokine production from activated white blood cells is lower than normal. Generally, cytokines are responsible for activation, proliferation or inhibition of proliferation, apoptosis, differentiation and chemotaxis. The aim of this study was to quantify the amounts of cytokines produced by stimulated whole blood culture (with a multi-antigen Pediacel vaccine) with 7 different doses of vaccine in order to establish the most suitable effective dose for future studies. Six male and 6 female healthy participants (age:  $26 \pm 3$  years; weight:  $66.7 \pm 11.2$  kg; BMI:  $22.7 \pm 2.4$  kg/m<sup>2</sup>) were recruited. Following an overnight fast from 21:00 and with no vigorous physical activity during the preceding 24 h, participants came to the laboratory between 09:00 and 10:00. A resting blood sample was collected and incubated with appropriate volumes of RPMI-1640 medium and vaccine for 20 h (5% CO<sub>2</sub>, 37°C). Supernatants were subsequently removed for analysis of multiple cytokine production (IL-2, IL-4, IL-6, IL-8, IL-10, TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\alpha$ , and IL-1 $\beta$ ) using an Evidence Investigator biochip array. In addition, IL-10 production was analysed by ELISA and the data obtained was then analysed for its correlation with data obtained from Evidence Investigator using Spearman correlation coefficient. Blood was also analysed for haematological parameters (haemoglobin concentration, haematocrit, red blood cell

count, and total and differential leukocyte counts). The main finding was that the most appropriate dose of stimulant was found to be between a 1,000-fold and 4,000-fold dilution of the original Pediacel vaccine. In addition, correlation between data obtained from Evidence Investigator and ELISA for IL-10 was moderately strong ( $r = 0.828$ ,  $n = 38$ ,  $P < 0.001$ ).

### 3.1 Introduction.

The immune response to infection involves activation of innate and adaptive immune response by the action of cytokines. Numerous cytokines are released from activated white blood cells and have stimulatory effects on other effector cells. A cytokine is defined as an inducible protein of molecular weight greater than 5000 Dalton which induces specific receptor-mediated effects on target cells or on the producer cell (Henderson and Nair, 1998). Generally, cytokines can be divided into 6 different families (Table 3.1). Besides, they may also be divided into two main types: pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines (e.g., IFN- $\gamma$ , TNF- $\alpha$ , and IL-2) are produced by Th1 cells while anti-inflammatory cytokines (e.g., IL-4 and IL-10) are produced by Th2 cells. However, some cytokines (e.g., IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) are produced by monocytes and macrophages. It is known that, when pathogen penetrates human body, pro-inflammatory cytokines will attack to kill the pathogen. This may cause an inflammation with signs such as pain, heat, redness, and swelling. This effect will be down-regulate by anti-inflammatory cytokines, where they will limit the sustained or excessive inflammatory reaction (Sultani et al., 2012). Thus, net effect of inflammatory response is determined by balance between pro- and anti-inflammatory cytokines. Nevertheless, it is important to aware that it may be misleading to clear-cut the classification of cytokine as either pro- or anti-inflammatory. This is because the type, duration, and also the extent of cellular activities induced by one particular cytokine can be influenced considerably by the nature of the target cells, the micro-environment of a cell, depending, for example, on the growth and activation state of the cells, the type of neighbouring cells, cytokine concentrations, the presence of other cytokines, and even on the temporal sequence of several cytokines acting on the same cell (Ibelgaufits, 2010).

Cytokines are unique proteins that can have both effector and regulatory activities which influence activation of antimicrobial functions including proliferation or inhibition of proliferation, apoptosis, differentiation, and chemotaxis (Paul and Seder, 1994) of immune cells. Cytokine production is triggered by immune function disturbance that can be induced by factors such as disease, exposure to pathogens, infections, tissue damage, psychological stress, and exercise. Table 3.2 shows the principal sources of the cytokines and their primary functions.

*Table 3.1: Cytokine families (Gleeson, 2006).*

<b>Family</b>	<b>Examples</b>
Interleukins (IL)	IL-1 to IL-7
Cytotoxic cytokines	Tumour necrosis factor (TNF)
Colony-stimulating factors (CSF)	IL-3, granulocyte-CSF
Interferons (IFN)	IFN- $\alpha$ , - $\beta$ , - $\gamma$
Growth factors (GF)	Fibroblast GF, platelet-derived GF
Chemokines	IL-8, Monocyte chemotactic protein-1 (MCP-1)



Table 3.2: Cytokines and their principal sources, and functions (King, 2010).

<b>Cytokines</b>	<b>Principal sources</b>	<b>Functions</b>
IL-1 $\alpha$ , IL-1 $\beta$	Macrophages and other antigen presenting cells (APCs).	Co-stimulation of APCs and T cells, inflammation and fever, acute phase response, and haematopoiesis.
IL-2	Activated Th1 cells and NK cells.	Proliferation of B cells and activated T cells, and NK functions.
IL-4	Th2 and mast cells.	B cell proliferation, eosinophil and mast cell growth and function, IgE and class II MHC expression on B cells, and inhibition of monokine and Th1 cytokine production.
IL-6	Activated Th2 cells, APCs, contracting skeletal muscle, and other somatic cells such as hepatocytes and adipocytes.	Acute phase response, B cell proliferation, thrombopoiesis, synergistic with IL-1 and TNF on T cells, inhibition of IL-1 $\beta$ and TNF- $\alpha$ production, and stimulation of IL-10, IL-1ra, and cortisol secretion.
IL-8	Macrophages, other somatic cells	Chemoattractant for neutrophils and T cells.
IL-10	Activated Th2 cells, CD8 <sup>+</sup> T and B cells, and macrophages.	Inhibits cytokine production, promotes B cell proliferation and antibody production, suppresses cellular immunity, and mast cell growth.
TNF- $\alpha$	Primarily activated macrophages and adipocytes.	Induces expression of other autocrine growth factors, increases cellular responsiveness to growth factors and induces signalling pathways that lead to proliferation.
IFN- $\gamma$	Activated Th1 and NK cells.	Induces class I MHC on all somatic cells, induces class II MHC on APCs and somatic cells, and activates macrophages, neutrophils, and NK cells.

The Evidence Investigator system (Randox, Crumlin, UK) has an ability to measure multiple cytokine production in a single sample in response to an antigen. The Evidence Investigator utilise the basic principles of enzyme linked immunosorbent assay (ELISA) in a sandwich chemiluminescence immunoassay. High level of cytokine in a sample will increase the binding of antibody labelled with horseradish peroxidase (HRP), thus increasing the chemiluminescence signal emitted. The light signal generated is detected using digital imaging technology and compared to that from a stored calibration curve based on the known concentration of a range of 9 standards. The concentration of analyte present in the sample is calculated from the calibration curve.

However, knowledge about the effective dose of the stimulant (antigen) to be used in a whole blood culture assay of antigen-stimulated cytokine production is needed. Choosing an effective dose is important because at a suitable dose (somewhere in the mid-range of the dose response curve) the effects of an intervention (either positive or negative) can be observed. Thus, this study aimed to quantify the amounts of cytokines produced by stimulated whole blood culture over a range of stimulant doses. This may help us to decide the appropriate dose within the effective dose range to be used in any future studies. In addition, we investigated the relationship between data collected from an Evidence Investigator system and a high sensitivity ELISA method for the IL-10 response to the chosen stimulant. The latter was a commercially available multi-antigen, virus-based vaccine called Pediacel (Sanofi Pasteur).

## **3.2 Methods.**

### 3.2.1 Participants.

Six male and 6 female healthy participants were recruited in this study from the students and staff of Loughborough University. Their mean age, weight, and body mass index (BMI) are  $26 \pm 3$  years,  $66.7 \pm 11.2$  kg, and  $22.7 \pm 2.4$  kg/m<sup>2</sup> respectively.

### 3.2.2 Procedures.

Participants were requested to come to the laboratory between 09:00 and 10:00 following an overnight fast from 21:00. However, they were permitted to drink plain water. They were also asked to refrain from any vigorous physical activity in the preceding 24 h. Blood samples were collected into K<sub>3</sub>EDTA and lithium heparin collection tubes as described in the general methods (Chapter 2, section 2.4.3).

### 3.2.3 Blood analysis.

#### *3.2.3.1 Haematological analysis.*

Haematological analyses were carried out as described in the general methods (Chapter 2, section 2.4.4).

#### *3.2.3.2 One hundred times diluted vaccine dose preparation.*

This was prepared as described in the general methods (Chapter 2, section 2.4.5.1).

### 3.2.3.3 Whole blood culture incubation and supernatant storage.

Required volumes of RPMI-1640 medium were added into the culture wells, followed by required volume of 100 x diluted vaccine (as shown in the Table 3.3). Different volumes of 100 x diluted vaccine added into the wells produced different stimulant doses which were labelled as dose 1 to 7. After that, 0.25 ml of blood sample was added and incubated for 20 h as described in the general methods (Chapter 2, section 2.4.5.2).

*Table 3.3: Volume of 100 x diluted vaccine and RPMI added to each well for each blood sample. To these volumes, 0.25 ml heparinised blood was added to give the respective dilution factor.*

<b>Well (Dose)</b>	<b>100 x diluted vaccine (µl)</b>	<b>RPMI-1640 medium (ml)</b>	<b>Dilution factor</b>
1	0	0.75	No vaccine
2	5	0.745	20, 000
3	10	0.74	10, 000
4	25	0.725	4, 000
5	50	0.7	2, 000
6	100	0.65	1, 000
7	500	0.25	200

### 3.2.3.4 Evidence Investigator cytokine array.

This was carried out as described in general methods (Chapter 2, section 2.4.5.3).

### 3.2.3.5 Enzyme Linked-Immuno-Sorbent Assay (ELISA) for IL-10.

This assay was conducted in duplicate using a human high sensitivity IL-10 ELISA kit (Diaclone, France). Firstly, 100 µl of standard diluent was added to the

standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1 and F2. Then, 200 µl standard (25 pg/ml) was pipetted into wells A1 and A2. Serial dilutions were made by transferring 100 µl from A1 and A2 to B1 and B2. This procedure was repeated for the subsequent wells to give a range of standard from 50.00 to 1.56 pg/ml. One hundred µl from the content of the last wells used (F1 and F2) was discarded. As a blank, wells G1 and G2 contained 100 µl standard diluent only.

After that, 100 µl sample (supernatant from blood culture) was added to the sample wells followed by 50 µl of diluted biotinylated anti-IL-10. Then the plate was covered with the plate cover and incubated for 2 h at room temperature (18 - 22°C) with slow shaking on the plate shaker (Denley, UK). After 2 h incubation, the plate was placed in the automated microplate washer (Anthos Labtec, France) which was set to the appropriate washing procedure, where liquid from each well was aspirated followed by dispensing of 0.3 ml of washing solution into each well, and then was aspirated again. This step was repeated two times.

Then, 100 µl of streptavidin-HRP solution 1 (prepared just before use) was added to all wells. Again, the plate was covered and incubated for 20 min at room temperature with slow shaking on the shaker followed by the washing procedure as described previously. Subsequently, 100 µl of amplifier dilution (prepared just before use) was added into the all wells and incubated for 15 min with slow shaking. After incubation, the plate was washed. Next, 100 µl of streptavidin-HRP solution 2 (prepared just before use) was distributed into the all wells. Again, the plate was incubated for 20 min on the shaker and washed. After washing, 100 µl of ready-to-use TMB substrate solution was added to the all wells. Afterwards, it was incubated in the dark for 5 min at room temperature. Finally, 100 µl of stop reagent (H<sub>2</sub>SO<sub>4</sub>) was

added to the all wells to stop the enzyme-substrate reaction. Immediately after that, the plate was read using an OpsysMR ELISA plate reader (Dynex, UK) at 450 nm wavelength.

#### 3.2.4 Data analysis.

Data were analysed using the Statistical Package for Social Sciences (SPSS) version 17 (SPSS Inc., USA). Descriptive analysis was used to measure mean and standard deviation (SD). Statistical significance of differences between means was analysed using paired samples t-test and analysis for correlation between the IL-10 concentrations determined by the two analytical methods was conducted using Spearman's correlation coefficient. All the data were expressed as mean  $\pm$  SD.

### **3.3 Results.**

Mean and SD for haematological parameters are presented in Table 3.4. All values fell within the normal range except for haematocrit value which was slightly lower. Overall, participants were considered healthy with no haematological problems. Figures 3.1 – 3.9 show the response of the measured cytokines to the different doses of stimulant (vaccine). Theoretically, the dose response curve should be S-shaped. This is because low dosage gives no or an insignificant response and at the upper end of the dose response curve higher dosage gives no further response (or even a reduced response), which may be attributed to the toxicity of the stimulant to blood leukocytes. As shown in the figures that follow, different cytokines give different range of dose active response. From these graphs, effective dose range can be determined and used in the future studies. The most suitable dose (dilution of vaccine) was defined as the dose that induced at least a 3-fold increase above the

unstimulated (dose 1) cytokine production but less than 50% of the maximal response. For the cytokines IL-2, IL-6, IL-8, IL-10, IFN- $\gamma$ , TNF- $\alpha$  and IL-1 $\beta$ , this was achieved with dose between 4 and 6, corresponding to a 1,000 – 4,000 fold dilution of the original Pediacel vaccine. However, IL-4 and IL-1 $\alpha$  production were only elevated substantially above basal level by dose 7 (200-fold dilution of vaccine).

*Table 3.4: Mean and SD values of haematological parameters.*

<b>Parameters</b>		<b>Observed values</b>	<b>Normal range</b>
Red blood cells ( $10^{12}/L$ )	Male	$4.6 \pm 0.9$	4.3 – 6.2
	Female	$3.9 \pm 0.9$	3.8 – 5.5
Haemoglobin (g/dL)	Male	$14.7 \pm 1.2$	13.2 – 16.2
	Female	$13.7 \pm 1.0$	12.0 – 15.2
Haematocrit (%)	Male	$38.4 \pm 7.6$	40 – 52
	Female	$33.0 \pm 6.5$	37 – 46
White blood cells ( $10^9/L$ )		$5.7 \pm 1.4$	4 – 11
Neutrophils ( $10^9/L$ )		$2.8 \pm 0.8$	2.0 – 8.0
Lymphocytes ( $10^9/L$ )		$2.1 \pm 0.5$	0.7 – 4.4
Monocytes ( $10^9/L$ )		$0.5 \pm 0.2$	0.1 – 0.8
Eosinophils ( $10^9/L$ )		$0.2 \pm 0.1$	0.1 – 0.4
Basophils ( $10^9/L$ )		$0.1 \pm 0.0$	0.0 – 0.1

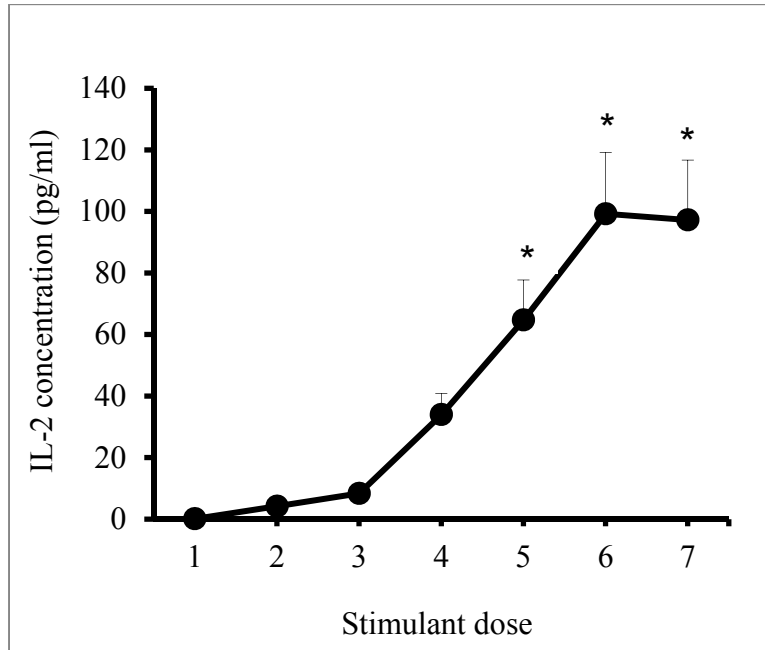


Figure 3.1: IL-2 responses to different stimulant dose.  
 \* Significantly different from dose 1 ( $P < 0.05$ ).

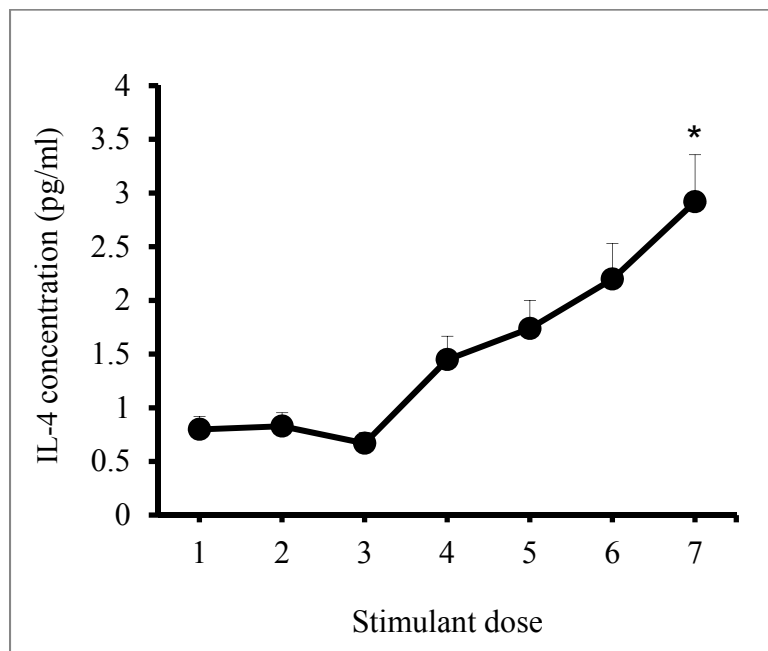


Figure 3.2: IL-4 responses to different stimulant dose.  
 \* Significantly different from dose 1 ( $P < 0.05$ ).



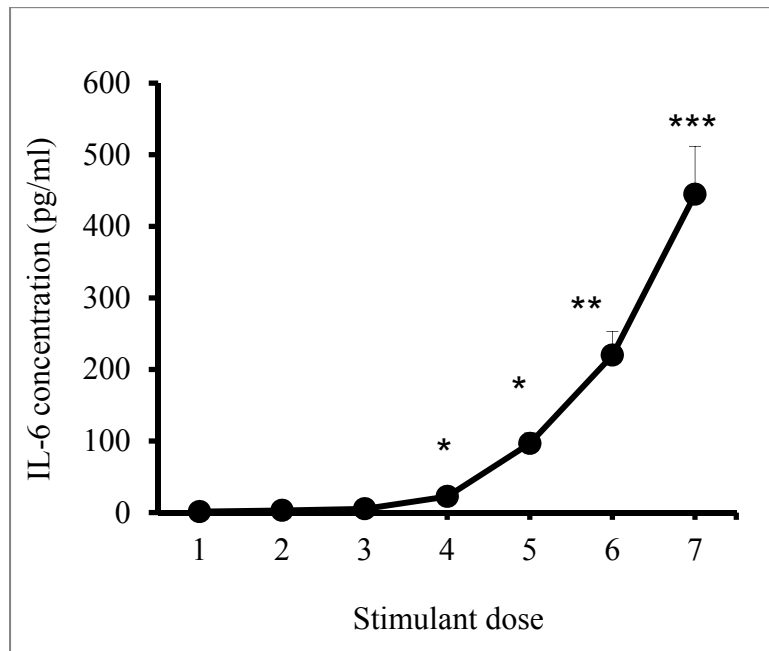


Figure 3.3: IL-6 responses to different stimulant dose.  
 \*, \*\*, \*\*\* Significantly different from dose 1 ( $P < 0.05$ ,  $P < 0.001$ , and  $P < 0.000$  respectively).

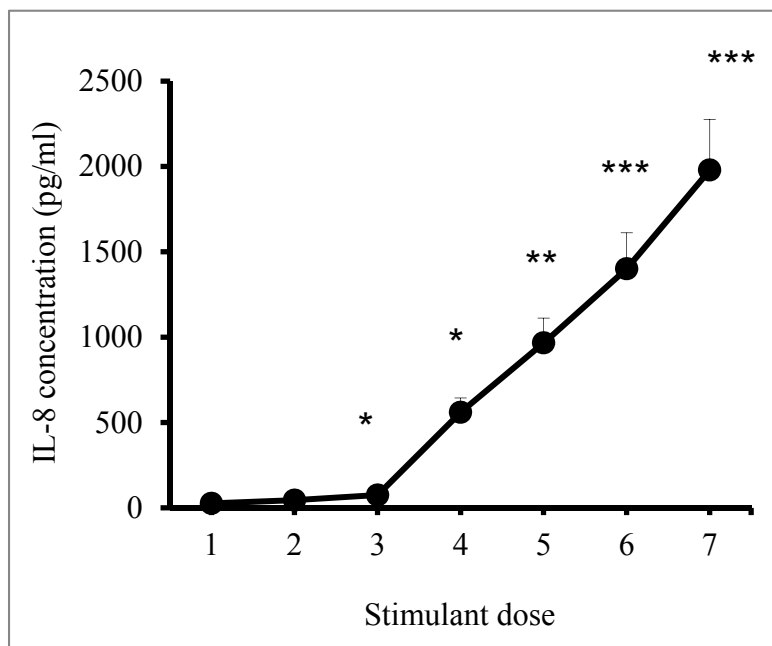


Figure 3.4: IL-8 responses to different stimulant dose.  
 \*, \*\*, \*\*\* Significantly different from dose 1 ( $P < 0.05$ ,  $P < 0.001$ , and  $P < 0.000$  respectively).

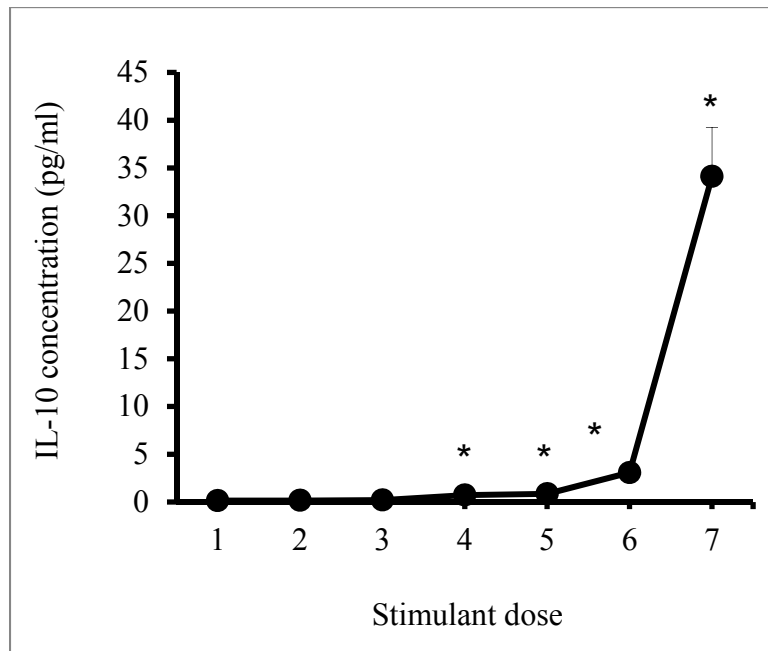


Figure 3.5: IL-10 responses to different stimulant dose.  
 \* Significantly different from dose 1 ( $P < 0.05$ ).

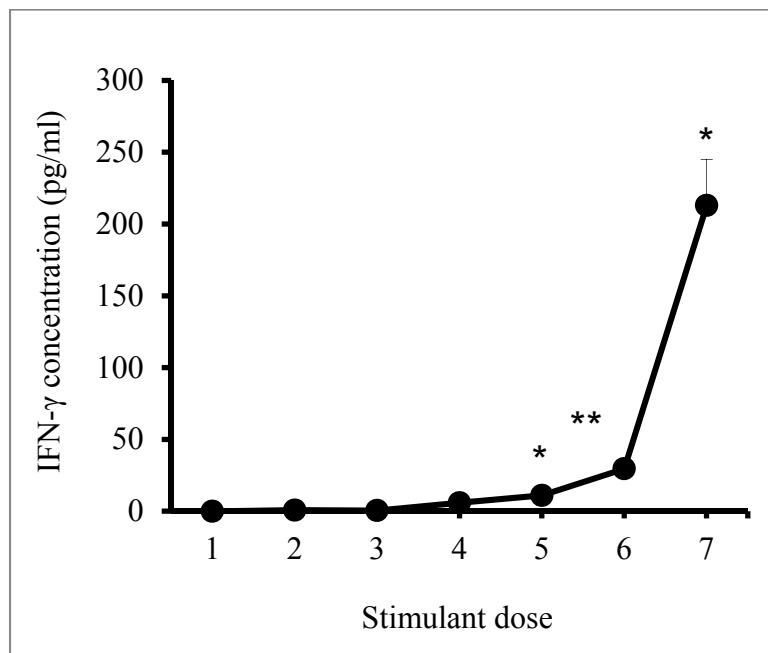


Figure 3.6: Interferon-gamma (IFN- $\gamma$ ) responses to different stimulant dose.  
 \*, \*\* Significantly different from dose 1 ( $P < 0.05$  and  $P < 0.001$ ).

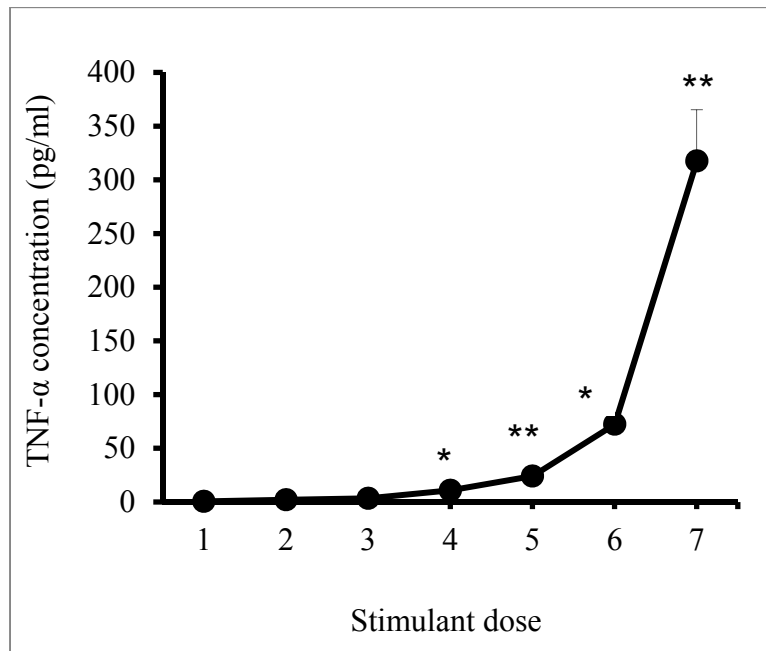


Figure 3.7: Tumor Necrosis Factor-alpha (TNF- $\alpha$ ) responses to different stimulant dose.

\*, \*\* Significantly different from dose 1 ( $P < 0.05$  and  $P < 0.001$ ).

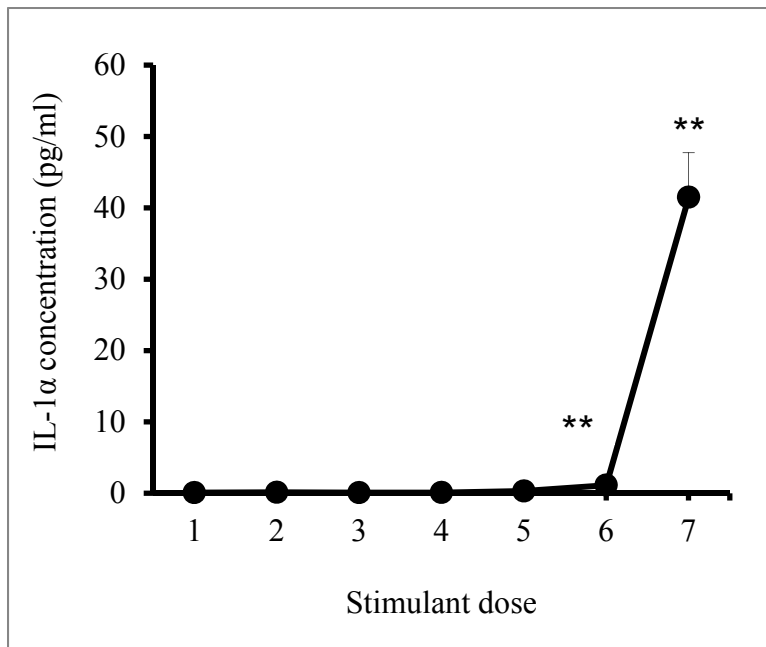


Figure 3.8: IL-1 $\alpha$  responses to different stimulant dose.

\*\* Significantly different from dose 1 ( $P < 0.001$ ).

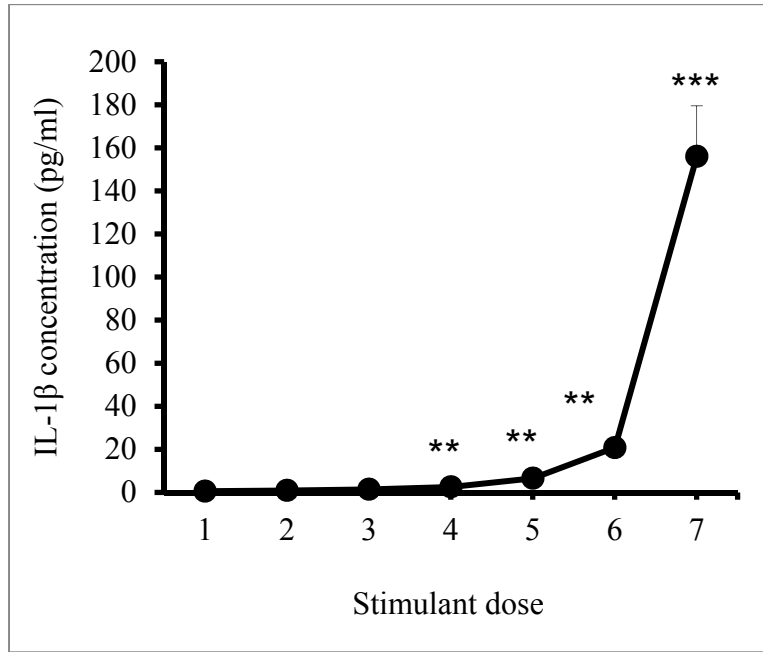


Figure 3.9: IL-1 $\beta$  responses to different stimulant dose.  
 \*\*, \*\*\* Significantly different from dose 1 ( $P < 0.001$  and  $P < 0.000$ ).

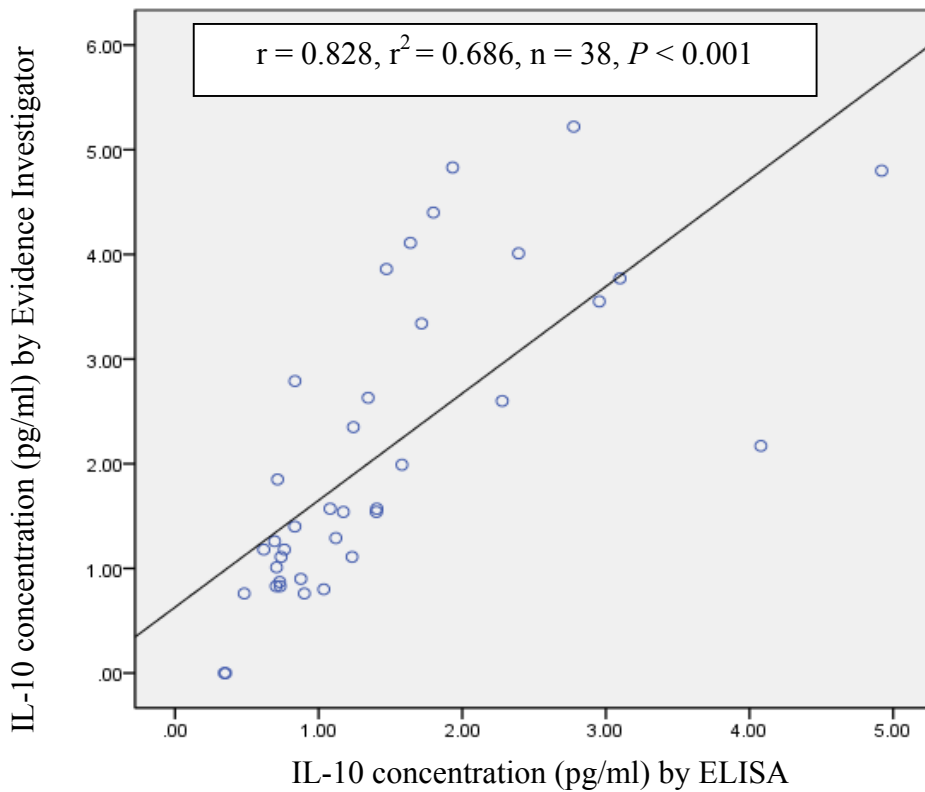


Figure 3.10: Correlation graph of the data obtained from Evidence Investigator and ELISA for IL-10.

Figure 3.10 shows a correlation graph of the data obtained from Evidence Investigator and ELISA for IL-10. Since the shape of the cluster starts off narrow and then get wider, it violated the assumption of homoscedasticity. Thus, Spearman's correlation coefficient was used instead of Pearson correlation coefficients to determine the relationship between data obtained from Evidence Investigator and ELISA for IL-10. There was a moderately strong, positive correlation between the two variables;  $r = 0.828$ ,  $n = 38$ ,  $P < 0.001$ .

### **3.4 Discussion and conclusion.**

From the results obtained, it appears that the effective dose range for most of the measured cytokines is between dose 4 and 6 (corresponding to a 1,000-4,000 fold range of dilution of the original Pediacel vaccine), hence providing a reference for the future studies. The correlation between data obtained from Evidence Investigator and ELISA was moderately strong. Thus, using Evidence Investigator for analysis is an acceptable alternative to ELISA. Multiple cytokine production after multiple antigen challenge in a single sample is a new technology which significantly helps scientists with their investigation. Previous methods using ELISA to give single cytokine production measurements at a time consumes a lot more time and cost. Moreover, multiple antigen challenge provides valuable information of the cytokines production since not all cytokines respond to the same antigen. The capacity of leukocytes to produce cytokines upon adequate challenge has potentially far reaching consequences for the entire functional capacity of the immune system. It is highly likely to reflect the capacity of an individual to defend itself against intruding microorganisms and hence is a suitable measure to examine the impact of nutritional interventions designed to boost immune function.

## CHAPTER 4

### **STUDY 2: The *in vitro* effects of various immunomodulatory nutritional compounds on antigen-stimulated whole blood culture cytokine production.**

#### **Abstract**

This study investigated the effects of *in vitro* exposure to low and high dose of several immunomodulators which include alcohol (ethanol), caffeine, curcumin, echinacea, kaloba, and quercetin on antigen-stimulated whole blood culture cytokine production. Whole blood samples were taken from 5 healthy male (age:  $32 \pm 12$  years; weight:  $75.7 \pm 6.1$  kg; BMI:  $24.3 \pm 1.5$  kg/m<sup>2</sup>) following an overnight fast with no vigorous activity during the preceding 24 h. The whole blood was then stimulated with vaccine dose 5 (based on previous study – Chapter 3) and low or high dose of immunomodulator in the culture plate. After 20 h incubation (5% CO<sub>2</sub>, 37°C), it was analysed using Evidence Investigator to determine the production of cytokines including IL-2, IL-4, IL-6, IL-8, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\alpha$ , and IL-1 $\beta$ . Caffeine and quercetin showed a tendency towards decrease cytokine production as the doses were increased. On the other hand, an upward trend was evident with kaloba, where a high dose of kaloba seemed to increase the cytokine production. Nevertheless, the effects of alcohol, curcumin, and echinacea on cytokine production were inconclusive. Interleukin-6, IL-8, TNF- $\alpha$ , and IL-1 $\beta$  production were relatively similar for all the cultures and it was speculated that the vaccine dose used was beyond their effective dose range, thus they were exhibiting a maximal response.

#### 4.1 Introduction.

Immunomodulation is a therapeutic approach in which we try to intervene in auto regulating processes of the host defence system. Immunomodulators can be defined as substances (drugs and nutrients) that alter the activities of the immune system via dynamic regulation of messenger molecules such as cytokines, adhesion molecules, nitric oxide, hormones, neurotransmitters, and other peptides. Immunomodulators can help to increase immunity of people with a low immune system, and also can help to normalize an overactive immune system. Generally, these compounds can be divided into immunosuppressants and immunostimulants (Haavisto, 2009). Numerous *in vitro* and *in vivo* studies have found that immunomodulators modulate the secretion of multiple cytokines (Spelman et al., 2006).

Altering cytokine expression with immunomodulators may offer some therapeutic potential. As reviewed by Del-Rio-Navarro and colleagues (2006), on average, immunostimulants reduce the incidence of acute respiratory tract infections in children by 40%. Several published studies have suggested that alcohol (Nelson and Kolls, 2002; Szabo, 199), caffeine (Horrigan et al., 2004 and 2006), curcumin (Bharti et al., 2010; Shirley et al., 2008) and quercetin (Chen, 2010; Chuang et al., 2010) as immunosuppressants while echinacea (Fusco et al., 2010; Bodinet et al., 2002) and kaloba (Kolodziej and Kiderlen, 2007; Trun et al., 2006) as immunostimulants. However, current evidence concerning their efficacy is limited. Hence, this present study intended to investigate the effects of alcohol, caffeine, curcumin, quercetin, echinacea and kaloba on antigen-stimulated whole blood culture cytokine production *in vitro*.

## **4.2 Methods.**

### 4.2.1 Participants.

Five healthy men were recruited as participants in this study. Mean age, weight, and BMI of the participants were  $32 \pm 12$  years,  $75.7 \pm 6.1$  kg, and  $24.3 \pm 1.5$  kg/m<sup>2</sup> respectively.

### 4.2.2 Procedures.

Participants were requested to come to the laboratory between 09:00 and 10:00 following an overnight fast from 21:00. However, they were permitted to drink plain water. They were also asked to refrain from any vigorous physical activity in the preceding 24 h. Blood samples were collected into K<sub>3</sub>EDTA and lithium heparin collection tubes as described in the general methods (Chapter 2, section 2.4.3).

### 4.2.3 Blood analysis.

#### *4.2.3.1 Haematological analysis.*

Haematological analyses were carried out as described in general methods (Chapter 2, section 2.4.4).

#### *4.2.3.2 One hundred times diluted vaccine dose preparation.*

This was prepared as described in general methods (Chapter 2, section 2.4.5.1).



#### *4.2.3.3 Immunomodulatory nutritional compounds preparation.*

##### Alcohol (Ethanol)

Alcohol low blood dose was targeted at 100 mg/dL. This concentration was 20% higher than the current UK legal limit blood alcohol concentration for drivers.

$$100 \text{ mg/dL} = 1 \text{ g/L}$$

$$\text{Alcohol solution needed} = 100 \times 1 \text{ g/L} = 100 \text{ g/L} = 1 \text{ g/10 ml}$$

As alcohol density is 0.79 g/ml, 1 g alcohol = 1.27 ml

Thus, 1.27 ml alcohol was added to 8.73 ml RPMI solution.

In the whole blood culture, low dose (1 g/L) of alcohol was achieved by adding 10  $\mu$ l of this solution into the culture wells while for high dose (10 g/L), 100  $\mu$ l was added.

##### Caffeine

Caffeine low blood dose was targeted at 9.71 mg/L (Fletcher and Bishop, 2011).

$$\text{Caffeine solution needed} = 100 \times 9.71 \text{ mg/L} = 971 \text{ mg/L} = 97.1 \text{ mg/100 ml}$$

Thus, 97.1 mg caffeine was added to 100 ml RPMI solution.

In the whole blood culture, low dose (9.71 mg/L) of caffeine was achieved by adding 10  $\mu$ l of this solution into the culture wells while for high dose (97.1 mg/L), 100  $\mu$ l was added.

## Curcumin

Curcumin low blood dose was targeted at 663 µg/L (Cheng et al., 2001).

Curcumin solution needed =  $100 \times 663 \text{ µg/L} = 66.3 \text{ mg/L}$

As curcumin has low solubility in water, the solution was made up in alcohol.

$66.3 \text{ mg} / 100 \text{ ml alcohol} = 663 \text{ mg/L}$

Then, this solution was 10 times diluted with RPMI to make curcumin solution at 66.3 mg/L.

In the whole blood culture, low dose (663 µg/L) of curcumin was achieved by adding 10 µl of this solution into the culture wells while for high dose (6630 µg/L), 100 µl was added.

## Quercetin

Quercetin low blood dose was targeted at 0.02 mg/ml (Nieman et al., 2007a).

Quercetin solution needed =  $100 \times 0.02 \text{ mg/ml} = 2 \text{ mg/ml}$

As quercetin solution has low solubility in water, the solution was made up in alcohol.

Then, this solution was 10 times diluted with RPMI to make quercetin solution at 200 mg/L.

In the whole blood culture, low dose (0.02 mg/ml) of quercetin was achieved by adding 10 µl of this solution into the culture wells while for high dose (0.2 mg/ml), 100 µl was added.

## Echinacea

Echinacea low blood dose was targeted at 10 mg/L. This dose was aimed based on the prescription of the supplement, as suggested by the manufacturer.

Echinacea solution needed =  $100 \times 10 \text{ mg/L} = 1000 \text{ mg/L} = 100 \text{ mg}/100 \text{ ml}$  RPMI solution.

In the whole blood culture, low dose (10 mg/L) of Echinacea was achieved by adding 10  $\mu\text{l}$  of this solution into the culture wells while for high dose (100 mg/L), 100  $\mu\text{l}$  was added.

## Kaloba

Kaloba low blood dose was targeted at 3  $\mu\text{g}/\text{ml}$  (Conrad et al., 2007).

Kaloba solution needed =  $100 \times 3 \text{ } \mu\text{g}/\text{ml} = 300 \text{ } \mu\text{g}/\text{ml}$

Thus, 20 mg Kaloba was added to 66.7 ml RPMI solution.

In the whole blood culture, low dose (3  $\mu\text{g}/\text{ml}$ ) of Kaloba was achieved by adding 10  $\mu\text{l}$  of this solution into the culture wells while for high dose (30  $\mu\text{g}/\text{ml}$ ), 100  $\mu\text{l}$  was added.

#### 4.2.3.4 Whole blood culture incubation and supernatant storage.

As shown in the Table 4.1, required volumes of RPMI-1640 medium were added into the culture wells, followed by 50  $\mu$ l of 100 x diluted vaccine (dose 5 – 2,000 time dilution of the original Pediacel vaccine). Next, the appropriate amount of immunomodulator solution was added, followed by 0.25 ml of blood. After gently mixed, they were incubated for 20 h as described in the general methods (Chapter 2, section 2.4.5.2).

*Table 4.1: Volume of RPMI, 100 x diluted vaccine, and immunostimulant solution added to each well for each blood sample. To these volumes, 0.25 ml heparinised blood was added.*

<b>Well</b>	<b>RPMI-1640 medium (<math>\mu</math>l)</b>	<b>Immunomodulators solution (<math>\mu</math>l)</b>	<b>Vaccine (50 <math>\mu</math>l)</b>
1	750	NO	NO
2	725	NO	YES
3	715	Alcohol (L) – 10 $\mu$ l	YES
4	625	Alcohol (H) – 100 $\mu$ l	YES
5	715	Caffeine (L) – 10 $\mu$ l	YES
6	625	Caffeine (H) – 100 $\mu$ l	YES
7	715	Curcumin (L) – 10 $\mu$ l	YES
8	625	Curcumin (H) – 100 $\mu$ l	YES
9	715	Echinacea (L) – 10 $\mu$ l	YES
10	625	Echinacea (H) – 100 $\mu$ l	YES
11	715	Kaloba (L) – 10 $\mu$ l	YES
12	625	Kaloba (H) – 100 $\mu$ l	YES
13	715	Quercetin (L) – 10 $\mu$ l	YES
14	625	Quercetin (H) – 100 $\mu$ l	YES
15	725	NO	YES

L = Low dose

H = High dose

#### 4.2.3.5 Evidence Investigator cytokine array.

This was carried out as described in the general methods (Chapter 2, section 2.4.5.3).

#### 4.2.4 Data analysis.

Data were analysed using the Statistical Package for Social Sciences (SPSS) version 17 (SPSS Inc., USA). Descriptive analysis was used to measure mean and standard deviation (SD). Statistical significance of differences between means was analysed using paired samples t-test. All the data were expressed as mean  $\pm$  SD.

### **4.3 Results.**

Table 4.2 presents the values of haematological parameters measured. The entire observed values fell within the normal range. This indicates that the participants recruited for the study were healthy and have no obvious haematological problem. Table 4.3 shows the concentrations of the cytokines produced in the whole blood culture after 20 h incubation with the vaccine dose 5 and immunomodulators at high or low dose. Control refers to the whole blood cultured with only vaccine without any added immunomodulators.

*Table 4.2: Mean and SD values of haematological parameters.*

<b>Parameters</b>	<b>Observed values</b>	<b>Normal range</b>
Red blood cells ( $10^{12}/L$ )	$4.9 \pm 0.4$	4.3 – 6.2
Haemoglobin (g/dL)	$14.7 \pm 1.4$	13.2 – 16.2
Haematocrit (%)	$47.3 \pm 4.1$	40.0 – 52.0
White blood cells ( $10^9/L$ )	$5.1 \pm 0.6$	4.0 – 11.0
Neutrophils ( $10^9/L$ )	$2.3 \pm 0.5$	2.0 – 8.0
Lymphocytes ( $10^9/L$ )	$1.9 \pm 0.2$	0.7 – 4.4
Monocytes ( $10^9/L$ )	$0.5 \pm 0.2$	0.1 – 0.8
Eosinophils ( $10^9/L$ )	$0.2 \pm 0.9$	0.1 – 0.4
Basophils ( $10^9/L$ )	$0.0 \pm 0.0$	0.0 – 0.1

Table 4.3: Antigen-stimulated (vaccine dose 5) whole blood culture cytokine production (pg/ml) in response to alcohol, caffeine, curcumin, quercetin, echinacea and kaloba at low and high doses.

	Dose	IL-2	IL-4	IL-6	IL-8	IL-10
CONTROL		2.3±1.3	5.7±2.3	830±35	2460±0	27.5±11.5
ALCOHOL	L (1 g/L)	2.2±1.1	4.1±1.9	850±0	2460±0	47.5±31.0
	H (10 g/L)	3.9±3.6	2.7±1.7	850±0	2460±0	70.6±74.7
CAFFEINE	L (9.71 mg/L)	2.5±1.3	5.6±3.5	850±0	2460±0	48.2±35.5
	H (97.1 mg/L)	1.5±0.6	3.9±1.3	850±0	2460±0	47.4±29.3
CURCUMIN	L (663 µg/L)	2.4±1.2	5.0±2.2	850±0	2460±0	27.4±9.9
	H (6630 µg/L)	6.2±5.5	3.0±0.8	850±0	2460±0	19.5±5.2
QUERCETIN	L (20 µg/ml)	3.2±2.2	3.1±0.2	850±0	2460±0	25.9±22.2
	H (200 µg/ml)	2.5±1.9	2.6±0.8	850±0	2460±0	44.7±54.2
ECHINACEA	L (10 mg/L)	2.9±1.1	4.6±2.0	850±0	2460±0	35.2±23.0
	H (100 mg/L)	3.0±1.3	4.8±2.0	850±0	2460±0	33.1±21.0
KALOBA	L (3 µg/ml)	3.6±3.1	3.7±2.3	850±0	2460±0	25.5±11.7
	H (30 µg/ml)	2.4±1.8	4.7±2.9	850±0	2460±0	51.9±39.9

Table 4.3 (continued).

	Dose	IFN- $\gamma$	TNF- $\alpha$	IL-1 $\alpha$	IL-1 $\beta$
CONTROL		792 $\pm$ 600	885 $\pm$ 0	175 $\pm$ 105	650 $\pm$ 0
ALCOHOL	L (1 g/L)	636 $\pm$ 525	885 $\pm$ 0	329 $\pm$ 188	650 $\pm$ 0
	H (10 g/L)	346 $\pm$ 257	885 $\pm$ 0	392 $\pm$ 177**	650 $\pm$ 0
CAFFEINE	L (9.71 mg/L)	579 $\pm$ 553	885 $\pm$ 0	293 $\pm$ 172*	650 $\pm$ 0
	H (97.1 mg/L)	84 $\pm$ 96	885 $\pm$ 0	234 $\pm$ 184	650 $\pm$ 0
CURCUMIN	L (663 $\mu$ g/L)	800 $\pm$ 562	885 $\pm$ 0	236 $\pm$ 115	650 $\pm$ 0
	H (6630 $\mu$ g/L)	329 $\pm$ 217	885 $\pm$ 0	332 $\pm$ 215	650 $\pm$ 0
QUERCETIN	L (20 $\mu$ g/ml)	196 $\pm$ 107	844 $\pm$ 83	193 $\pm$ 208	650 $\pm$ 0
	H (200 $\mu$ g/ml)	52 $\pm$ 48	885 $\pm$ 0	177 $\pm$ 219	650 $\pm$ 0
ECHINACEA	L (10 mg/L)	741 $\pm$ 531	885 $\pm$ 0	340 $\pm$ 173*	650 $\pm$ 0
	H (100 mg/L)	741 $\pm$ 527	885 $\pm$ 0	260 $\pm$ 171**	650 $\pm$ 0
KALOBA	L (3 $\mu$ g/ml)	768 $\pm$ 558	885 $\pm$ 0	237 $\pm$ 167	650 $\pm$ 0
	H (30 $\mu$ g/ml)	860 $\pm$ 532	885 $\pm$ 0	299 $\pm$ 190**	650 $\pm$ 0

\*, \*\* significantly different from the control (antigen stimulated whole blood culture without immunomodulators) value ( $P < 0.05$  and  $P < 0.001$ , respectively).



#### 4.4 Discussion.

Conspicuously, a somewhat similar concentration of cytokine was produced throughout the culture plate for IL-6, IL-8, TNF- $\alpha$ , and IL-1 $\beta$ , suggesting that they were having their maximal response; and the response was above the upper range of the assay used. The best explanation for this may be because the dose of the vaccine used in this study is beyond their effective dose range. It is known that cytokines response to certain vaccine dose is not similar. In this case, somehow vaccine dose 5 is beyond their effective dose range but not for other cytokines (IL-2, IL-4, IL-10, IFN- $\gamma$  and IL-1 $\alpha$ ). Such dose of vaccine was chosen based on the previous pilot study (Chapter 3). Hence, in this study, we are unable to see the effects of the immunomodulators on IL-6, IL-8, TNF- $\alpha$ , and IL-1 $\beta$  production *in vitro*.

In general, we found that the productions of most of the measured cytokines were not significantly affected by the immunomodulators that we examined. However, caffeine and quercetin showed a tendency towards decreased cytokine production as the doses were increased. On the other hand, an upward trend was evident with Kaloba, where a high dose of Kaloba seemed to increase the cytokine production. Nevertheless, in this study, the direction of the effects of alcohol, curcumin, and echinacea on cytokine production were inconclusive.

It has been pointed out that chronic alcohol consumption alters the cellular (Watson, 1988) and humoral immune function (Cook, 1998). This present study, however, failed to confirm previous findings, where we found no significant effects of alcohol (ethanol) on whole blood culture except for IL-1 $\alpha$  (with high dose) ( $P < 0.001$ ) (Table 4.3). There was also no apparent tendency for any effects of alcohol on the production of other cytokines. Hence, it is speculated that the alcohol concentration used in this study was not enough to induce immune depression in the whole blood culture. Alcohol impaired immune function in several ways which include impair the chemotaxis; the ability of neutrophils to migrate to

sites of injury and infection (Bautista 2001) and alter the production of macrophages and cytokines (Szabo 1999). Alcohol also cause rise of circulating pro-inflammatory cytokine, shift the balance of Th1/Th2, increase oxidative stress, and inhibit antigen presenting capacity of monocytes (Szabo, 1997).

There were no significant differences (compared with control) in cytokine production in response to caffeine and quercetin with low or high dose (Table 4.3). However, the results showed a tendency towards reduced cytokine production with high doses of both caffeine and quercetin. Previous studies have found that caffeine and quercetin act as immunosuppressants (Horrigan et al., 2004 and 2005; Chen et al., 2010; Chuang et al., 2010). Thus, this finding is not in contradiction to those previous findings.

Mechanism of action of caffeine was debated. However, it is well established that caffeine inhibits cyclic adenosine monophosphate (cAMP)-phosphodiesterase. But, this only occurs with high concentration of caffeine, higher than average human consumption (Fredholm et al., 1999). Yet, growing evidence suggests that mechanism of action of caffeine is mediated by antagonism of adenosine receptors (Fredholm et al., 1999; Mandel, 2002). Generally, activated adenosine receptors will lead to suppress of pro-inflammatory cytokine (especially TNF- $\alpha$ ) (Hasko et al., 2000; Link et al., 2000), thus if caffeine act to antagonise adenosine receptor (by blocking the action of endogenous adenosine), this will result in increased TNF- $\alpha$  production. However, one study found that caffeine decreased TNF- $\alpha$  production (Horrigan et al., 2004), thus they hypothesised that the suppressive effect of caffeine on TNF- $\alpha$  production observed in their study may be due to the inhibition of cAMP-phosphodiesterase, a resultant increase in intracellular cAMP concentrations, and activation of protein kinase A.

Potential mechanism of action for anti-inflammatory action of quercetin (blocked TNF- $\alpha$  mediated inflammation or insulin resistance in adipocytes) are proposed by interfering TNF- $\alpha$  receptor (TNFR) binding, suppressing TNF- $\alpha$ -TNFR signalling, and altering the activity of proteins involved in inflammation or glucose and lipid metabolism (Chuang et al., 2010). Quercetin also was proposed to exert its effects in inflammation prevention and insulin resistant is via inhibition of TNF- $\alpha$  mediated reactive oxygen species (ROS - increases inflammatory gene expression) production (Goossens et al., 1999).

There were also no significant effects of curcumin and echinacea on cytokine productions (Table 4.3). These findings make it inconclusive as to whether they are immunostimulant or immunosuppressant. Again, this may be because the dose used was not enough to induce a significant effect on the cytokine production. However, previous studies have suggested that curcumin is an immunosuppressant (Shirley et al., 2008; Bharti et al., 2010) and echinacea is an immunostimulant (Bodinet et al., 2002; Fusco et al., 2010). It was proposed that, though it is yet unclear, curcumin's anticancer potential is due to its ability to suppress the proliferation of cancer cells (Matchanickal and Rafi, 2006). Curcumin was found to down-regulate the activity of two major transcription factors (nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and activator protein 1 (AP-1)), scavenge reactive oxygen species (ROS), suppress mitogen-activated protein kinases generated by inflammatory stimuli, suppress the expression of pro-inflammatory enzymes (cyclooxygenases and lipoxygenases), induce apoptosis in the cancer cells, and induce Phase II detoxification enzymes (Matchanickal and Rafi, 2006).

The exact mechanism(s) of how echinacea exert its effects is unclear. However it has been shown that echinacea extracts modulate macrophage cytokine and reactive oxygen species (ROS) production (Rininger et al., 2000; Bodinet et al., 2002; Goel et al., 2002;

Morazzoni *et al.*, 2005; Zhai *et al.*, 2007). Furthermore, echinacea's antimicrobial activities is exert either through increasing macrophage intracellular defense (Steinmuler *et al.*, 1993), or increasing macrophage antimicrobial behaviours in two models of bacterial infection (Roesler *et al.*, 1991; Steinmuller *et al.*, 1993). Studies also found that echinacea supplemented mice showed an enhanced phagocytosis in macrophages (Goel *et al.*, 2002; Pedalino *et al.*, 2004).

We found no significant differences in cytokine production in response to Kaloba in this study. Nevertheless, there was an evident upward trend of Kaloba on cytokine production (Table 4.3) where a high dose of Kaloba seemed to increase the cytokine production. This is in agreement with previous findings which suggested Kaloba as an immunostimulant (Trun *et al.*, 2006; Kolodziej and Kiderlen, 2007).

Kaloba was reported to have multifactorial mode of action; antiviral and cytoprotective properties, antibacterial properties, secretomotory properties and inhibition of 'sickness behaviour'. Several *in vitro* studies found that Kaloba triggers stimulation of IFN synthesis (Trun *et al.*, 2006; Kayser *et al.*, 2007) and improve phagocyte function (Conrad *et al.*, 2007). It also increased the ciliary beat frequency, thus increasing the transportation of mucous and pathogen from the respiratory tracts. This in turn reduced pathogen count, removed the nutrient medium for their subsequent proliferation and substantially improved respiration (Neugebauer *et al.*, 2005).

#### **4.5 Conclusion.**

There were no significant differences in the production of IL-2, IL-4, IL-10, IFN- $\gamma$ , and IL-1 $\alpha$  in antigen-stimulated whole blood culture in the presence of the selected immunomodulators. Nevertheless, we found that caffeine and quercetin have potential as

immunosuppressants and Kaloba as immunostimulant. The production of IL-6, IL-8, TNF- $\alpha$ , and IL-1 $\beta$  were stable throughout the culture plate, indicating that the vaccine dose (dose 5) used in this study is beyond their effective range dose, where they were having their maximal response. Thus, further study is warranted with a lower vaccine dose to examine any beneficial effects of the immunomodulators on the production of these cytokines.

## CHAPTER 5

### **Study 3: The effects of Kaloba supplementation on the immune response to prolonged exercise.**

#### **Abstract**

The purpose of this study was to examine the effects of Kaloba supplementation on immune response to prolonged exercise in 9 healthy male recreational athletes (age:  $21 \pm 5$  years; weight:  $73.7 \pm 5.6$  kg; BMI:  $22.4 \pm 1.5$  kg/m<sup>2</sup>). In this study, participants cycled for 90 min at 60%  $\dot{V}O_2$ max on two occasions (two trials separated at least by 7 days). Participants came to the laboratory in the morning after an overnight fast (plain water was permitted) during each trial. The second trial was performed after 7 days Kaloba (Schwabe GmbH, Germany) supplementation. Kaloba was taken as 1 tablet 3 times daily (morning, midday, and evening). Blood and unstimulated saliva samples were collected pre-, post-, and 1 h post-exercise. Participants also completed the Wisconsin Upper Respiratory Symptom Survey-21 (WURSS-21) for 7 days after each trial. The whole blood sample was exposed to vaccine dose 5 (based on previous study – Chapter 3) and incubated for 20 h (5% CO<sub>2</sub>, 37°C) before being analysed for cytokines (IL-2, IL-6, IL-8, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\alpha$ , and IL-1 $\beta$ ) production with the Evidence Investigator. Total leukocytes and differential counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) were also measured. Saliva samples were analysed for the S-IgA by ELISA. Data analysis was carried out using SPSS (two-way ANOVA with repeated measure and paired t-test). However, for data sets that were not normal distributed, non-parametric tests were used (Friedman test and Wilcoxon test). The main findings of this study were that prolonged moderate intensity exercise significantly decreased S-IgA concentration and secretion rate post-exercise but did not affect the saliva flow rate. However, the values returned to baseline by 1 h post-exercise. Total leukocyte count was

significantly increased post-exercise, and was further increased 1 h post-exercise. This was mainly due to a rise in the neutrophil count. Nevertheless, there were no significant differences between control and Kaloba trials in cytokine production, leukocyte counts, S-IgA response, and the WURSS-21 questionnaire (numbers of sickness days, number of sickness symptoms and severity of sickness symptoms).

## 5.1 Introduction.

In Chapter 4, it was found that Kaloba showed a tendency to act as an immunostimulant. Thus, in this study, the intention was to further investigate the effects of Kaloba supplementation on the immune response to prolonged exercise in athletes. To date, the effectiveness of Kaloba supplementation in enhancing athletes' immune function is very scarce in the literature. It is well known that athletes, who engage in heavy training and competition, are vulnerable to infections due to impaired immune function. This was explained by the 'open window' and 'J curve' theories (Nieman, 2000). Exercise-induced immune dysfunction (especially prolonged, high intensity exercise) is associated with the alteration in stress hormone and cytokine secretion. Luna and colleagues (2010) had investigated immune response induced by *Pelargonium sidoides* (Kaloba) extract in serum and nasal mucosa of athletes after exhaustive exercise. In that study, participants performed a high-intensity running session ( $\sim 85\%$  of  $\dot{V}O_2\text{max}$ ) after 28 days of supplementation (3 x 30 drops/day of a solution of 80 g of extract/100 ml in solution). They found that saliva secretory IgA concentration was increased suggesting that *Pelargonium sidoides* able to modulate the immune response of athletes during intense physical activity. However, the post-exercise sample was collected 48 h after exercise completed. The authors did not justify the selection of this sampling time point and it would be of interest to know if the IgA response to exercise was altered in the immediate post-exercise recovery period.

Kaloba used in this study was manufactured in Germany by Schwabe GmbH. It was in the form of tablet where each film-coated tablet contains 20 mg of extract (as dry extract) from the roots of *Pelargonium sidoides* (EPs 7630). The extraction solvent used was 11% ethanol (w/w). In Germany, this product has become popular for the treatment of acute bronchitis, common cold, sinusitis, pharyngitis, and tonsillitis (Kolodziej and Schulz, 2003).



*Pelargonium sidoides* is a plant species that is indigenous to South Africa and was traditionally believed and used to treat dysentery, diarrhoea, colds, wounds, fatigue, fevers, hepatic complaints, generalized malaise, and respiratory tract infections such as tuberculosis, bronchitis, and sinusitis. Several clinical trials conducted on adults and children have subsequently confirmed some of these benefits (Lizogub et al., 2007; Matthys and Funk, 2008; Bachert et al., 2009; Kamin et al., 2010a and 2010b; Matthys et al., 2010).

Kaloba has been reported to have multifactorial mode of action; antiviral and cytoprotective properties, antibacterial properties, secretomotor properties and inhibition of 'sickness behaviour'. Several *in vitro* studies found that Kaloba triggers stimulation of IFN synthesis (Trun et al., 2006; Kayser et al., 2007) and improves phagocyte function (Conrad et al., 2007). It was also shown to increase the ciliary beat frequency, thus increasing the transportation of mucous and pathogen from the respiratory tracts. This in turn reduced pathogen count, removed the nutrient medium for their subsequent proliferation and substantially improved respiration (Neugebauer et al., 2005).

Thus, the aim of the present study was to investigate the effects of Kaloba intake on immune responses to exercise in individuals engaged in regular endurance sport training.

## **5.2 Methods.**

### **5.2.1 Participants.**

Nine healthy active men participated in this study. All participants were recruited from Loughborough University students. Their mean age, weight and BMI were  $22 \pm 5$  years,  $73.7 \pm 5.6$  kg, and  $22.4 \pm 1.5$  kg/m<sup>2</sup>, respectively. They were required to abstain from ingesting products containing *Pelargonium sidoides* and also any other supplements that are known to affect immune function such as probiotics, etc. during the study period.

### 5.2.2 Procedures.

Participants visited the laboratory on 4 occasions. The first 2 visits were for the preliminary tests which included a maximum oxygen uptake ( $\dot{V}O_2\text{max}$ ) test and familiarisation test, while the following 2 visits were the experimental trials.

#### 5.2.2.1 Preliminary tests.

Maximum oxygen uptake ( $\dot{V}O_2\text{max}$ ) and familiarisation tests were carried out as described in the general methods (Chapter 2, section 2.2 and 2.3 respectively).

#### 5.2.2.2 Experimental trials.

Approximately one week after the familiarisation trial, participants visited the lab for the first experimental trial. Participants came to the laboratory in the morning at 9:00 after an overnight fast (but they were permitted to drink plain water) from 22:00. There were also asked to record their diet during the 24 h period before the overnight fast. Participants were asked to follow the same diet as before the second experimental trial.

Each experimental trial consist of cycling at 60%  $\dot{V}O_2\text{max}$  for 90 min. Saliva and blood samples were collected (as described in general methods; Chapter 2, section 2.4.1 and 2.4.3) pre-exercise, immediately post-exercise, and 1 h post-exercise. Before the 1 h post-exercise sample was collected, participants were asked to rest in a comfortable room without doing any physical activity. Heart rate and RPE were collected pre-exercise, and at 20<sup>th</sup>, 60<sup>th</sup>, 80<sup>th</sup>, and 90<sup>th</sup> min of exercise while expired gas was collected at 20<sup>th</sup> and 60<sup>th</sup> min of exercise.

At least 1 day after the first experimental trial, participants started taking 1 Kaloba tablet (each film-coated tablet contains 20 mg of root extract), three times daily for 7 days. After 7 days supplementation, participants performed the second experimental trial which

was exactly the same with the first experimental trial. After each experimental trial, participants completed the Wisconsin Upper Respiratory Symptom Survey-21 (WURSS-21) (Barrett et al., 2002) for 7 days.

A placebo controlled, crossover design study is known to avoid bias in the results; however, a pre- and post- supplementation design was used in the present study because of time and resource constraints. As the main outcome parameters (blood cells, plasma cytokines production, and salivary IgA response) measured were not subjective and could not be consciously controlled or influenced by the participants or researchers, the pre- and post-supplementation design was deemed acceptable. Thus, either study design should have no effect on the outcomes measured in this present study. However, if subjective parameters were to be important outcome measures (e.g. participants' perceptions), a placebo controlled, crossover design would be much more appropriate.

### 5.2.3 Blood analysis.

#### 5.2.3.1 *Haematological analysis.*

Haematological analyses were carried out as described in the general methods (Chapter 2, section 2.44).

#### 5.2.3.2 *One hundred times diluted vaccine dose preparation.*

This was prepared as described in the general methods (Chapter 2, section 2.4.5.1).

#### 5.2.3.3 *Whole blood culture incubation and supernatant storage.*

Heparinised blood was cultured and incubated for 20 h as described in the general methods (Chapter 2, section 2.4.5.2).

#### 5.2.3.4 Evidence Investigator cytokine array.

Cytokine analysis was carried out as described in the general methods (Chapter 2, section 2.4.5.3).

#### 5.2.4 Saliva Analysis.

Saliva samples were analysed for S-IgA level by using a Salimetrics S-IgA ELISA kit as described in the general methods (Chapter 2, section 2.4.2.1).

#### 5.2.5 Data analysis.

Data were analysed using the Statistical Package for Social Sciences (SPSS) version 17 (SPSS Inc., USA). Two-way ANOVA with repeated measures was used for data sets that were normally distributed. For data sets that were not normally distributed, non-parametric tests were used (Friedman test and Wilcoxon test). All the data were expressed as mean  $\pm$  SD.

### 5.3 Results.

#### 5.3.1 Physiological parameters.

Table 5.1 shows the physiological parameters measured in this study. Rating of perceived exertion and HR were significantly increased over the time ( $P < 0.05$ ), nevertheless, they were not significantly different between the two trials. In each trial, the  $\dot{V}O_2$  value was not significantly different between the two time points ( $P > 0.05$ ). There was also no significant difference in oxygen uptake between trials at both time points.

### 5.3.2 Haematological parameters.

Total leukocyte counts were significantly increased post-exercise and were further increased 1 h post-exercise (Table 5.2). This was mainly due to a rise in neutrophils. Besides, the monocyte and basophil counts were also significantly increased over the time.

*Table 5.1: Physiological parameters (n = 9).*

<b>Parameters</b>	<b>Value (Mean ± SD)</b>		<b>P value between trials</b>
$\dot{V}O_2\text{max}$ (ml.kg <sup>-1</sup> .min <sup>-1</sup> )	56.1 ± 7.8		
60% $\dot{V}O_2\text{max}$ (ml.kg <sup>-1</sup> .min <sup>-1</sup> )	34.4 ± 4.8		
Work Rate at 60% $\dot{V}O_2\text{max}$ (W)	184.0 ± 25.0		
Rating of perceived exertion at the end of the trial (Borg's unit)	Non-Kaloba Trial	13	<i>P</i> = 0.053
	Kaloba Trial	12	
Heart rate at the end of the trial (Beats.min <sup>-1</sup> )	Non-Kaloba Trial	147 ± 13	<i>P</i> = 0.411
	Kaloba Trial	146 ± 13	
$\dot{V}O_2$ at 20-min (ml. kg <sup>-1</sup> .min <sup>-1</sup> )	Non-Kaloba Trial	33.2 ± 3.7	<i>P</i> = 0.172
	Kaloba Trial	34.4 ± 3.0	
$\dot{V}O_2$ at 60-min (ml. kg <sup>-1</sup> .min <sup>-1</sup> )	Non-Kaloba Trial	34.7 ± 4.0	<i>P</i> = 0.551
	Kaloba Trial	35.2 ± 3.3	

Table 5.2: Mean and SD values of haematological parameters in both non-Kaloba and Kaloba trials.

\*\*\*\*\* Significantly different from respective pre-exercise value ( $P < 0.05$ ,  $P < 0.001$ , and  $P < 0.000$  respectively).

<sup>+</sup>, <sup>++</sup> Significantly different from respective post-exercise value ( $P < 0.05$  and  $P < 0.001$  respectively).

Parameters	Trial	Pre-exercise	Post-exercise	1h post-exercise
Red blood cells ( $10^{12}/L$ )	Non-Kaloba	4.7±0.3	4.7±0.2	4.7±0.3
	Kaloba	4.6±0.3	4.8±0.2**	4.7±0.3 <sup>+</sup>
Haemoglobin (g/dL)	Non-Kaloba	14.8±1	15.1±1.1	14.9±1.3
	Kaloba	14.2±0.6	14.8±0.4****	14.5±0.6* <sup>+,++</sup>
Haematocrit (%)	Non-Kaloba	45.9±3	46.8±1.6	46.1±2.3
	Kaloba	45.7±2.2	47.6±1.6**	46.6±2.5
Total leukocytes ( $10^9/L$ )	Non-Kaloba	5.5±1.5	10±2.5**	12.7±3**** <sup>+</sup>
	Kaloba	5.1±1.7	9.1±2.2**	11.2±2.1**** <sup>+,+</sup>
Neutrophils ( $10^9/L$ )	Non-Kaloba	2.5±1	6.5±2.3**	9.5±2.4**** <sup>+,++</sup>
	Kaloba	2.5±1.1	5.9±1.9**	8.6±1.9**** <sup>+,++</sup>
Lymphocytes ( $10^9/L$ )	Non-Kaloba	2.2±0.7	2.4±0.6	2±0.7
	Kaloba	2±0.5	2.3±0.9	1.7±0.5
Monocytes ( $10^9/L$ )	Non-Kaloba	0.5±0.2	0.8±0.3*	0.9±0.3**
	Kaloba	0.5±0.2	0.6±0.1*	0.7±0.1**
Eosinophils ( $10^9/L$ )	Non-Kaloba	0.2±0.2	0.2±0.1	0.2±0.2
	Kaloba	0.2±0.2	0.2±0.1	0.2±0.1
Basophils ( $10^9/L$ )	Non-Kaloba	0.03±0.05	0.08±0.06*	0.1±0.07*
	Kaloba	0.01±0.02	0.06±0.06*	0.08±0.04**

### 5.3.3 Saliva secretory immunoglobulin A (S-IgA).

There were significant main effects of time for S-IgA concentration ( $P = 0.007$ ) and secretion rate ( $P = 0.006$ ), but not for saliva flow rate ( $P > 0.05$ ) (Figures 5.2, 5.3, and 5.4). Following exercise, both S-IgA secretion rate and S-IgA level were decreased but then increased 1 h post-exercise. However, there were no significant differences between the two trials (non-Kaloba and Kaloba) in S-IgA level, S-IgA secretion rate, and saliva flow rate ( $P > 0.05$ ).

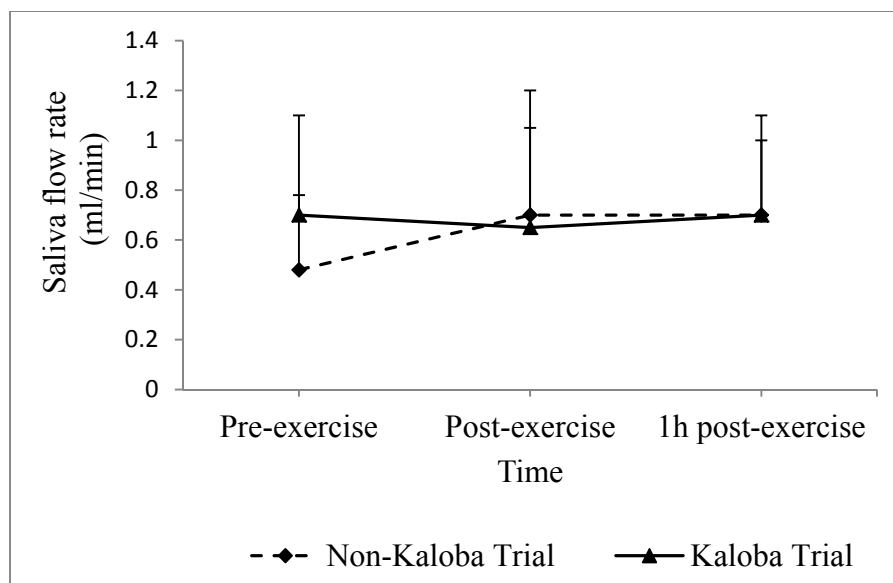


Figure 5.1: Saliva flow rate (ml/min) in non-Kaloba and Kaloba trials.

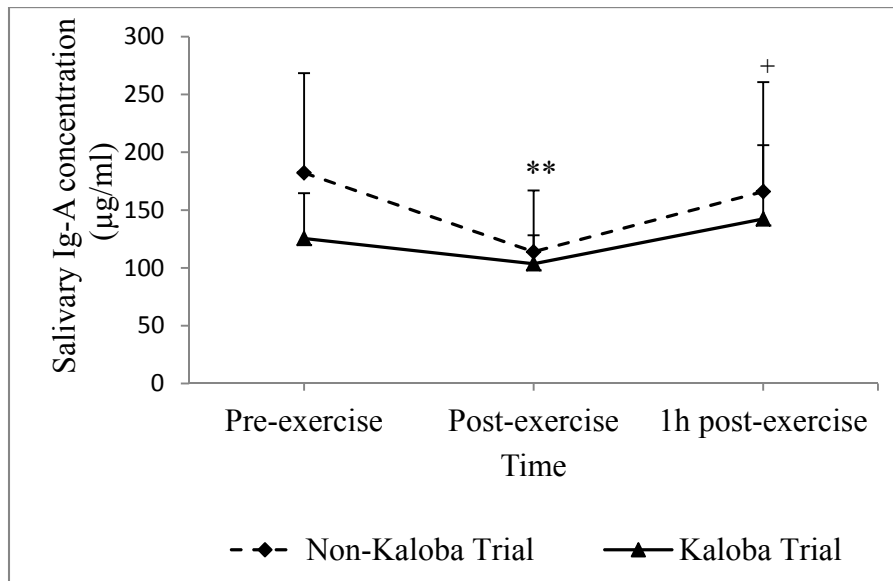


Figure 5.2: Salivary Ig-A concentration ( $\mu\text{g/ml}$ ) in non-Kaloba and Kaloba trials.

\*\* Significantly different from respective pre-exercise value ( $P < 0.001$ ).

+ Significantly different from respective post-exercise value ( $P < 0.05$ ).

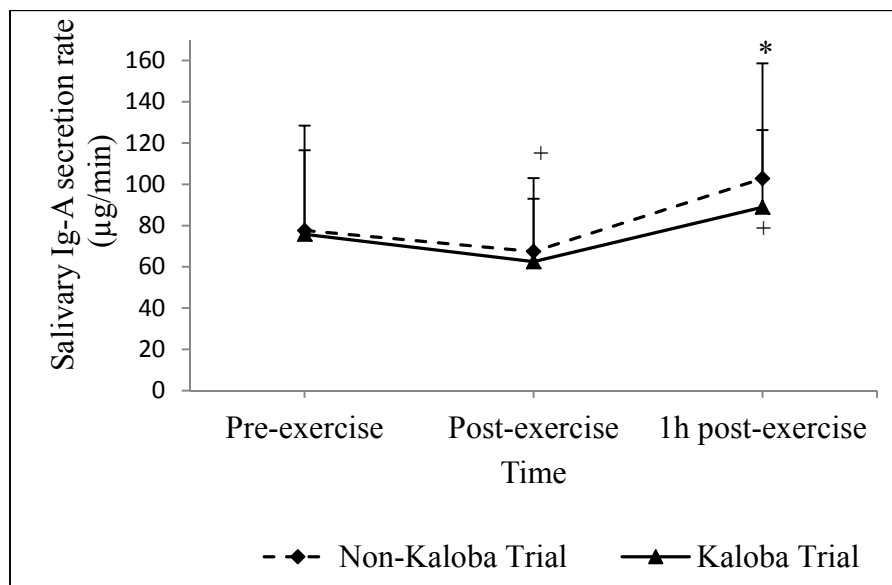


Figure 5.3: Salivary Ig-A secretion rate ( $\mu\text{g/min}$ ) in non-Kaloba and Kaloba trials.

\* Significantly different from respective pre-exercise value ( $P < 0.05$ ).

+ Significantly different from respective post-exercise value ( $P < 0.05$ ).



### 5.3.4 Cytokine production.

Distribution of this data was not normal, thus a non-parametric test (Friedman test) was used to see the significant main effects of time throughout the trials separately. There was no significant main effect of time ( $P > 0.05$ ) on cytokine production throughout both trials. Another non parametric test (Wilcoxon test) was then used to examine possible differences in cytokine production between the two trials at each time point. There were no significant differences between trials at any time point ( $P > 0.05$ ).

### 5.3.5 Questionnaire.

As illustrated in Table 5.3, there were no significant differences in days of sickness, number of symptoms, and symptom scores (severity of the sickness) between both two trials.

*Table 5.3: Wisconsin Upper Respiratory Symptom Survey-21 (WURSS-21) results.*

<b>Parameters</b>	<b>Value (Mean <math>\pm</math> SD)</b>		<b><i>P</i> value between trials</b>
Days of sickness (Duration of sickness)	Non-Kaloba Trial	3.4 $\pm$ 3.0	$P = 0.290$
	Kaloba Trial	2.4 $\pm$ 2.9	
Number of symptoms	Non-Kaloba Trial	3.6 $\pm$ 3.1	$P = 0.053$
	Kaloba Trial	2.0 $\pm$ 2.2	
Symptom score (Severity of sickness)	Non-Kaloba Trial	33.2 $\pm$ 3.7	$P = 0.172$
	Kaloba Trial	34.4 $\pm$ 3.0	

## 5.4 Discussion.

The main finding of this study was that prolonged exercise at moderate intensity decreased S-IgA concentration and secretion rate post-exercise, but the values returned back to baseline by 1 h post-exercise. Prolonged exercise also induced an increase in circulating leukocytes which further increased 1 h post-exercise; this was mainly due to a rise in neutrophil counts. Cycling for 90 min at moderate intensity (60%  $\text{VO}_2\text{max}$ ) led to a decrease in S-IgA secretion rate and concentration post-exercise but then significantly increased 1 h post exercise (Figure 5.2). This is in agreement with previous studies following prolonged exercise (Walsh et al., 2002; Laing et al., 2005). The present study also found no significant effects of exercise on saliva flow rate. However, Allgrove et al. (2009) found that during prolonged cycling at 65%  $\text{VO}_2\text{max}$ , saliva flow rate decreased, S-IgA concentration increased, and S-IgA secretion rate remained unchanged. In fact, numerous studies have reported significant decreases in S-IgA after exercise (Gleeson et al., 1999; Nieman et al., 2002), while others have reported significant increases (Blannin et al., 1998; Sari-Sarraf et al., 2007) or no change (Mackinnon and Hooper, 1994; Li and Gleeson, 2004). This discrepancy may be attributed to the different method of expressing S-IgA, nutritional status of the individual, and the exercise protocol employed.

It is well known that a low level of S-IgA in athletes was associated with higher risk of URTI (Gleeson et al., 1999; Fahlman and Engels, 2005). However, little is known about how exercise influences salivary responses. Theoretically, S-IgA secretion rate is dependent on the production of IgA by the plasma cells in the submucosa and/or the IgA transcytosis rate across the epithelial cell which is determined by polymeric Ig receptor (pIgR) availability (Bosch et al., 2002). During chronic stress, prolonged sympathetic nervous system (SNS) activation may down regulate IgA synthesis and expression of pIgR, thus decreasing the

secretion of IgA. Nonetheless, in the present study, Kaloba did not influence salivary responses in athletes.

In the present study, moderate, prolonged exercise resulted in increased numbers of circulating leukocytes, neutrophils, monocytes, and basophils counts post-exercise, and they had not returned to the pre-exercise value 1 h post-exercise. This leukocytosis was mainly due to a rise in the blood neutrophil count. This is consistent with previous findings (Nieman et al., 2005; Pacque et al., 2007). Exercise-induced leukocytosis is largely due to demargination of the leukocytes that were adhered to the blood vessel walls at rest (called the margined pool of leukocytes) into the circulating pool (Gleeson, 2006b). This demargination is believed to be due to the exercise-induced increase in cardiac output which produces higher mechanical force, as well as the increase in shear stress within the capillaries. Furthermore, previously 'dormant' or 'closed' capillaries will also open up in the lungs and working muscles due to elevated blood flow, therefore releasing their leukocyte store into the circulating pool. Increased plasma catecholamines also may play a role in leukocytosis during exercise via reducing the adherence of leukocytes to the vascular endothelium. The size of leukocytosis is dependent on the severity of work rate; while its characteristics are dependent on intensity, duration, and type of exercise. It was found that magnitude of leukocytosis produced by prolonged exercise is larger than short term high intensity exercise, and is due to release of neutrophils from bone marrow.

Despite evidence from several *in vitro* studies about positive beneficial effects of Kaloba, there are very few published studies about its supplementation effects on the immune response in athletes to date. This study found no effects of Kaloba on athletes' immune response after prolonged exercise. We speculated that the dose of the Kaloba and supplementation period was not enough. Luna and colleagues (2010) suggest that 28 days of

*Pelargonium sidoides* supplementation (3 x 30 drops/day) is able to modulate the immune response associated with the upper airway mucosa in athletes submitted to intense physical activity. They found a relative increase in S-IgA concentration post-exercise, however the saliva samples were collected 48 h after the exercise was completed. This may raise some issue since post-exercise effect on S-IgA level may have been disappeared after 48 h or the S-IgA level measured may be attributed to other factors. It would be much more meaningful if the post-exercise samples were collected immediately, 1 h or 2 h post-exercise.

## **5.5 Conclusion.**

In brief, 7 days Kaloba supplementation had no effect on any of the parameters measured: salivary IgA response, blood cell counts, antigen-stimulated cytokine production, and respiratory tract infection symptoms. The most possible explanation for this is the concentration/dose and length of supplementation of Kaloba in this study was not enough to induce any significant effects of the Kaloba. Nevertheless, prolonged exercise at moderate intensity decreased S-IgA concentration and secretion rate post-exercise, but the values returned back to baseline by 1 h post-exercise. Prolonged exercise also induced a rise in circulating leukocyte counts post-exercise and further increased them 1 h post-exercise; this was mainly due to a rise in blood neutrophil counts.

## CHAPTER 6

### **Study 4: Effects of a 14-strain probiotic supplement on salivary antimicrobial proteins at rest and in response to an acute bout of prolonged exercise.**

#### **Abstract**

This study was designed to determine the effects of a period of supplementation with a 14-strain probiotic on salivary antimicrobial proteins at rest and in response to an acute bout of prolonged exercise in recreationally active, healthy adults. In this study, 11 participants (age:  $22 \pm 1$  years, weight:  $69.5 \pm 12.2$  kg, BMI:  $23.0 \pm 1.8$  kg/m<sup>2</sup>) cycled for 2 h at 60%  $\text{VO}_{2\text{max}}$  on two occasions (two trials separated by 30 days). During each trial, participants came to the lab in the morning, 2 h after having their breakfast. They were asked to have a similar breakfast before each trial. The second trial was performed after 30 days of supplementation with a 14-strain probiotic. The probiotic was in capsule form and taken as 1 capsule 3 times daily (morning, midday, and evening). Timed, unstimulated saliva samples were collected pre-, post-, 1 h post-, and 2 h post-exercise. Saliva samples were analysed for secretory immunoglobulin A (S-IgA),  $\alpha$ -amylase, lactoferrin, and lysozyme concentrations. Data analysis was carried out using SPSS (two-way ANOVA with repeated measures). However, for data sets that were not normally distributed, non-parametric tests were used (Friedman test and Wilcoxon test). Results showed that 30 days of supplementation with the 14-strain probiotic did not alter salivary antimicrobial proteins at rest and in response to an acute bout of prolonged exercise. However, prolonged exercise significantly increased lactoferrin concentration and  $\alpha$ -amylase activity post-exercise. It was concluded that the supplementation period and/or the dose/concentration of the 14-strain probiotic used in this study were insufficient to induce any beneficial effects on athletes' salivary antimicrobial

proteins at rest and in response to an acute bout of prolonged exercise, or simply that this probiotic supplement was ineffective.

## 6.1 Introduction.

As defined by World Health Organisation (WHO), probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO, 2006). Lilly and Stillwell were reported to be the first to use the term probiotics in 1965, to describe substances secreted by one organism which stimulate the growth of another (Gupta and Garg, 2009). The efficacy of single strains of probiotic on immune function in both healthy individuals and clinical patients has been widely investigated. For example, two studies reported that regular ingestion of single strain probiotic (*Lactobacillus casei*) may modify the salivary secretory immunoglobulin A (S-IgA) level (O'Connell et al., 2010; Gleeson et al., 2011a).

Nevertheless, studies on the effects of mixtures of probiotic strains are far fewer compared to those that have examined single strain probiotic supplements. Some have reported that probiotic mixtures are more effective in modifying immune function and preventing respiratory tract infection (Chapman et al., 2011). However, it is unknown if this is a result of synergistic interactions between strains or a consequences of the higher probiotic dose used in some studies. Most of multi-strain probiotic studies did not directly compare between a mixture and its component strains. This makes us unable to make a definite conclusion about the efficacy of the probiotic mixtures or their component strains. Potential modes of action of probiotics include (1) modulation of the intestinal immune system, (2) displacement of potential pathogens through competitive exclusion and (3) production of antimicrobial agents (Rowland et al., 2010), so intuitively, one might expect a combination of bacterial strains to be more effective than a single strain.

The multi-strain probiotic supplement used in this study contained 14 strains of beneficial microorganisms at a concentration of 10 billion per gram (with a minimum of 2

billion probiotic microorganisms per capsule). The microorganisms it contained were *Lactobacillus acidophilus*, *Lactobacillus delbrueckii ssp. bulgaricus*, *Lactococcus lactis ssp. lactis*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius ssp. salivarius*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bacillus subtilis*, and *Streptococcus thermophilus*. Several multi-strain probiotic studies have reported reduced number of days with fever, duration of cold episode, total symptom score, reduced incidence of respiratory tract infections and reduction in gastrointestinal infections (Winkler et al., 2005; de Vrese et al., 2006; Lin et al., 2009). However, their effect on salivary antimicrobial proteins has not been investigated. Thus, the purpose of the present study was to investigate the efficacy of a multi-strain probiotic supplement on recreational athletes' salivary antimicrobial proteins at rest and in response to an acute bout of prolonged exercise.

## **6.2 Methods.**

### **6.2.1 Participants.**

Eleven healthy active Loughborough University students were involved in this study. Their mean age, weight and BMI were  $22 \pm 1$  years,  $69.5 \pm 12.2$  kg, and  $23.0 \pm 1.8$  kg/m<sup>2</sup> respectively. During the study period, participants abstained from probiotic products and also other supplements that believed to affect immune function.

### **6.2.2 Procedures.**

Participants visited the laboratory on 3 occasions. The first visit was for a maximum oxygen uptake ( $\dot{V}O_{2\max}$ ) test, while the following 2 visits were the experimental trials.



### 6.2.2.1 Preliminary test.

Maximum oxygen uptake ( $\dot{V}O_{2\max}$ ) test was carried out as described in the general methods (Chapter 2, section 2.2).

### 6.2.2.2 Experimental trials.

Participants came to the laboratory in the morning (approximately at 11:00 am), 2 h after having their breakfast. They were asked to consume a similar breakfast before the second experimental trial. The first experimental trial consisted of cycling at 60%  $\dot{V}O_{2\max}$  for 2 h. Participants were given plain water (3 ml/kg body weight) to drink before exercise, during exercise (20<sup>th</sup>, 40<sup>th</sup>, 60<sup>th</sup>, 80<sup>th</sup> and 100<sup>th</sup> min of exercise), after the second saliva collection, and after the third saliva collection. Rating of perceived exertion, HR and expired gas were collected during exercise. Saliva samples were collected (as described in the general methods; Chapter 2, section 2.4.1) pre-, immediately post-, 1 h post- and 2 h post-exercise. Saliva samples were analysed for S-IgA, lactoferrin,  $\alpha$ -amylase, and lysozyme concentrations. Participants then, start taking the 14-strain probiotic supplementation for 30 days before coming to the laboratory for the second trial (which followed exactly the same protocol as the first experimental trial).

A placebo controlled, crossover design study is known to avoid bias in the results; however, a pre- and post- supplementation design was used in the present study because of time and resource constraints. As the main outcome parameters (salivary antimicrobial protein responses) measured were not subjective and could be consciously controlled or influenced by the participants or researchers the pre- and post- supplementation design was deemed acceptable. Thus, either study design should have no effect on the outcomes measured in this present study. However, if subjective parameters were to be important

outcome measures (e.g. participants' perceptions), a placebo controlled, crossover design would be much more appropriate.

### 6.2.3 Saliva Analysis.

#### *6.2.3.1 Saliva secretory Ig A (S-IgA) analysis.*

Salivary-IgA was analysed as described in the general methods (Chapter 2, section 2.4.2.1).

#### *6.2.3.2 Alpha ( $\alpha$ )-amylase analysis.*

Alpha-amylase was analysed as described in the general methods (Chapter 2, section 2.4.2.4).

#### *6.2.3.3 Lysozyme analysis.*

Lysozyme was analysed as described in the general methods (Chapter 2, section 2.4.2.2).

#### *6.2.3.4 Lactoferrin analysis.*

Lactoferrin was analysed as described in the general methods (Chapter 2, section 2.4.2.3).

#### *6.2.3.5 Nitrite and Nitrate Analysis.*

To perform the assay for nitrite, 100  $\mu$ l of standards (standard 1 – 8) were added in duplicate to the first two rows of wells. Then, 200  $\mu$ l of water or assay buffer was added to the blank wells. After that, 100  $\mu$ l of sample (x 2 dilution) was added to the chosen wells. Then, 50  $\mu$ l of Griess Reagent R1 was added followed by addition of 50  $\mu$ l Griess reagent R2

to each of the wells except the blank wells. After 10 min, the absorbance was measured with spectrophotometer Varioskan Flash (Thermo Scientific, UK) at 540 nm wavelength.

To perform the assay for total nitrate + nitrite, 100 µl of standards (standard 1 – 8) were added in duplicate to the first two rows of wells. Then, 200 µl of water or assay buffer was added to the blank wells. After that, 80 µl of sample (x 4 dilution) was added to the chosen wells. Then, 10 µl of the Enzyme Cofactor Mixture was added, followed by 10 µl of the Nitrate Reductase Mixture to each of the wells except the blank wells. Next, the plate was covered and incubated at room temperature for 2 h. After incubation, 50 µl of Griess Reagent R1 was added followed by addition of 50 µl Griess reagent R2 to each of the wells except the blank wells. After 10 min, the absorbance was measured with spectrophotometer Varioskan Flash (Thermo Scientific, UK) at 540 nm wavelength.

Calculations:

The absorbance value of the blank wells was averaged, and this value was subtracted from the absorbance values of all the other wells. Then, the standard curves were plotted. The nitrate standard curve was used for determination of total nitrate + nitrite concentration, whereas the nitrite standard curve was used for the determination of nitrite alone. The following equations were used to calculate the nitrite and nitrate concentrations:

$$[\text{Nitrate} + \text{Nitrite}] (\mu\text{l}) = \left( \frac{A_{540} - y\text{-intercept}}{\text{slope}} \right) \left( \frac{200 \mu\text{l}}{\text{volume of sample used} (\mu\text{l})} \right) \times \text{dilution}$$

$$[\text{Nitrite}] (\mu\text{l}) = \left( \frac{A_{540} - y\text{-intercept}}{\text{slope}} \right) \left( \frac{200 \mu\text{l}}{\text{volume of sample used} (\mu\text{l})} \right) \times \text{dilution}$$

$$[\text{Nitrate}] (\mu\text{l}) = (\text{Nitrate} + \text{Nitrite}) - (\text{Nitrite})$$

#### 6.2.4 Data analysis.

Data were analysed using the Statistical Package for Social Sciences (SPSS) version 17 (SPSS Inc., USA). Descriptive analysis was used to measure mean and standard deviation (SD). Statistical significance of differences between means was analysed using paired samples t-test. Two-Way ANOVA with repeated measure was used. However, whenever the data was not normally distributed, the Friedman test was used to examine the main effect of time in each trial and the Wilcoxon signed rank test was used to find the differences between trials in each time point value. All the data were expressed as mean  $\pm$  SD. The accepted level of significance was  $P < 0.05$ .

### **6.3 Results.**

#### 6.3.1 Physiological parameters.

Table 6.1 shows the physiological parameters measured in this study. Rating of perceived exertion and HR were significantly increased over the time ( $P < 0.05$ ), although, they were not significantly different between trials. However, the rate of oxygen uptake during the two trials was significantly different ( $P < 0.001$ ).

Table 6.1: Physiological parameters (n = 9).

Parameters	Value (Mean $\pm$ SD)		P value between trials
VO <sub>2</sub> max (ml.kg <sup>-1</sup> .min <sup>-1</sup> )	47.5 $\pm$ 3.8		
60% VO <sub>2</sub> max (ml.kg <sup>-1</sup> .min <sup>-1</sup> )	28.5 $\pm$ 2.3		
Work Rate at 60% VO <sub>2</sub> max (W)	136 $\pm$ 24		
Rating of perceived exertion at the end of the trial (Borg's unit)	Non-probiotic Trial	13	<i>P</i> = 0.503
	Probiotic Trial	12	
Heart rate at the end of the trial (Beats.min <sup>-1</sup> )	Non-probiotic Trial	150 $\pm$ 11	<i>P</i> = 0.625
	Probiotic Trial	151 $\pm$ 9	
Average VO <sub>2</sub> during the trial (ml.min <sup>-1</sup> )	Non-probiotic Trial	29.2 $\pm$ 3.3 (61.6% VO <sub>2</sub> max)	<i>P</i> < 0.01
	Probiotic Trial	26.7 $\pm$ 3.1 (56.3% VO <sub>2</sub> max)	

### 6.3.2 Saliva flow rate, S-IgA concentration, and secretion rate.

There were no significant main effects of time or treatment on saliva flow rate, S-IgA concentration, and S-IgA secretion rate (Figure 6.2 – 6.4). There were also no significant interaction effect for the probiotic on the saliva flow rate, S-IgA concentration, and secretion rate.

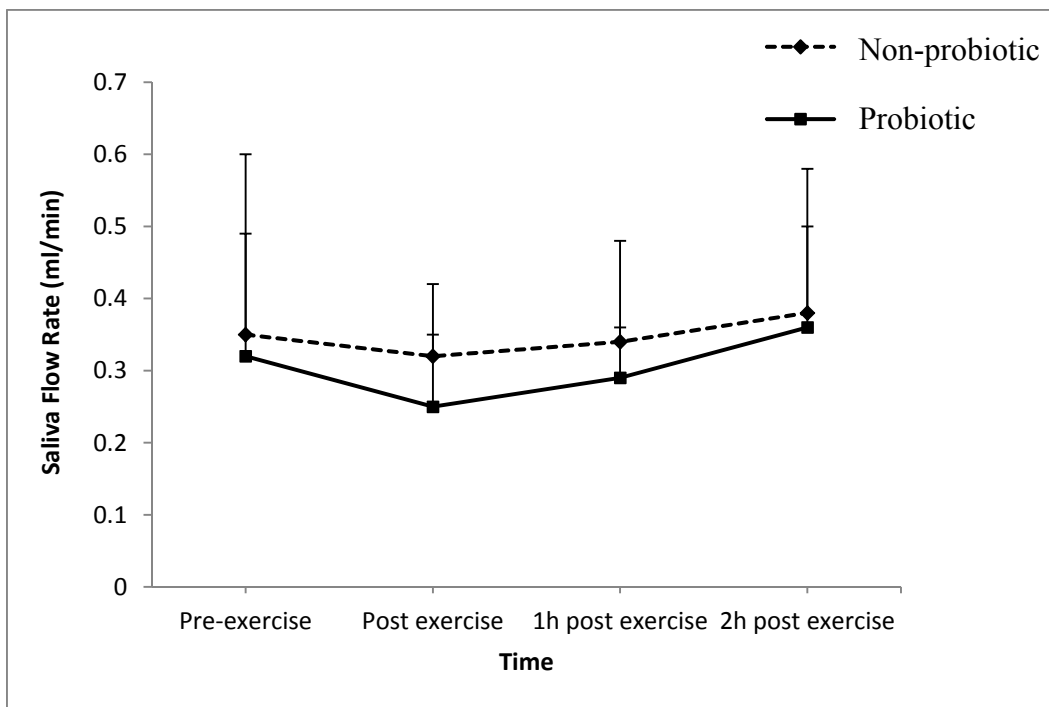


Figure 6.1: Saliva flow rate in both trials.

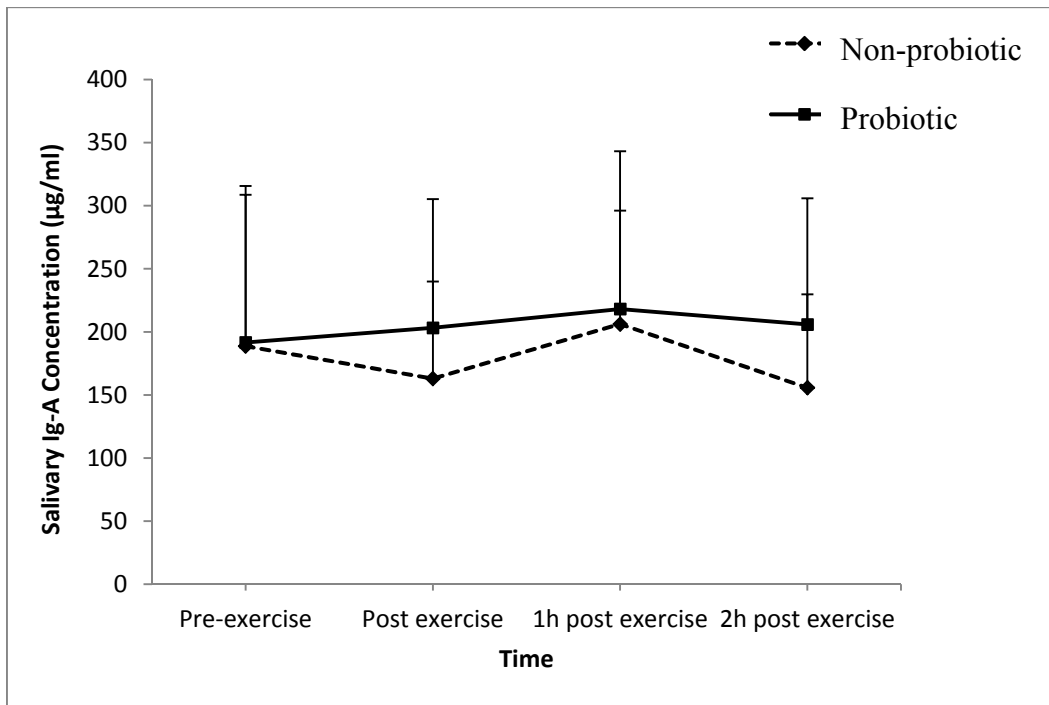


Figure 6.2: S-IgA concentration in both trials.

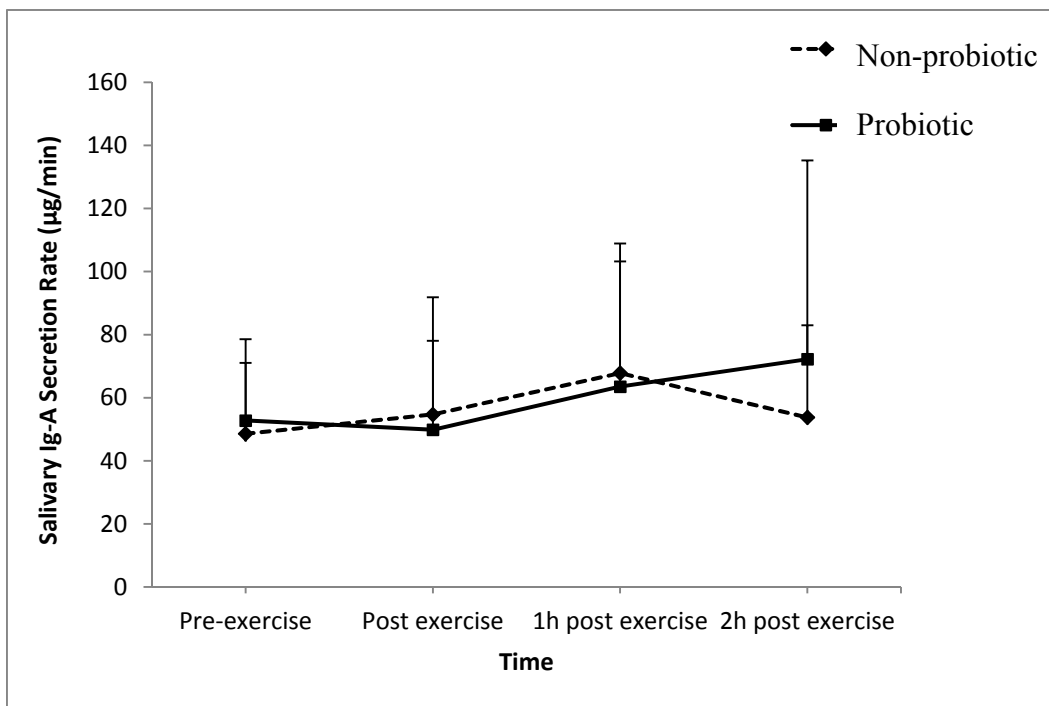


Figure 6.3: S-IgA secretion rate in both trials.

### 6.3.3 Lactoferrin.

There was a significant main effect of time on lactoferrin production in both trials ( $P < 0.05$ ) (Figure 6.5). However, there was no significant effect of the probiotic supplementation on lactoferrin production.

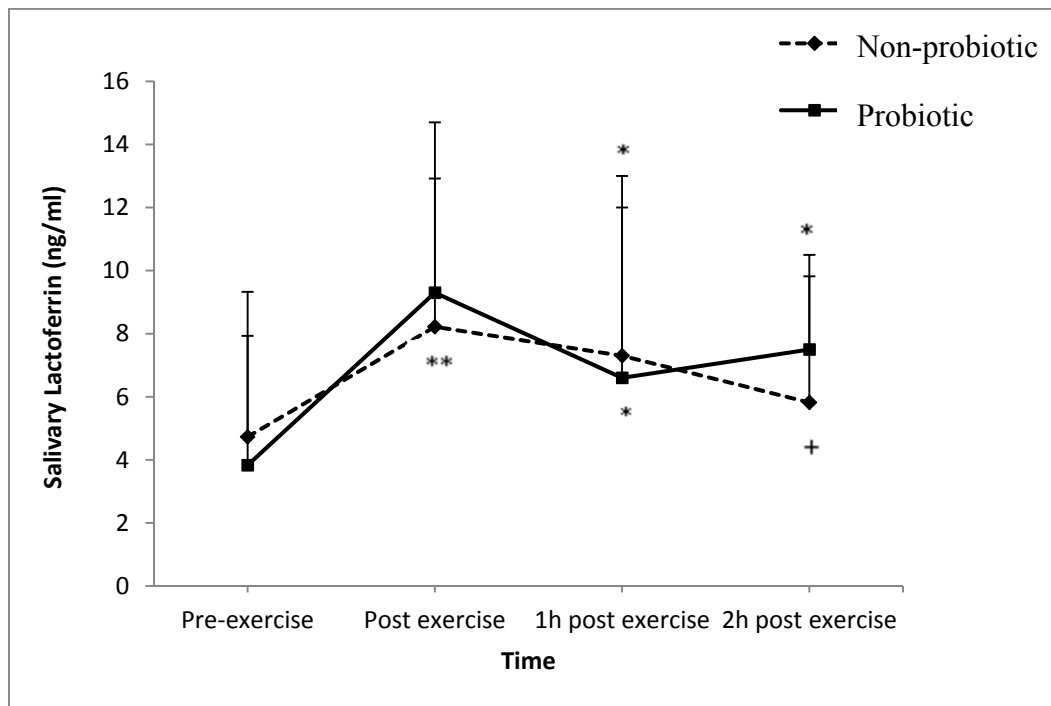


Figure 6.4: Lactoferrin concentration in both trials.

\*, \*\* Significant difference compared to respective pre-exercise value ( $P < 0.05$  and  $P < 0.001$  respectively).

+ Significant difference compared to respective 1h post-exercise value ( $P < 0.05$ ).



#### 6.3.4 Lysozyme.

There was no significant main effect of time on lysozyme concentration in both trials (Figure 6.6). There was also no significant effect of the probiotic on lysozyme concentration.

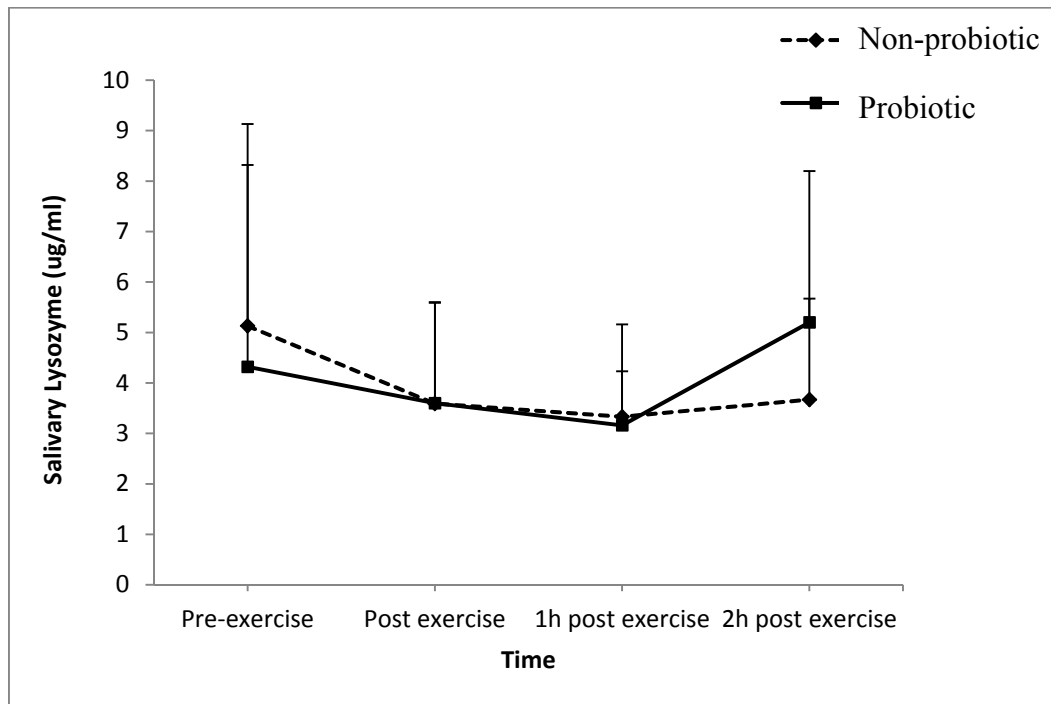


Figure 6.5: Lysozyme concentration in both trials.

### 6.3.5 Alpha-amylase.

There was significant main effect of time on  $\alpha$ -amylase concentration ( $P < 0.05$ ) (Figure 6.7). However, there was no significant effect ( $P > 0.05$ ) of the probiotic on salivary  $\alpha$ -amylase concentration.

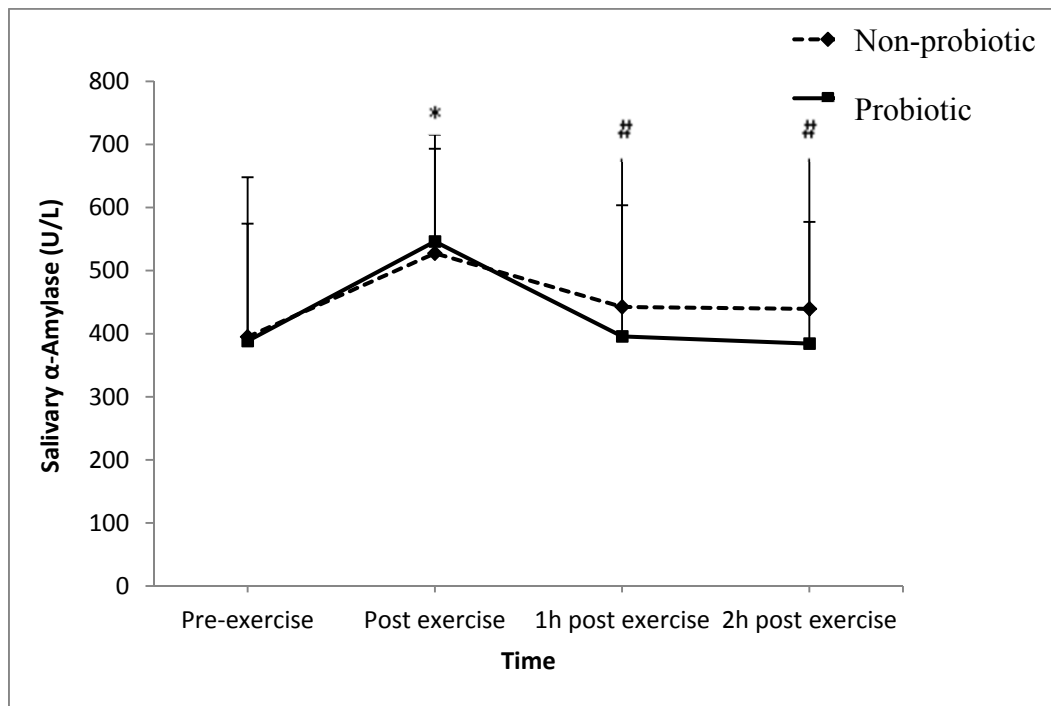


Figure 6.6:  $\alpha$ -amylase concentration in both trials.

\* Significant difference compared to respective pre-exercise value ( $P < 0.05$ ).

# Significant difference compared to respective post-exercise value ( $P < 0.05$ ).

### 6.3.6 Nitrite and Nitrate.

As shown in the Table 6.2, there was no significant effect of trial on the saliva nitrite and nitrate concentrations.

*Table 6.2: Nitrite and Nitrate concentration in both trials.*

	<b>Non-probiotic Trial</b>	<b>Probiotic Trial</b>	<b>P value</b>
Nitrite	21.7 ± 7.6	16.8 ± 5.4	0.319
Nitrate	56.3 ± 14.1	41.5 ± 7.6	0.294

## 6.4 Discussion.

Average oxygen uptake ( $\text{VO}_2$ ) in both trials was significantly different (Table 6.1). However, RPE and HR were not significantly different between the trials. This suggests that the two trials were conducted in the similar condition, but the difference in the  $\text{VO}_2$  value might be due to one of these reasons; the effects of familiarisation, technical error in the measurement of the  $\text{VO}_2$  or difference in pre-exercise nitrite/nitrate concentration in both trials.

The effect of familiarisation was unlikely because the two trials were separated by at least 5 weeks. Technical error in the measurement of  $\text{VO}_2$  was also unlikely because it was done properly with the appropriate calibration procedures. It has been suggested that nitrate in the food (some of which will be converted by bacteria to nitrite upon ingestion, with the rest will be converted to nitrite in the alimentary tract) increases the efficiency of adenosine triphosphate (ATP) production in mitochondria (Larsen et al., 2011). This has previously been shown to lead to a lower  $\text{VO}_2$  during submaximal exercise and hence improved exercise performance (Bailey et al., 2009 and 2010; Lansley et al., 2011; Christensen et al., 2012;

Jones et al., 2012). Thus, it was hypothesised that the differences in the  $\dot{V}O_2$  between the two trials in the present study might be related to this condition, where the probiotic bacteria ingested may have increased the reduction of nitrate to nitrite. To test this hypothesis, nitrite and nitrate analyses of saliva samples were carried out. However, as can be seen in Table 6.2, the pre-exercise saliva nitrite and nitrate concentrations were not different between the trials. This suggests that the 14-strain probiotic did not have any effect on the reduction of nitrate to nitrite. Thus, the reason why the  $\dot{V}O_2$  values were different between trials is unclear. However, since the present study used pre- and post- supplementation design, this finding may be attributed to some other factor (e.g. change in diet, training, environment etc.) over time.

Prolonged exercise increased lactoferrin (Figure 6.4) and  $\alpha$ -amylase (Figure 6.6) concentration post-exercise but did not affect saliva flow rate (Figure 6.1), S-IgA concentration (Figure 6.2), S-IgA secretion rate (Figure 6.3), and lysozyme (Figure 6.5) concentration. As discussed before (Chapter 5, section 5.4), findings about the effects of exercise on S-IgA were inconsistent due to several reasons and it is quite controversial. However, several studies have reported that S-IgA concentration is reduced after intense exercise (Steerenberg et al., 1997; Fahlman et al., 2011), increased after moderate or lower intensity exercise (Williams et al., 2001; Dorrington et al., 2003; Li and Gleeson, 2004) or not affected by exercise intensity (Blannin et al., 1998; McDowell et al., 1991). The later studies were in agreement with the present study. It is known that S-IgA is the predominant immunoglobulin (Ig) in external secretions and is an important component of saliva. It is involved in immune exclusion (preventing antigens and microbes from adhering to and penetrating the epithelium), intracellular neutralization (interrupting replication of intracellular pathogens during transcytosis through epithelial cells), and immune excretion

(binding to antigens in the lamina propria facilitating their excretion through the epithelium back into the lumen) (Lamm, 1998).

On the other hand, previous studies reported an increase in  $\alpha$ -amylase activity in saliva and that this effect is related to the exercise intensity (Walsh et al., 1999; Bishop et al., 2000). One study reported that  $\alpha$ -amylase secretion was increased after cycling at 75%  $\dot{V}O_2\text{max}$  and after incremental exercise to exhaustion (Allgrove et al., 2008). Li and Gleeson (2004) also reported an increase in  $\alpha$ -amylase activity after strenuous exercise. Alpha-amylase accounts for about half of the total protein in saliva. It is an enzyme that breaks down starch into maltose, and also functions to interrupt the adherence and growth of certain bacteria (Scannapieco et al., 1993). It has been suggested that  $\alpha$ -amylase can be a tool for evaluating the relationship between sympathetic nervous system and mucosal immunity following psychological and/or physical stress (Walsh et al., 1999). This is because  $\alpha$ -amylase is regulated by neuronal pathways and has been suggested to reflect changes in plasma noradrenaline and increased sympathetic activity under stressful conditions including exercise (Chatterton et al., 1996). In the present study, 90 min cycling at 60%  $\dot{V}O_2\text{max}$  increased  $\alpha$ -amylase activity suggesting an increased in sympathetic activity due to stressful exercise.

Lactoferrin and lysozyme both possess antimicrobial activity (bactericide and fungicide) and are part of the innate defence, mainly at mucosa (Sanchez et al., 1992). Following an intense training session, saliva lactoferrin (Cox et al., 1999) and lysozyme (Inoue et al., 2004) concentrations were reported to be decreased. However, in contrast, others have reported that lactoferrin (West et al., 2008) and lysozyme (Allgrove et al., 2008; West et al., 2008) concentrations were increased after an exhaustive bout of exercise. The present study found that 90 min cycling at 60%  $\dot{V}O_2\text{max}$  significantly increased saliva

lactoferrin (Figure 7.4) concentration but had no effect on lysozyme (Figure 7.5) concentration. Exercise-induced increases in the concentration of AMPs are most likely related to sympathetic nervous system activity (Allgrove et al., 2008). Damage to epithelial cells through hyperventilation and subsequent exposure to environmental irritants, and the activation of neutrophils that follows may have led to increased secretion of these AMPs. Following physical damage (Dorschner et al., 2001) and contact with microbes (Duits et al., 2003), epithelial cells increase their expression of AMPs.

This study found that 30 days supplementation of the 14-strain probiotic did not affect the saliva AMPs measured. Previous studies with 4 weeks (Clancy et al., 2010; Cox et al., 2010) and 3 weeks (Tiollier et al., 2007) of probiotic supplementation were also found to have no effect on the saliva S-IgA concentration. However, S-IgA concentration was reported to be increased after 8 weeks and 16 weeks of a single strain probiotic supplementation (Gleeson et al., 2011a) and Kotani and colleagues (2010) also reported an accelerated S-IgA secretion after 12 weeks of daily ingestion of a single strain probiotic. It appears that the supplementation period (30 days) and the dosage of the probiotic used in the present study were not enough to induce any beneficial effects of the probiotic.

To date, there were no published studies (PubMed search) regarding the effects of mixtures of probiotics on AMPs. Nevertheless, there have been a few published studies reporting beneficial effects of multi-strain probiotics on incidence and duration of respiratory tract infection. Winkler and colleagues (2005) found that a mixture of probiotics decreased the incidence of infection by 13.6%. They also reported lower symptom severity and fewer numbers of days with fever in the multi-strain probiotic group compared to the placebo group. Similarly, de Vrese et al., (2006) reported reduced number of days with fever, duration of cold episode, and total symptom score in a mixtures probiotic group compared to a placebo

group. A meta-analysis in a recent review article found that there was a marginal beneficial effect of probiotics on the prevention of the common cold (Kang et al., 2013).

It is known that probiotics modulate immune function through their effects on epithelial cell function, including epithelial cell barrier function, epithelial cytokine secretion, and their antibacterial effects relating to colonization of the epithelial layer (Boiriviant and Strober, 2007). Probiotics also interact with the receptors on M cells and gastro intestinal tract and so may influence aspects of systemic immune function (Kudsk, 2002). From the present study results, it may be worth investigating the effects of this multi-strain probiotic on salivary responses but at a higher dose and for a longer supplementation period.

## **6.5 Conclusion.**

The present study found that 30 days supplementation of the 14-strain probiotic did not alter salivary antimicrobial proteins at rest and in response to an acute bout of prolonged exercise. However, prolonged exercise significantly increased lactoferrin and  $\alpha$ -amylase concentration post-exercise. It appears that 30 days supplementation period and the dose/concentration of the 14-strain probiotic used in this study was not enough to induce any beneficial effects on athletes' salivary antimicrobial proteins at rest and in response to an acute bout of prolonged exercise.

## CHAPTER 7

### **Study 5: Effects of a *Lactobacillus* probiotic on common cold infection incidence and mucosal immunity in endurance athletes.**

#### **Abstract**

The purpose of this study was to assess evidence of health and immune benefit by consumption of a *Lactobacillus* probiotic in highly physically active healthy people. In a single-centre, population-based, randomized, double-blind placebo-controlled trial, 267 student endurance sport athletes were randomly assigned to a liquid *Lactobacillus* probiotic (PRO) or placebo (PLA) supplement which was ingested twice daily for 16 weeks. Subjects completed validated questionnaires on respiratory tract infection (URTI) symptoms on a daily basis and on physical activity status at weekly intervals during the study period. Saliva samples were collected at entry and at 4, 8, 12 and 16 weeks of the intervention and analysed for antimicrobial protein (AMP) concentrations. Blood samples were collected before and after 16 weeks of the intervention for haematological analysis. Any difference in the proportion of subjects who presented with one or more episodes of URTI during the trial between the PRO and PLA groups was assessed by a chi-squared test. Comparison between the treatments for the number of URTI episodes was carried out using a Mann-Whitney test. Changes in saliva immune parameters before and after 4, 8, 12 and 16 weeks of the treatment period were analysed using two-way ANOVA with *post hoc* Sidak tests. The impact of URTI episodes on training volume was also evaluated by comparing physical activity levels (MET-h/week) on weeks when an URTI episode was present with the average MET-h/week when the subjects were healthy. There was no significant difference in URTI incidence between both treatment groups. When mean ( $\pm$  SEM) MET-h/week physical activity during healthy (no URTI symptoms) weeks was compared with mean physical activity during weeks when



an URTI episode occurred, there was a significant decrease in both treatment groups (PRO  $65.1 \pm 3.4$  vs.  $56.6 \pm 4.5$  MET-h/week,  $P = 0.001$ ; PLA  $71.5 \pm 3.0$  vs.  $47.2 \pm 3.4$  MET-h/week,  $P < 0.001$ ). When normalized, the extent of the reduction in training load was somewhat lower on PRO (13%) than PLA (33%). No significant treatment or interaction effect of regular probiotic was observed for saliva antimicrobial proteins or their secretion rates except for salivary secretory immunoglobulin A (S-IgA) concentration, which was significantly lower at baseline in PRO than in PLA ( $P = 0.048$ ). In summary, regular ingestion of a *Lactobacillus* probiotic tended to reduce the extent to which training was negatively affected in endurance athletes when infection was present, and increased both saliva S-IgA concentration and secretion rate over time but did not appear to influence URTI incidence or the duration and severity of URTI episodes. Two major confounding factors, namely the unexpectedly low incidence of URTI during the winter period and the lower baseline S-IgA in the PRO group may have prevented potential beneficial effects of probiotic supplementation from being identified.

## 7.1 Introduction.

Probiotics are food supplements that contain live microorganisms which when administered in adequate amounts confer a health benefit on the host. Some probiotics are incorporated into food supplements including yogurts, kefir, dark chocolate, microalgae and tempeh (Edward, 2011). There is now a reasonable body of evidence that regular consumption of probiotics can modify the population of the gut microbiota and influence immune function (Matsuzaki, 1998; Gill and Cross, 2002; Gill and Prasad, 2008; Mengheri, 2008; Borchers et al., 2009; Minocha, 2009) though it should be noted that such effects are strain specific. Suggested possible mechanism of actions of probiotics include modulation of the intestinal immune system, and displacement of potential pathogens through competitive exclusion or production of antimicrobial agents (Rowland et al., 2010). Documented health effects of probiotics include treatment of travellers' diarrhoea, relief of milk allergy in infants, reduction in the risk of atopic diseases, treatment of some inflammatory conditions, increased resistance to enteric pathogens, promotion of anti-tumour activity, and alleviation of some allergic and respiratory disorders in children (Hatakka et al., 2001; Kopp-Hoolihan, 2001; Saxelin et al., 2005; Rowland et al., 2010). Furthermore, it has been reported that probiotic supplementation enhances host resistance to upper respiratory tract infection (URTI) in the general population (Turchet et al., 2003; Winkler et al., 2005; de Vrese et al., 2006; Berggren et al., 2011) and several recent studies have indicated that some *Lactobacillus* probiotics can reduce URTI incidence in athletes (Cox et al., 2010; Gleeson et al., 2011a; West et al., 2011) who are generally considered to be a marginally immunocompromised population due to the depressive effects of hard physical exercise, psychological stress, sleep disturbance and negative energy balance on their immune system (Gleeson, 2007; Walsh et al., 2011).

The mucosal membranes covering the oral cavity, respiratory, gastrointestinal, and genitourinary tracts are continuously exposed to pathogenic microorganisms, thus effective protection of mucosal surfaces is important in conferring resistance to infection and depends on several components of the innate and acquired immune system. Antimicrobial proteins (AMPs) including salivary secretory IgA (S-IgA), lactoferrin and lysozyme are well established humoral factors of the innate immune system with antibacterial, antiviral and fungicidal properties (West et al., 2006; Zheng et al., 2012). Reduced levels of salivary AMPs in athletes may contribute to their increased risk of URTI (Fahlman and Engels, 2005; Gleeson et al., 1999, West et al., 2006; Neville et al., 2008; Gleeson et al., 2011b; Walsh et al., 2011). Salivary IgA levels have been observed to fall during intensive periods of athletic or military training (Gleeson et al., 1999; Fahlman and Engels, 2005; Tiollier et al., 2007; Neville et al., 2008) but some studies suggest that probiotic supplements may increase S-IgA (O'Connell et al., 2010) or help to maintain S-IgA during periods of intensive physical activity (Tiollier et al., 2007; Gleeson et al 2011a). However, the effects of probiotic supplementation on other AMPs have not been extensively studied even though exercise induced reduction in their concentrations has previously been reported (Cox et al., 1999; Innoue et al., 2004).

The aims of the present study were to examine the effects of 16 weeks of daily oral supplementation of a probiotic containing a Gram-positive *Lactobacillus* bacterium on infection incidence and salivary AMPs (S-IgA, lactoferrin, lysozyme and  $\alpha$ -amylase) and their secretion rates in a cohort of university-based endurance athletes during a period of winter training and competition. Reporting URTI episodes at a phone interview or using weekly as opposed to daily questionnaires has raised some obvious concerns about their reliability (Barrett et al., 2002). Daily questionnaires have been shown to be effective in several investigations involving athletic populations (Peters and Bateman, 1983; Robson-

Ansley et al., 2012). Hence, in the present study we used a validated daily questionnaire (Jackson et al., 1958) to establish the occurrence of URTI episodes and used the modification of Predy et al. (2005) to determine the total number of days with URTI symptoms.

## **7.2 Methods.**

### **7.2.1 Participants.**

Two hundred and sixty seven subjects who were engaged in regular sports training (predominantly endurance-based activities such as running, cycling, swimming, triathlon, team games and racquet sports) volunteered to participate in the study. Subjects ranged from recreationally active to Olympic triathletes and their self-reported training loads averaged 10 h/week. Subjects were required to complete a comprehensive health-screening questionnaire prior to starting the study and had not taken any regular medication, antibiotics or probiotics in the 3 months prior to the study. All subjects were fully informed about the rationale for the study and of all experimental procedures to be undertaken. Subjects provided written consent to participate in the study, which had earlier received the approval (document number R11-P128) of Loughborough University ethical advisory committee. Subjects were enrolled after having fulfilled all inclusion criteria, and presenting none of the exclusion criteria (determined by both questionnaire and interview).

Subjects could be included if they were currently healthy, had been involved in endurance training for at least 2 years, engaged in at least 3 sessions and at least 3 h of total moderate/high-intensity training time per week and were between 18 – 40 years of age. Subjects representing one or more of the following criteria were excluded from participation: smoking or use of any medication, currently taking probiotic supplements, suffered from or had a history of cardiac, hepatic, renal, pulmonary neurological, gastrointestinal,

haematological or psychiatric illness; objected to the prescription of diet (abstinence from fermented milk products other than the daily supplement).

A total of 267 healthy individuals were recruited as subjects and randomly assigned to one of two treatments (probiotic or placebo) with stratification by gender and type of sport (A: endurance sports such as triathlon, swimming, cycling and distance running; B: team games such as football, rugby and hockey; C: individual sports such as tennis, squash and badminton). Under double-blind procedures 128 received the probiotic and 139 received the placebo preparation. Of these 267 subjects 83 were females and 184 were males with the mean age of the study cohort at recruitment being  $20.6 \pm 2.8$  years (mean  $\pm$  SD).

Sample size estimation of at least 96 subjects per treatment group was based on an expected rate of  $2.1 \pm 1.2$  URTI episodes (mean  $\pm$  SD) during 4 winter months (Gleeson et al., 2011a), a target 20% reduction in number of episodes (to  $1.7 \pm 1.0$  URTI episodes), statistical power of 80% and type I error of 5%. Software called GPower 3.1 was used for the power analysis. We initially recruited 267 volunteers to account for an estimated 25% drop-out rate over the study period.

### 7.2.2 First visit.

For the first visit to the laboratory, subjects arrived in the morning at 08:30 – 10:30 following an overnight fast of approximately 12 h and their body mass and height were recorded. Information about the study was given to them and they then signed an informed consent form. Subjects then sat quietly for 10 min and completed a health-screening questionnaire and inclusion/exclusion criteria questionnaire before providing an unstimulated saliva sample as described in the general methods (Chapter 2, section 2.4.1)

Subsequently, a resting venous blood sample (5 ml) was collected into Vacutainer tube containing K<sub>3</sub>EDTA as described in the general methods (Chapter 2, section 2.4.3). Haematological analysis was immediately carried out on this sample as described in the general methods (Chapter 2, section 2.4.4). Subjects eligible for inclusion in the study were randomly assigned to the probiotic or placebo group and asked to start taking the supplement the next day.

### 7.2.3 Study intervention.

Probiotic (PRO) and placebo (PLA) supplements were supplied as fermented milk in sealed pots of 65 ml with date stamped expiry. The PRO drink contained a minimum of  $6.5 \times 10^9$  live cells of *Lactobacillus* bacteria in each pot. The PLA was identical in taste and colour to the PRO but contained no bacteria. The supplements were stored at 3 – 7°C and a fresh supply was provided by the manufacturer every 2 weeks. The name of the specific strain of bacteria and the name of the manufacturer cannot be disclosed at the present time due to a confidentiality agreement. Subjects returned to the laboratory every 2 – 3 weeks to receive a fresh supply of supplement. A compliance log of sample collection was taken. Subjects consumed the supplement twice per day; one 65 ml pot with breakfast and one with the evening meal for 16 weeks. Subjects were asked to keep a record of any days when they missed taking the supplement.

### 7.2.4 Study protocol.

During the 4-month intervention period with PRO or PLA subjects were requested to continue with their normal training programs. Other supplements (e.g. vitamins, minerals) and consumption of additional probiotics or any fermented dairy products (e.g. yoghurt, sour cream, crème fraiche) were not permitted during this period. Subjects completed a validated health (URTI symptoms) questionnaire (Jackson et al., 1958) on a daily basis. They were not

required to abstain from medication when they were suffering from illness symptoms but they were required, on a weekly basis, to report any un-prescribed medications taken, visits to the doctor or any prescribed medications.

The illness symptoms listed on the questionnaire were: sneezing, headache, malaise, nasal discharge, nasal obstruction, sore throat, cough, ear ache, hoarseness, fever, chilliness and joint aches and pains. The non-numerical severity ratings of mild, moderate and severe of severity of symptoms were scored as 1, 2 or 3, respectively to provide a quantitative means of data analysis and the total symptom score for every subject each day was calculated as a sum of multiplied numbers of symptoms experienced by the numerical severity ratings. An URTI was deemed present when (i) total symptom score was  $\geq 15$  on any two consecutive days and (ii) when a subject positively indicated suffering a common cold on  $\geq 3$  days (Jackson et al., 1958). Subjects were also asked to rate the impact of illness symptoms on their ability to train (above normal, at the same level, below normal or training stopped). The total number of URTI symptom days was also determined as the number of days with a symptom score of  $\geq 5$  according to Predy et al. (2005).

Subjects also completed a standard short form of International Physical Activity Questionnaire (IPAQ; <http://www.ipaq.ki.se/downloads.htm>) at weekly intervals, thus providing a quantitative information on training loads in metabolic equivalents (MET)-h/week (Craig et al., 2003). Subjects attended the laboratory every 4 weeks following an overnight fast. Subjects were required to abstain from any strenuous physical activity for 24 h before coming to the laboratory. During these visits body mass was recorded/calculated and an unstimulated saliva sample was collected. Venous blood samples were collected during the final visit and analysed for haematological variables as described for the first visit to the laboratory. The intra-assay coefficient of variation for all blood variables was less than 3.0%.

### 7.2.5 Saliva analysis.

The saliva volume collected and flow rate were estimated as described in the general methods (Chapter 2, section 2.4.1.1). Saliva samples were analysed for S-IgA,  $\alpha$ -amylase activity, lactoferrin and lysozyme as described in the general methods (Chapter 2, section 2.4.2). Secretion rates for each of the salivary AMPs were calculated as described in the general methods (Chapter 2, section 2.4.1.1).

### 7.2.6 Data analysis.

Any difference in the proportion of subjects who presented with one or more episodes of URTI during the trial between the PRO and PLA groups was assessed by a chi-squared test. Comparison between the treatments for the number of URTI episodes was carried out using a Mann Whitney test. For subjects who experienced one or more URTI episodes, comparison between the treatments for total symptom severity score and the mean duration of URTI episodes was carried out using unpaired t tests. Comparison between the treatments for the number of days with an URTI symptom score  $\geq 5$  (Predy et al., 2005) was carried out using an unpaired t test. Changes in saliva immune parameters before and after 4, 8, 12 and 16 weeks of the treatment period were analysed using two-way ANOVA. Saliva AMP changes over time within the PRO and PLA group were assessed using repeated-measures ANOVA with *post hoc* Sidak tests. We also evaluated the impact of URTI episodes on training volume by comparing physical activity levels (MET-h/week) on weeks when an URTI episode was present with the average MET-h/week when the subjects were healthy.

To fully appreciate the design of the current study additional analyses were performed focussing on time in weeks as independent parameter and incidence of common-cold infection, concentration and secretion rate of AMPs, and finally, physical activity as dependent parameter. Stepwise dummy regression was applied using a dummy factor to



identify the treatment (1: Probiotic; 0: Placebo), week number from the start and the combination of the two as an indicator for the change during the interval of the study as independent factors (Kleinbaum et al., 1998). This analysis enables not only to monitor a potential difference in incidence between both groups, but also a change in the dependent factor during the interval of the present study (change in time). As dependent parameters the incidence of common-cold infection, or the change in concentration or secretion rate of AMPs, or change in physical activity were taken. The change in parameter was calculated by subtracting the starting value per person per parameter from the values at the various intervals within the experimental design of the present study.

Whenever feasible, not only a linear regression was performed, but also a quadratic or logarithmic. Poisson regression was done with respect to the incidence of common-cold infection to appreciate the discrete distribution of the dependent parameter and normal regression for the outcome in salivary AMPs. To minimize the impact by outliers on the regression outcome robust regression was performed as well (Maronna et al., 2006).

With respect to the incidence of common-cold infection stratification in outcome was also done to minimize the impact by confounding factors. Three various approaches were used:

- Interval (decreasing versus increasing incidence)
- Gender (males versus females)
- Type of sport (team games, individual sports, and endurance sports)

Since the incidence over time revealed a parabolic curve, the time interval to be used in the incidence of common-cold infection was split into two segments as well: a decreasing and an increasing fraction. This reflects the sinusoidal character of the common cold infection pattern in general.

Data are presented as mean  $\pm$  SEM. A *P* value of 0.05 or less was considered to show statistical significance, using two-sided testing.

### **7.3 Results.**

#### 7.3.1 Adherence to the study.

Of the 267 subjects, 239 subjects (116 PRO, 123 PLA) completed the full 16 weeks of the study. The number of dropouts was smaller than anticipated and there were 12 dropouts on PRO and 16 on PLA (Table 7.1). Many of the subjects were athletes involved in national and international level competition and it was difficult or impossible for them to take the liquid supplement to competitions where air travel is needed due to airport security restrictions. Several subjects withdrew due to this issue. Some subjects also went abroad on vacation during the Christmas period which resulted in them dropping out of the study for the same reason or because we were unable to get the supplements to them. Other subjects withdrew due to injury or persistent non-respiratory illness (preventing them from performing training) or due to undisclosed reasons. Adherence to the intervention was good: subjects who completed the study reported that they missed taking the supplement on average only on 3 days (range 0 – 14 days). Saliva samples were obtained on all 5 visits from 237 subjects. Blood samples were obtained pre- and post-intervention from 237 subjects, although sufficient blood volume to permit analyses of all variables was only obtained from 233 subjects.

*Table 7.1: Flow diagram showing numbers of subjects in weeks 1 and 16.*

	<b>Total</b>	<b>PRO</b>	<b>PLA</b>
<b>Subjects, week 1</b>	267	128 (91M, 37F)	139 (94M, 45F)
<b>Subjects, week 16</b>	239	116 (84M, 32F)	123 (85M, 38F)

M = males; F = females

### 7.3.2 Patterns in baseline characteristics.

Baseline characteristics of the subjects who completed the study, including body mass, height, body mass index (BMI) and self-reported weekly training duration for the PRO and PLA groups are shown in Table 7.2. The baseline values were similar with no statistically significant difference between the two treatment groups, except for BMI which was higher on PRO than on PLA ( $P < 0.01$ ).

*Table 7.2: Baseline characteristics of the subjects who completed the study.*

	<b>PRO</b>	<b>PLA</b>
<b>Number (M, F)</b>	116 (84, 32)	123 (85, 38)
<b>Sport (A, B, C)</b>	70, 12, 34	66, 13, 44
<b>Age (years)</b>	20.8 ± 0.3	20.5 ± 0.2
<b>Body mass (kg)</b>	74.0 ± 1.1	73.1 ± 1.0
<b>Height (cm)</b>	175.8 ± 0.9	177.3 ± 0.8
<b>BMI (kg/m<sup>2</sup>)</b>	23.8 ± 0.2*	23.2 ± 0.2
<b>Training (h/week)</b>	9.6 ± 0.4	9.7 ± 0.4

Values are expressed as mean ± SEM. M = males; F = females. A = Endurance sports; B = Games players; C = Individual sports. \* Significantly different from PLA,  $P < 0.01$ .

### 7.3.3 Physical activity levels.

Mean training loads were not different for the PRO and PLA groups:  $66.6 \pm 3.1$  and  $67.6 \pm 2.6$  MET-h/week, respectively ( $P = 0.817$ ). This is equivalent to about  $11 \pm 1$  h of moderate-vigorous activity per week (Figure 7.1). There was a significant drop in MET-h/week in both groups from week 4 till weeks 8 – 10 after which the activity increased again to almost the same level as at the start. However, when a distinction was made in this between men and women, the decrease in physical activity for the women was significantly ( $P < 0.01$ ) greater in the PRO group than in the PLA group. This observation was not encountered in that for the men.

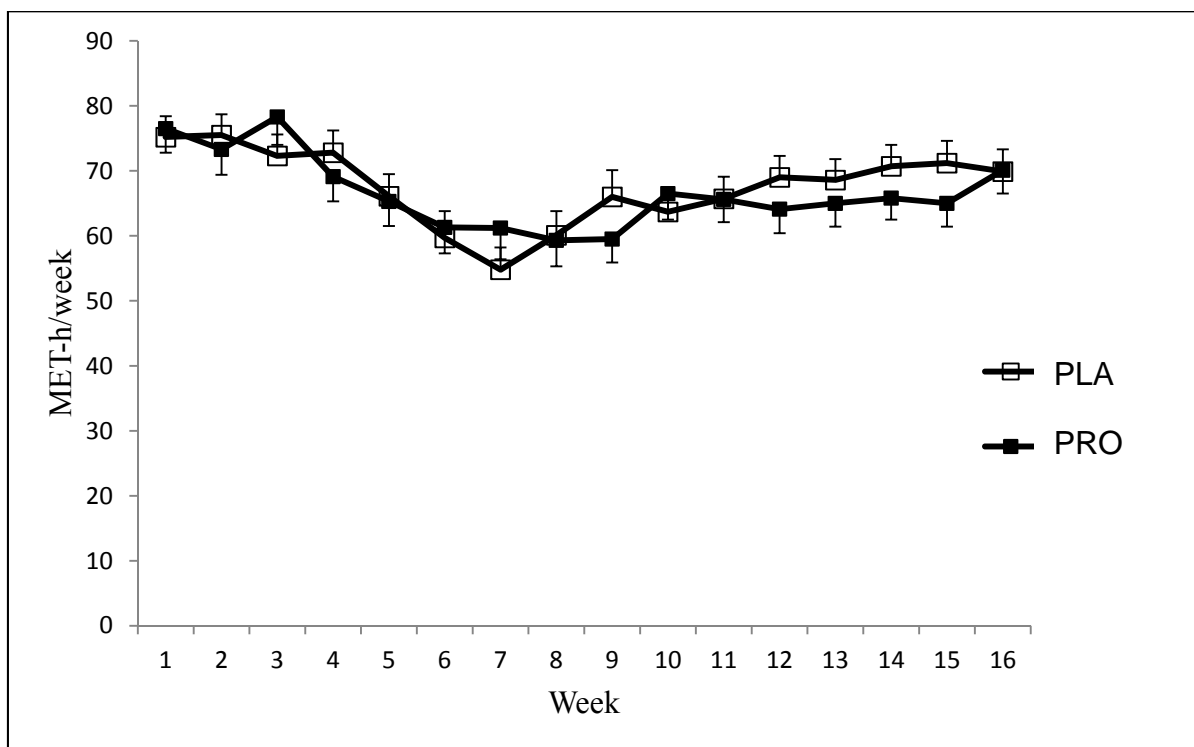


Figure 7.1: Training loads in MET-h/week over the 16-week study period for subjects who completed the study. Data are mean and SEM. No difference between treatments.

#### 7.3.4 URTI incidence.

Analysis of the URTI symptom questionnaires indicated that  $4.0 \pm 0.1$  % of the cohort experienced an URTI episode each week (Figure 7.2). One hundred and thirty six subjects did not experience a single URTI episode during the study period whereas 103 subjects experienced at least one URTI episode during the study period. The proportion of subjects from the PLA group who experienced one or more weeks with URTI symptoms was 0.41 whereas the proportion from the PRO group was 0.46. The difference in proportions was not significant (chi-squared test statistic = 0.618,  $P = 0.437$ ).

The mean number of URTI episodes, number of days with URTI symptom score  $\geq 5$  and mean total symptom score and duration of URTI episodes were not different between PRO and PLA (Table 7.3). The proportion of subjects whose training was negatively affected when URTI was present was not different between PRO and PLA (0.63 and 0.76; chi-squared test statistic = 2.085,  $P = 0.196$ ). When mean MET-h/week physical activity during healthy (no URTI symptoms) weeks was compared with mean physical activity during weeks when an URTI episode occurred, there was a significant decrease in both treatment groups [PRO  $65.1 \pm 3.4$  vs.  $56.6 \pm 4.5$  MET-h/week,  $P = 0.001$ ; PLA  $71.5 \pm 3.0$  vs.  $47.2 \pm 3.4$  MET-h/week,  $P < 0.001$ ]. When normalized, the extent of the reduction in training load was somewhat lower on PRO (13%) than PLA (33%).

The number of URTI episodes was positively correlated ( $P < 0.001$ ) with age and not with sex. The URTI total symptom score was not significantly higher in females than in males but the duration of URTI episodes was significantly ( $P < 0.03$ ) higher in females than in males. The number of days with URTI symptom score above 5 was not significantly higher in females than in males ( $P = 0.08$ ). There were no differences in the week number of the first URTI episode between males and females.

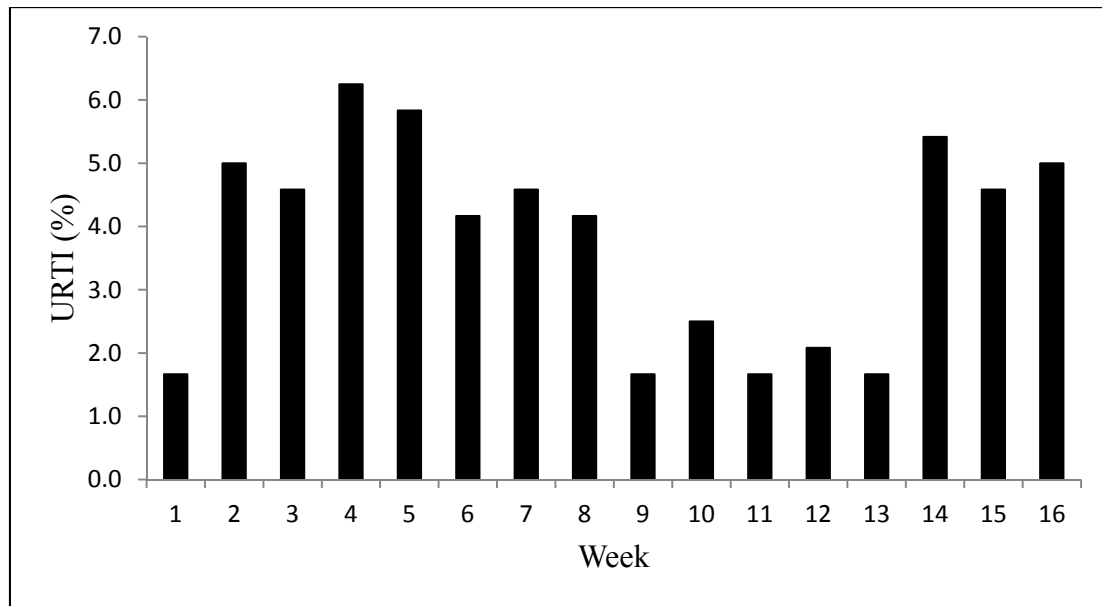


Figure 7.2: Percentage of the cohort reporting a URTI episode for each week of the study period.

Table 7.3: Number of URTI episodes, number of days with URTI symptom score  $\geq 5$ , and mean total symptom score and duration for URTI episodes in the 16-week period.

	PRO	PLA	P
<b>URTI episodes</b>	0.70 $\pm$ 0.08	0.59 $\pm$ 0.08	0.321
<b>Days with score <math>\geq 5</math></b>	6 $\pm$ 1	5 $\pm$ 1	0.404
<b>Total symptom score</b>	100.0 $\pm$ 7.2	94.9 $\pm$ 6.9	0.740
<b>URTI episode duration</b>	13.5 $\pm$ 0.8	12.5 $\pm$ 0.7	0.534

Values are expressed as mean  $\pm$  SEM.

### 7.3.5 Medication and visits to the doctor during URTI episodes.

During weeks when an URTI episode was present, the proportion of subjects who took medication was similar in the PRO and PLA groups (0.23 and 0.12, respectively; chi-squared test = 2.020,  $P = 0.198$ ) and the proportion who went to see their doctor was also similar in the PRO and PLA groups (0.02 and 0.06, respectively; chi-squared test = 1.166,  $P = 0.353$ ).

### 7.3.6 Outcome of the dummy stepwise regression analysis for URTI incidence.

Dummy stepwise Poisson regression revealed that the difference in incidence of common-cold infections was not significantly different between persons consuming the Probiotic drink or the Placebo during the interval of the present study (Figure 7.3), acknowledging the parabolic shape of the curves for both PRO as well as PLA.

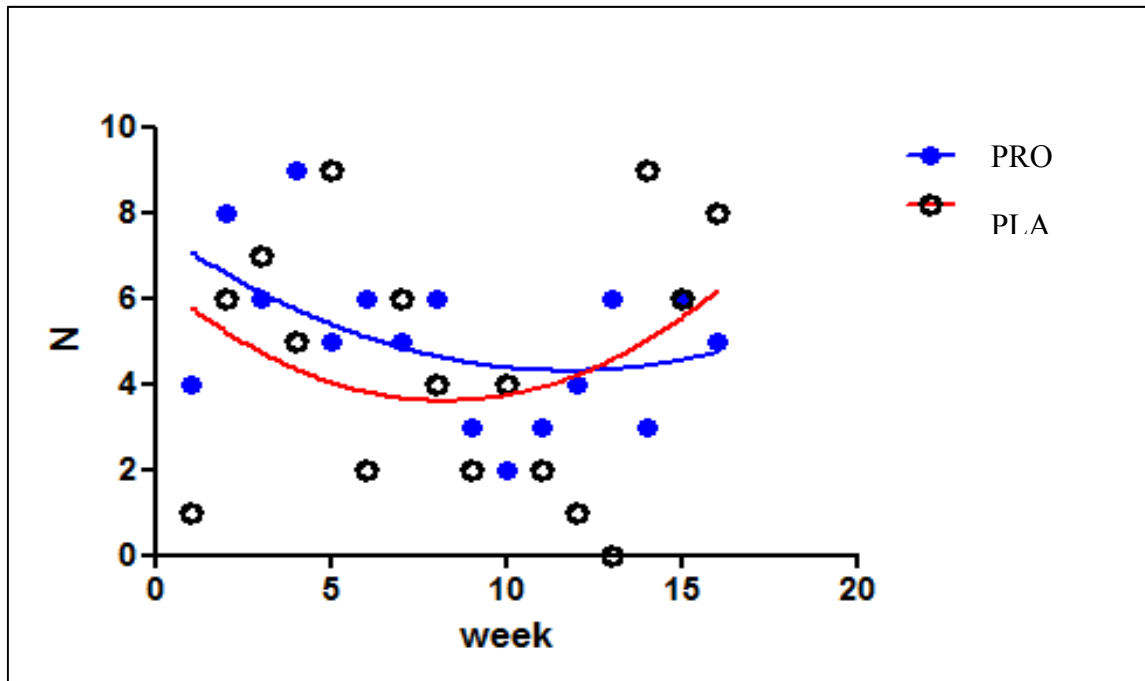


Figure 7.3: Number of common cold (URTI) episodes during the study period.

#### 7.3.6.1 Stratification I: time interval.

Interestingly though, when the time interval of the study was separated in a significantly (week 2 – 10;  $P < 0.01$ ) descending and significantly (week 11 – 16;  $P < 0.01$ ) ascending part, the difference in the latter with respect to the number of incidences became higher in the PLA than in the PRO group, although this difference did not reach significance. In contrast the incidence in the PRO group was higher, though not significantly, than that in the PLA group during the descending phase which was already apparent at the start of the intervention. This suggests a time-dependent effect by PRO on the incidence of the common-

cold infection. Moreover, when starting during a descent in incidence the room for improvement by the treatment could be too limited to detect any effect.

### 7.3.6.2 Stratification II: gender.

When the outcome for the male subjects only was examined (distinguished from those in the females) a significantly ( $P < 0.05$ ) higher incidence in common-cold infections was observed in the PRO than in the PLA group throughout the interval of the study (Figure 7.4). However, this effect was found to be outlier-dependent ( $P = 0.06$ ) as demonstrated via robust regression. For female subjects the situation was found to be opposite: throughout the interval the number of episodes was higher in the PLA than in the PRO group ( $P = 0.05$ ) (Figure 7.5), again disappearing after robust regression ( $P = 0.11$ ).

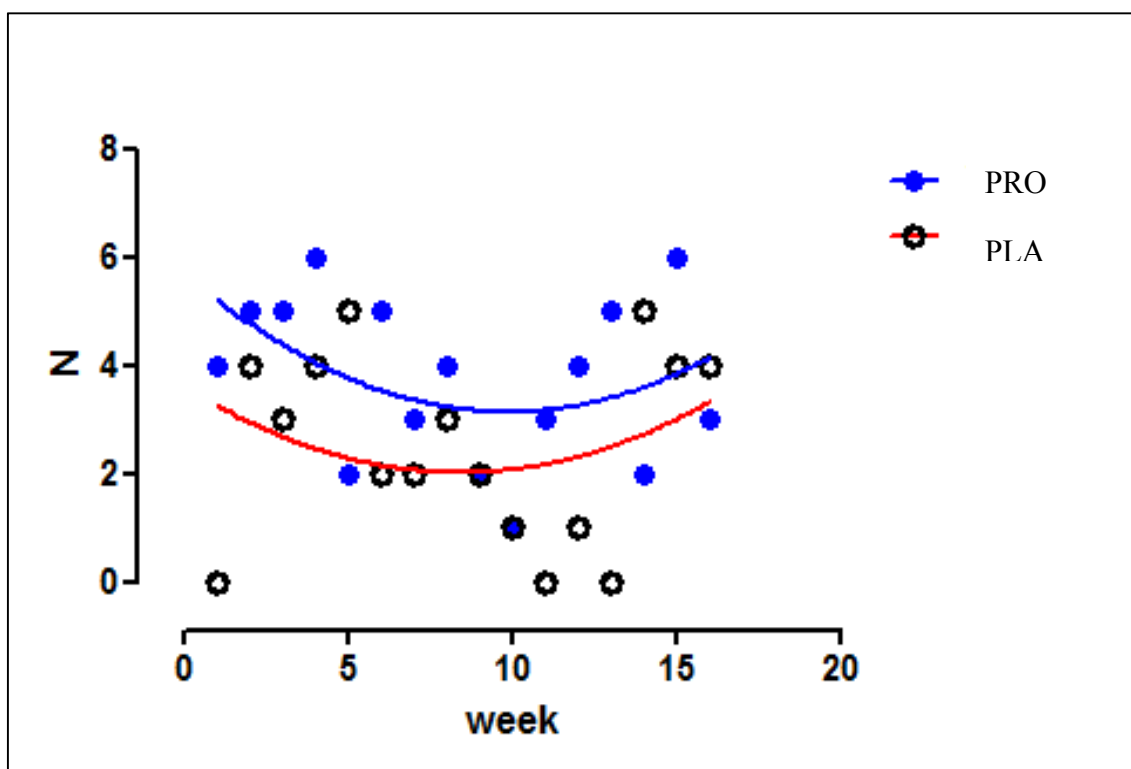


Figure 7.4: Number of common cold (URTI) episodes in males during the study period.



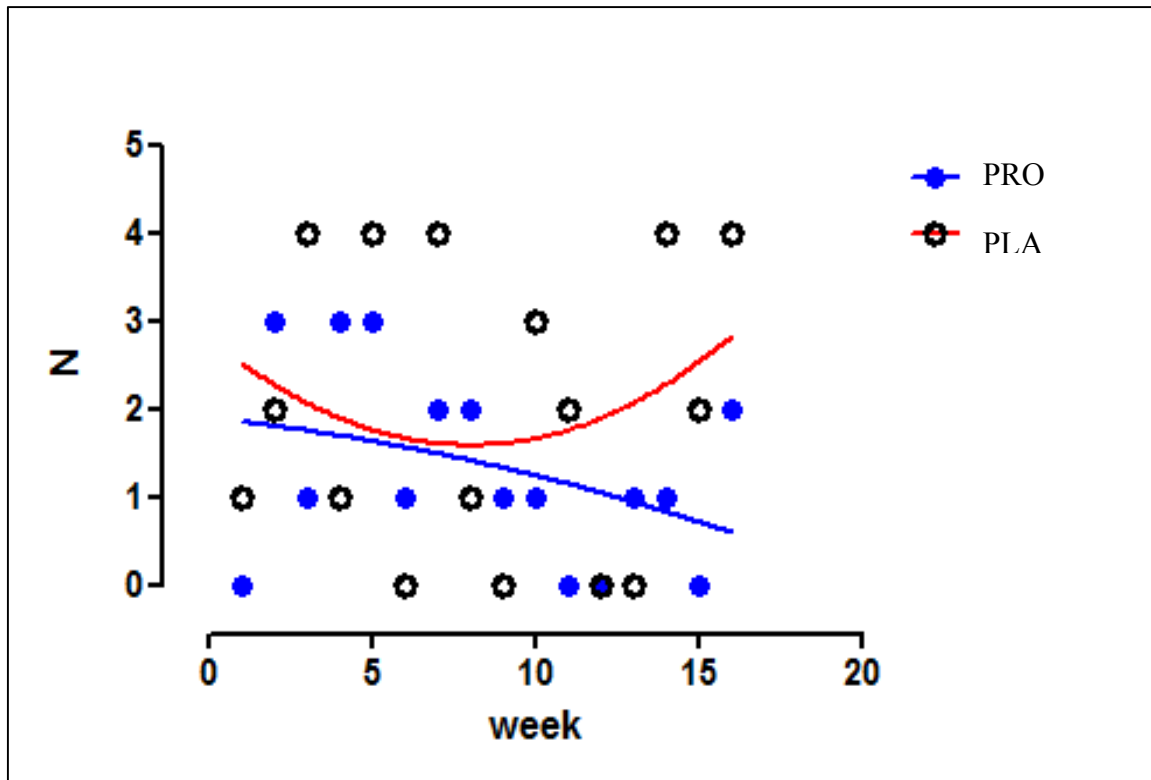


Figure 7.5: Number of common cold (URTI) episodes in females during the study period.

### 7.3.6.3 Stratification III: type of sport.

When the type of sport was considered: team game players, individual sport players and endurance sport athletes, no impact on the outcome was observed. In all three sport types there was no difference in the incidence of common-cold infections between the PRO and PLA groups. A potentially beneficial effect by consumption of PRO was the strongest in the endurance sport athletes, but again the effect was not statistically significant.

### 7.3.7 Salivary antimicrobial proteins.

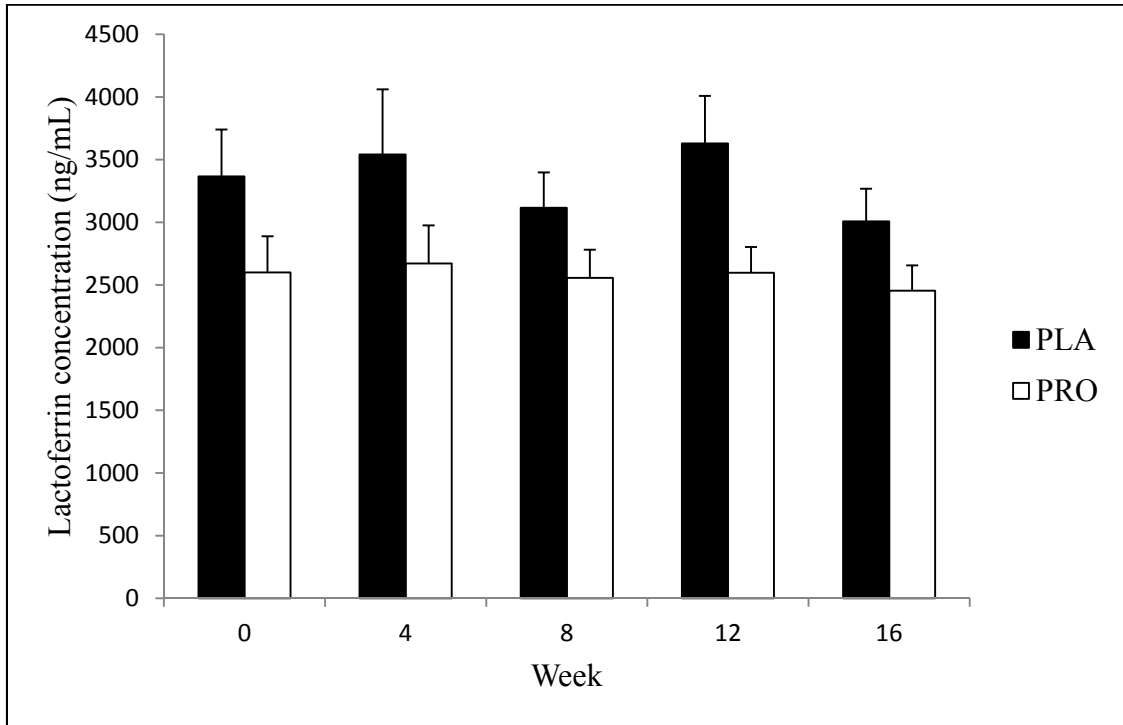
#### 7.3.7.1 Lactoferrin.

Saliva lactoferrin concentration (Figure 7.6A) was not different on PRO and PLA (time, interaction and treatment effects:  $F_{(3,779,842.752)} = 0.846$ ,  $P = 0.491$ ;  $F_{(3,779,842.752)} = 0.299$ ,  $P = 0.869$ ;  $F_{(1,223)} = 3.510$ ,  $P = 0.062$ , respectively). Similarly, saliva lactoferrin

secretion rate (Figure 7.6B) was not different between PRO and PLA (interaction and treatment effects:  $F_{(3,685,821.699)} = 0.363, P = 0.819$ ;  $F_{(1,223)} = 0.566, P = 0.453$ , respectively).

No significant effect of time for lactoferrin concentration was found in PRO or PLA ( $F_{(3,594,388.100)} = 0.170, P = 0.941$ ;  $F_{(3,729,428.846)} = 0.973, P = 0.418$ ). A significant main effect of time for the lactoferrin secretion rate was found ( $F_{(3,685,821.699)} = 6.101, P < 0.001$ ). Within group one way ANOVA revealed no effect of time in PRO ( $F_{(3,511,379.146)} = 1.862, P = 0.125$ ) but a significant effect of time was found in PLA ( $F_{(3,551,408.398)} = 4.588, P = 0.002$ ) with values higher than baseline at weeks 12 and 16 ( $P \leq 0.022$ ). However, there were no significant differences in this between PRO and PLA.

A



B

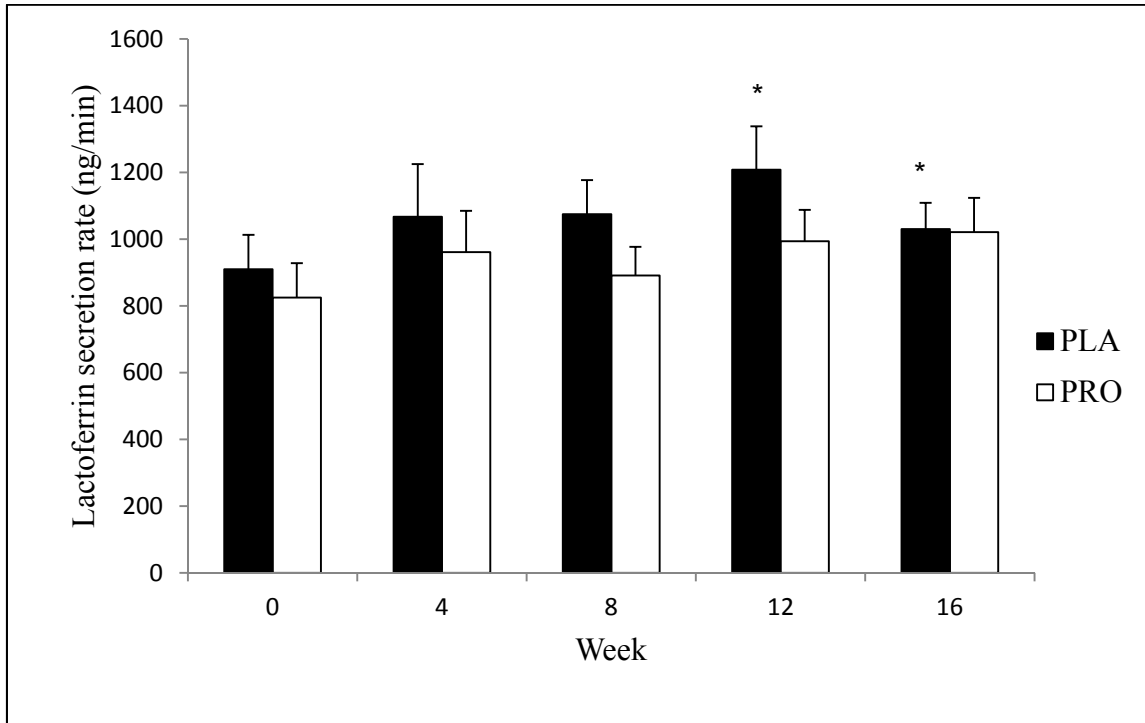


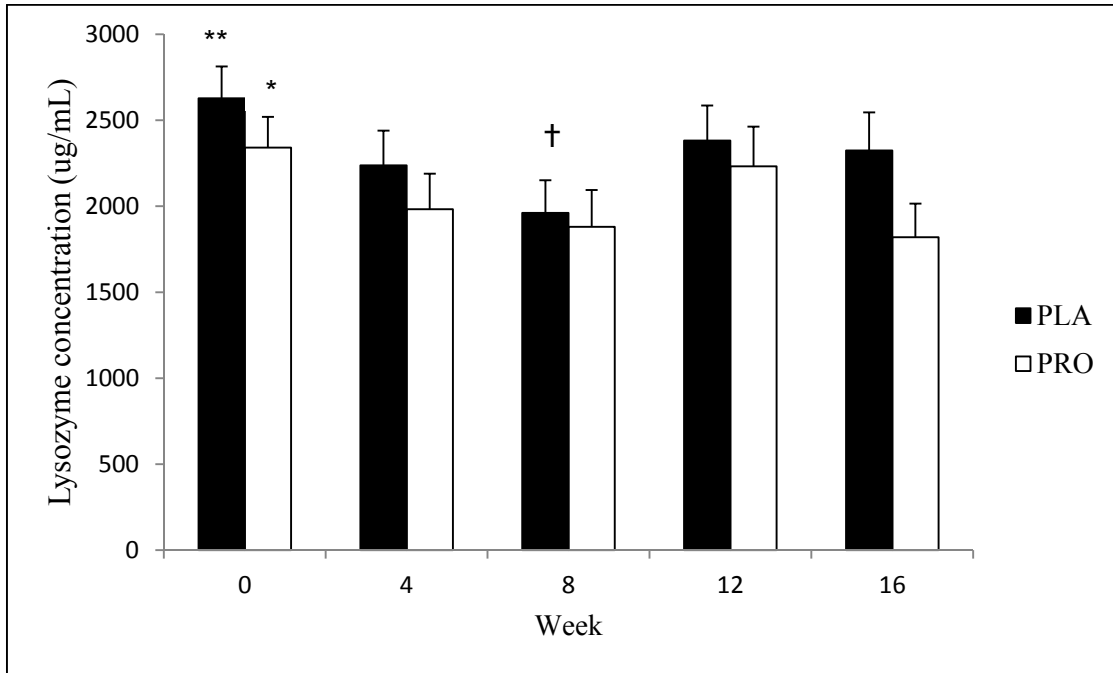
Figure 7.6: Saliva lactoferrin concentration (A) and secretion rate (B) before and after 4, 8, 12 and 16 weeks of the study. \* different from week 0 in the PLA trial,  $P \leq 0.022$ . (PRO,  $n = 111$ ; PLA,  $n = 121$ ).

### 7.3.7.2 Lysozyme.

No significant differences were observed in saliva lysozyme concentration (Figure 7.7A) between PRO and PLA (interaction and treatment effects:  $F_{(3.740, 755.536)} = 0.188$ ,  $P = 0.937$ ,  $F_{(1,202)} = 3.675$ ,  $P = 0.057$ , respectively). No significant differences were found in saliva lysozyme secretion rate (Figure 7.7B) between PRO and PLA (interaction and treatment effects:  $F_{(3.635, 734.260)} = 0.312$ ,  $P = 0.853$ ,  $F_{(1,202)} = 1.447$ ,  $P = 0.230$ , respectively).

A significant main effect of time for the lysozyme concentration was observed ( $F_{(3.740, 755.536)} = 14.949$ ,  $P < 0.001$ ). Within group one way ANOVA revealed a significant effect of time was found for lysozyme concentration in PRO ( $F_{(4, 392)} = 6.833$ ,  $P < 0.001$ ) with values higher at baseline than at weeks 8 and 16 ( $P \leq 0.001$ ). Similarly, a significant effect of time was found for lysozyme concentration in PLA ( $F_{(3.687, 383.498)} = 8.387$ ,  $P < 0.001$ ) with values higher at baseline than at weeks 4, 8 and 16 and between week 8 and 12 ( $P \leq 0.031$ ). Additionally, a significant main effect of time for the lysozyme secretion rate was found ( $F_{(3.635, 734.260)} = 4.586$ ,  $P = 0.002$ ). Within group one way ANOVA revealed no significant effect of time for lysozyme secretion rate in PRO ( $F_{(3.521, 345.071)} = 1.719$ ,  $P = 0.154$ ). A significant effect of time for lysozyme secretion rate was found in PLA ( $F_{(3.525, 366.621)} = 3.271$ ,  $P = 0.016$ ) with a lower value at week 8 than at week 12.

A



B

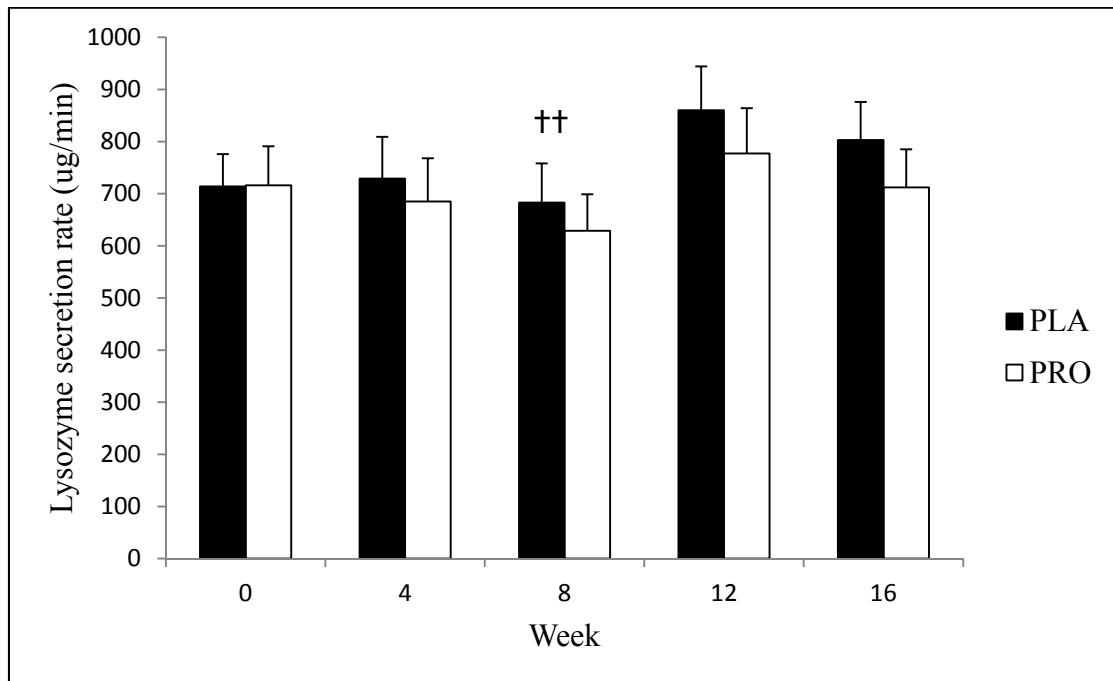


Figure 7.7: Saliva lysozyme concentration (A) and secretion rate (B) before and after 4, 8, 12 and 16 weeks of the study. \* different from weeks 8 and 16 in the PRO trial,  $P \leq 0.001$ ; \*\* different from weeks 4, 8 and 16 in the PLA trial,  $P \leq 0.031$ ; † different from week 12 in the PLA trial,  $P = 0.003$ ; †† different from week 12 in the PLA trial; (PRO,  $n = 108$ ; PLA,  $n = 114$ ).

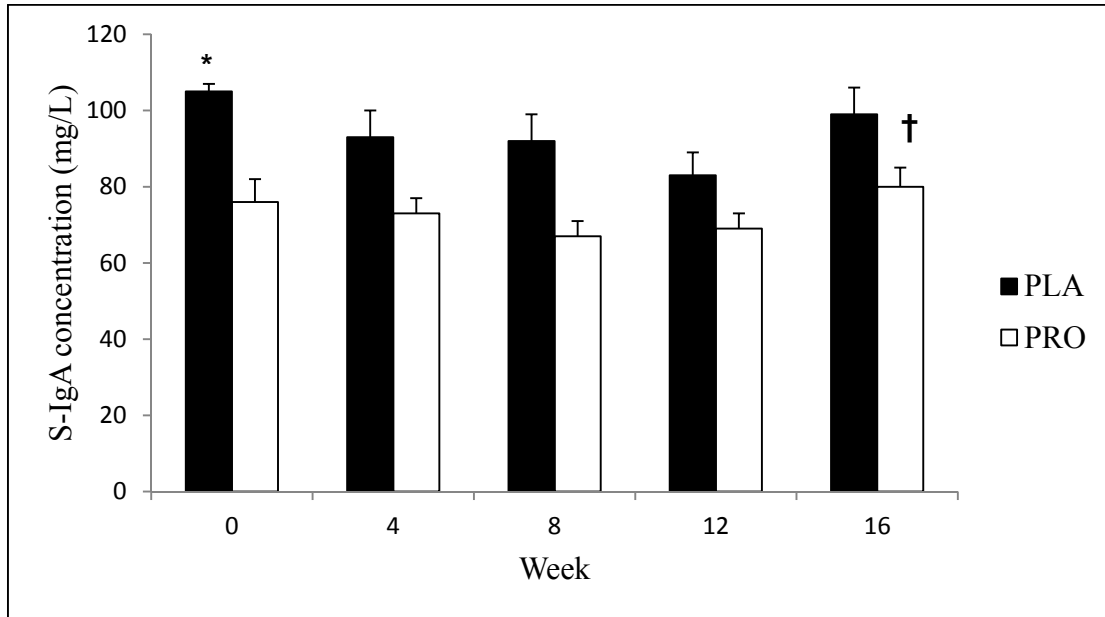
### 7.3.7.3 Saliva S-IgA.

Saliva S-IgA concentration was higher on PLA than PRO (significant treatment effect:  $F_{(1,228)} = 3.949$ ,  $P = 0.048$ ), due to  $t = 0$  differences. This difference was evident at baseline but not at any other time point (Figure 7.8A). No significant differences were found in saliva S-IgA secretion rate (Figure 7.8B) between PRO and PLA (interaction and treatment effects:  $F_{(3.730, 850.391)} = 0.861$ ,  $P = 0.481$ ,  $F_{(1,228)} = 0.600$ ,  $P = 0.439$ , respectively).

A significant main effect of time for the S-IgA concentration was observed ( $F_{(4, 912)} = 6.462$ ,  $P < 0.001$ ). Within group one way ANOVA revealed a significant time effect for S-IgA in PLA ( $F_{(4, 472)} = 3.624$ ,  $P = 0.006$ ), but *post hoc* Sidak test showed no differences. A significant effect of time for S-IgA in PRO was observed ( $F_{(4, 440)} = 3.919$ ,  $P = 0.004$ ) with values higher at week 16 than at weeks 8 and 12 ( $P \leq 0.022$ ).

Similarly, a significant main effect of time for the S-IgA secretion rate was found ( $F_{(3.730, 850.391)} = 15.690$ ,  $P < 0.001$ ). Within group one way ANOVA revealed a significant effect of time in PRO ( $F_{(3.555, 390.999)} = 10.534$ ,  $P < 0.001$ ) with values higher at week 16 than at other time points and with week 12 higher than at baseline ( $P \leq 0.032$ ). Similarly, a significant time effect was found in PLA ( $F_{(4, 472)} = 6.092$ ,  $P < 0.001$ ) with values higher at week 16 than at weeks 0, 4, 8 ( $P \leq 0.013$ ).

A



B

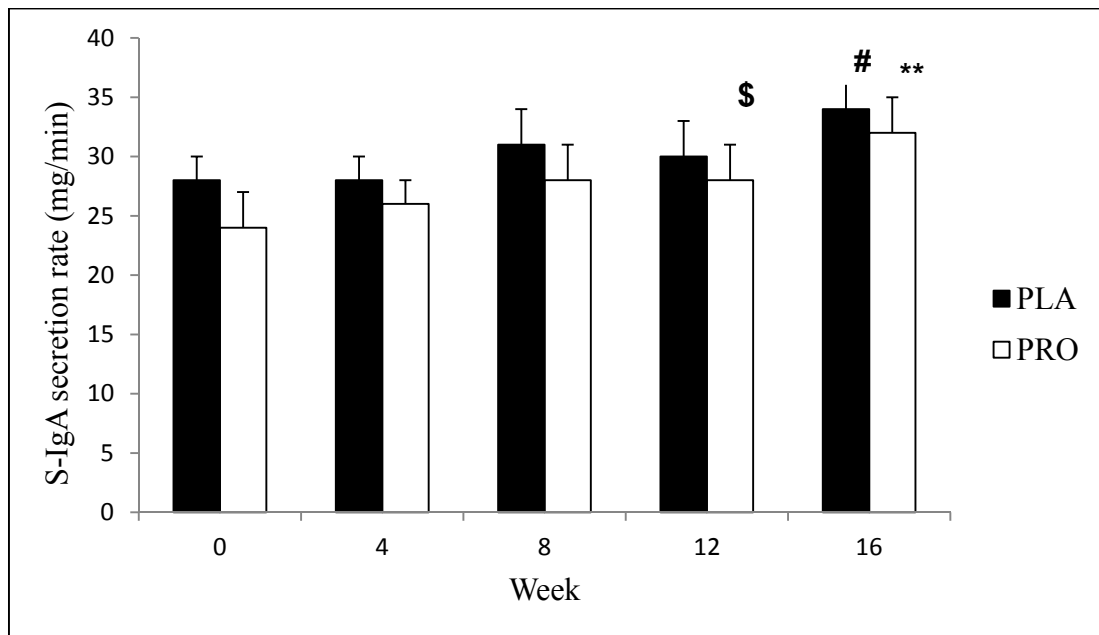


Figure 7.8: Saliva IgA concentration (A) and secretion rate (B) before and after 4, 8, 12 and 16 weeks of the study. \*  $P < 0.05$  vs PRO; † different from weeks 8 and 12 in PRO,  $P \leq 0.022$ ; \*\* different from other time points in PRO,  $P \leq 0.013$ ; \$ different from week 0 in PRO,  $P = 0.032$ ; # different from weeks 0, 4 and 8 in PLA,  $P \leq 0.013$ . (PRO,  $n = 113$ ; PLA,  $n = 120$ ).

#### 7.3.7.4 Alpha-amylase.

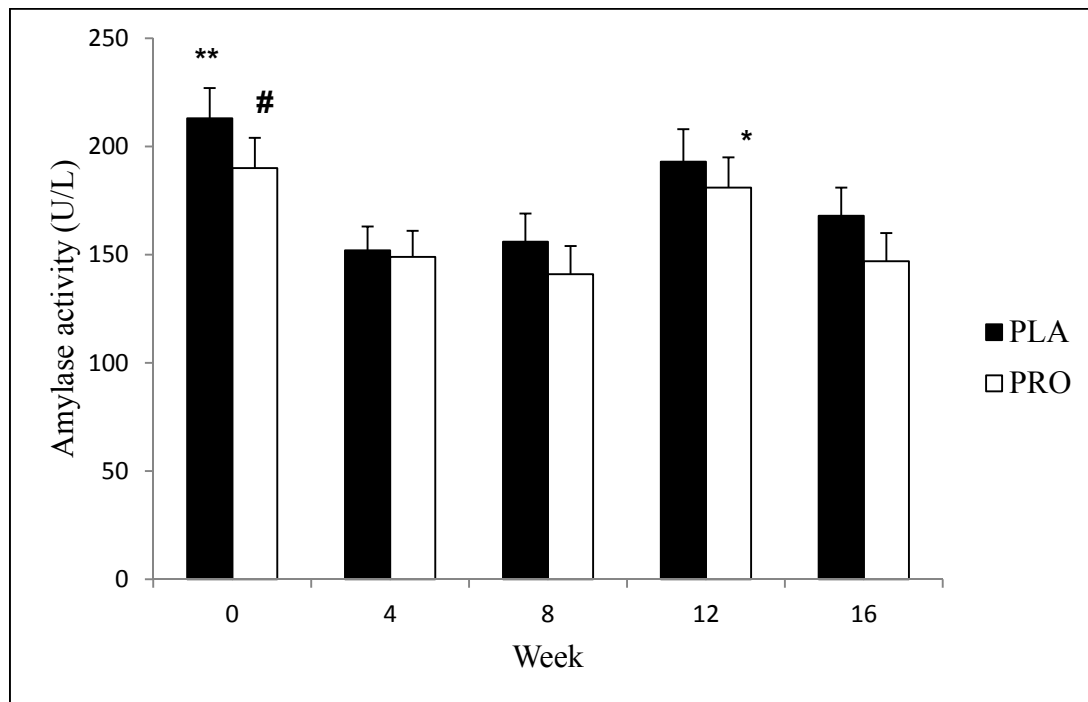
Saliva  $\alpha$ -amylase activity (Figure 7.9A) was not different between PRO and PLA (interaction and treatment effects:  $F_{(3.780,827.852)} = 0.445$ ,  $P = 0.765$ ;  $F_{(1,219)} = 1.159$ ,  $P = 0.283$ , respectively), Similarly, saliva  $\alpha$ -amylase secretion rate (Figure 7.9B) was not different between PRO and PLA (interaction and treatment effects:  $F_{(3.764,824.335)} = 0.616$ ,  $P = 0.641$ ;  $F_{(1,219)} = 0.106$ ,  $P = 0.745$ , respectively).

A significant main effect of time for the  $\alpha$ -amylase activity was found ( $F_{(3.780, 827.852)} = 16.084$ ,  $P < 0.001$ ). Within group one way ANOVA revealed a significant effect of time in PRO ( $F_{(3.646, 386.423)} = 8.486$ ,  $P < 0.001$ ) with values higher at baseline than at weeks 4, 8 and 16 ( $P \leq 0.014$ ) and higher at week 12 than at week 16 ( $P = 0.035$ ). Likewise, a significant effect of time was found in PLA ( $F_{(4, 452)} = 8.068$ ,  $P < 0.001$ ) with a higher value at baseline than at weeks 4, 8 and 16 ( $P < 0.001$ ).

Similarly, a significant main effect of time for the  $\alpha$ -amylase secretion rate was observed to be different between PRO and PLA ( $F_{(3.764, 824.335)} = 7.050$ ,  $P < 0.001$ ). Within group one way ANOVA revealed a significant effect of time in PLA ( $F_{(4, 452)} = 2.940$ ,  $P = 0.020$ ) but *post hoc* Sidak tests revealed no differences. A significant effect of time was found in PRO ( $F_{(3.695, 391.622)} = 4.742$ ,  $P = 0.001$ ) with values higher at week 12 than at weeks 4 and 8 ( $P \leq 0.019$ ).



A



B

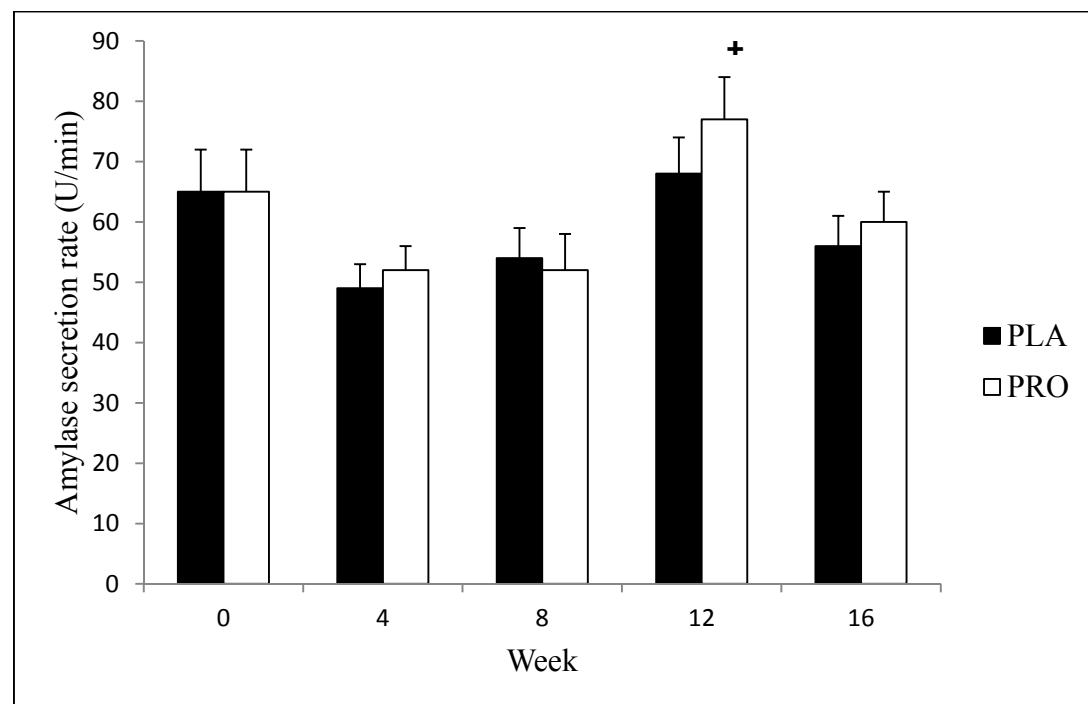


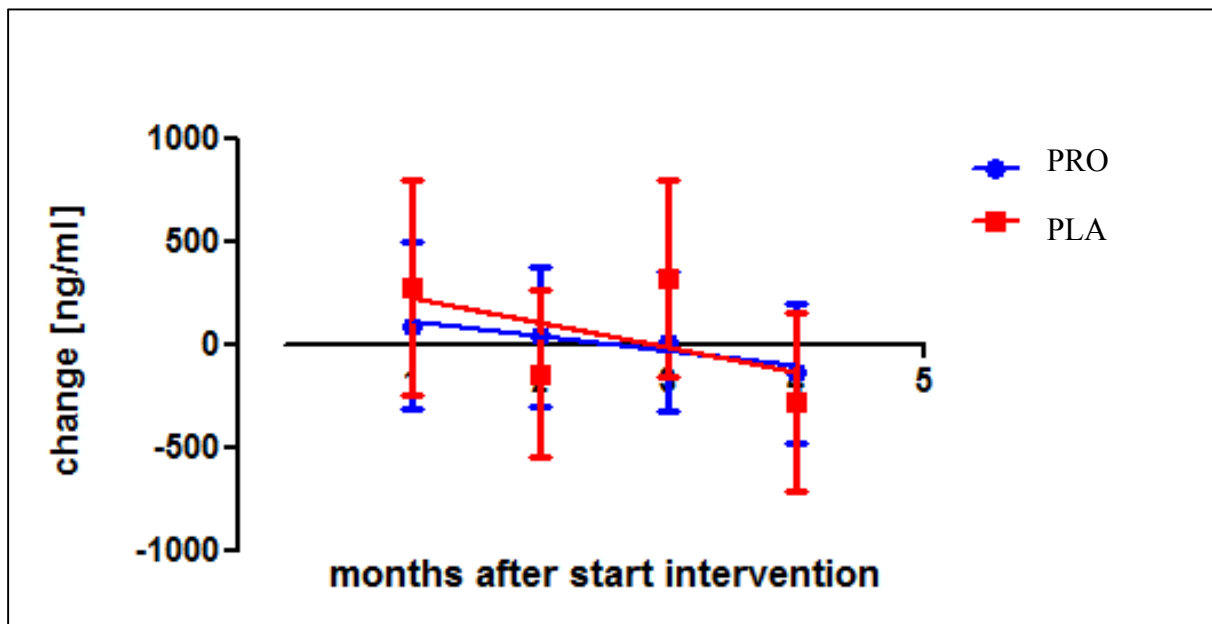
Figure 7.9: Saliva amylase activity (A) and secretion rate (B) before and after 4, 8, 12 and 16 weeks of the study. \* different from week 16 in PRO,  $P = 0.035$ ; # different from weeks 4, 8 and 16 in PRO,  $P \leq 0.014$ ; \*\* different from weeks 4, 8 and 16 in PLA,  $P < 0.001$ ; † different from weeks 4 and 8 in PRO,  $P \leq 0.019$ . (PRO,  $n = 109$ ; PLA,  $n = 114$ ).

### 7.3.8 Outcome of the dummy stepwise regression analysis for salivary antimicrobial proteins.

#### 7.3.8.1 *Lactoferrin*.

Not only was the absolute value at the start of the study, but also the change in either the concentration or the secretion rate not significantly different between both groups (Figure 7.10). Lactoferrin concentrations (Figure 7.10A) remained stable in both groups throughout the interval of the study whereas the secretion rate (Figure 7.10B) increased, non-significantly, in both.

A



B

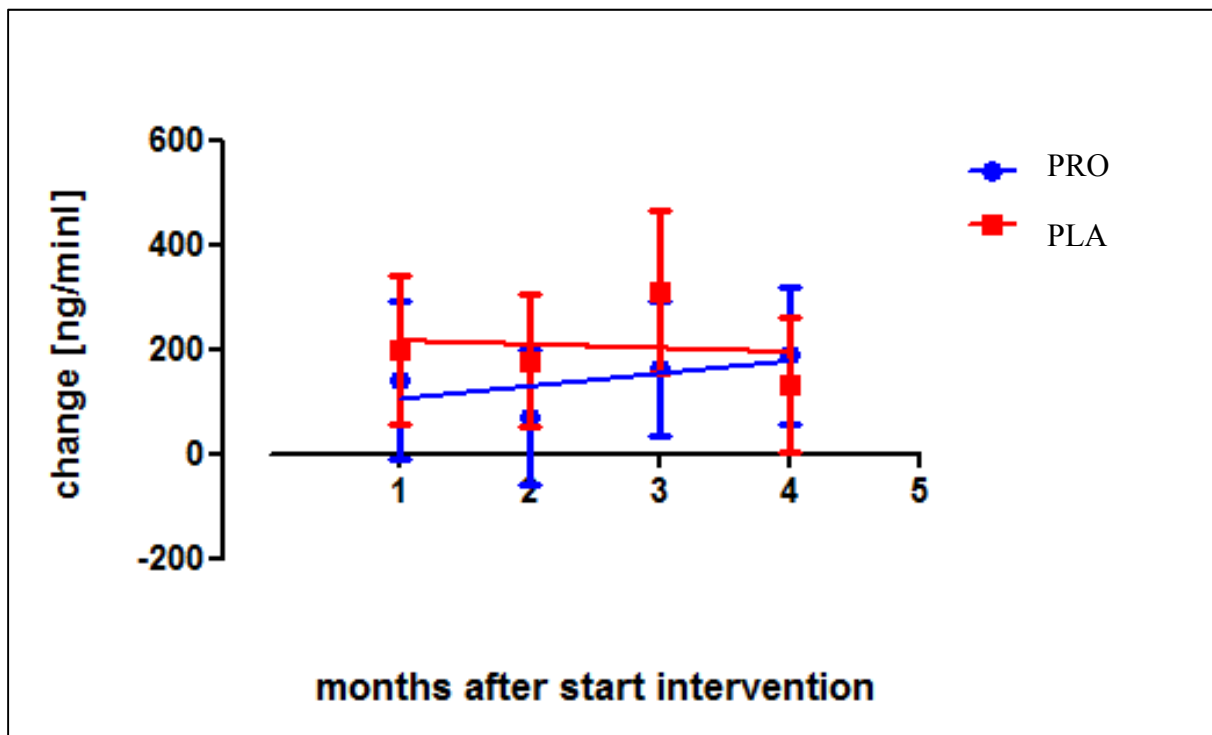
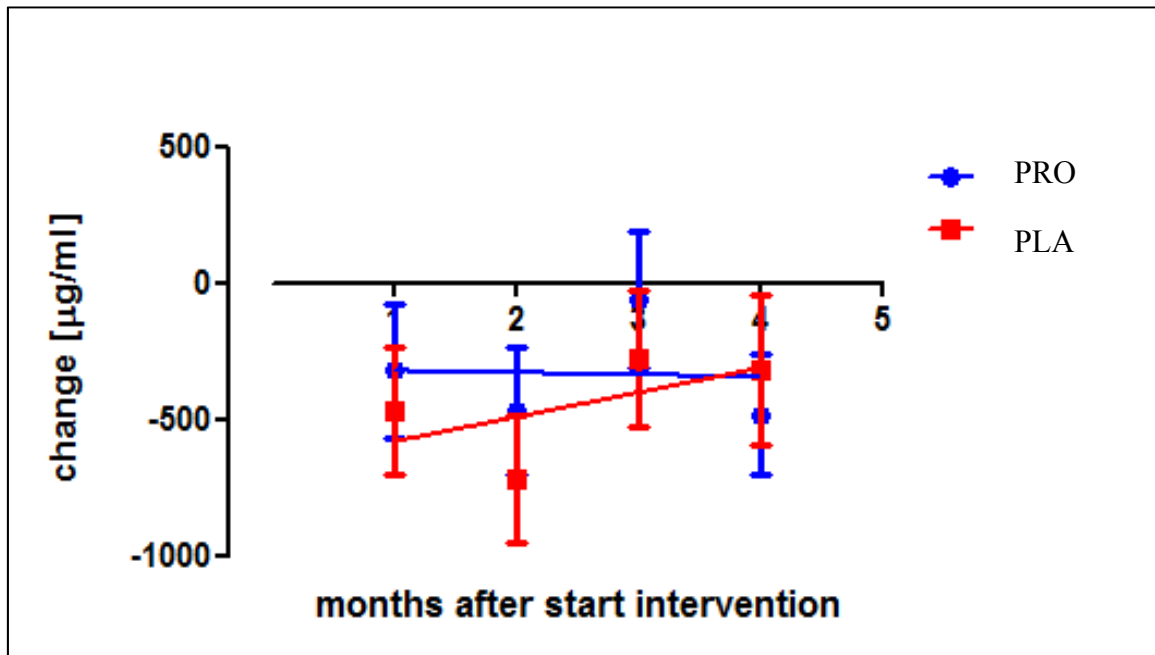


Figure 7.10: Change in salivary lactoferrin concentration (A) and secretion rate (B) over the study period.

### 7.3.8.2 Lysozyme.

With the absolute levels being not different at the start of the study, the decrease in lysozyme concentrations was similar in both groups, till the end of the intervention period (Figure 7.11A). However, the secretion rate of lysozyme was significantly ( $P < 0.03$ ) higher in the PLA than in the PRO group throughout the interval of the intervention (Figure 7.11B).

A



B

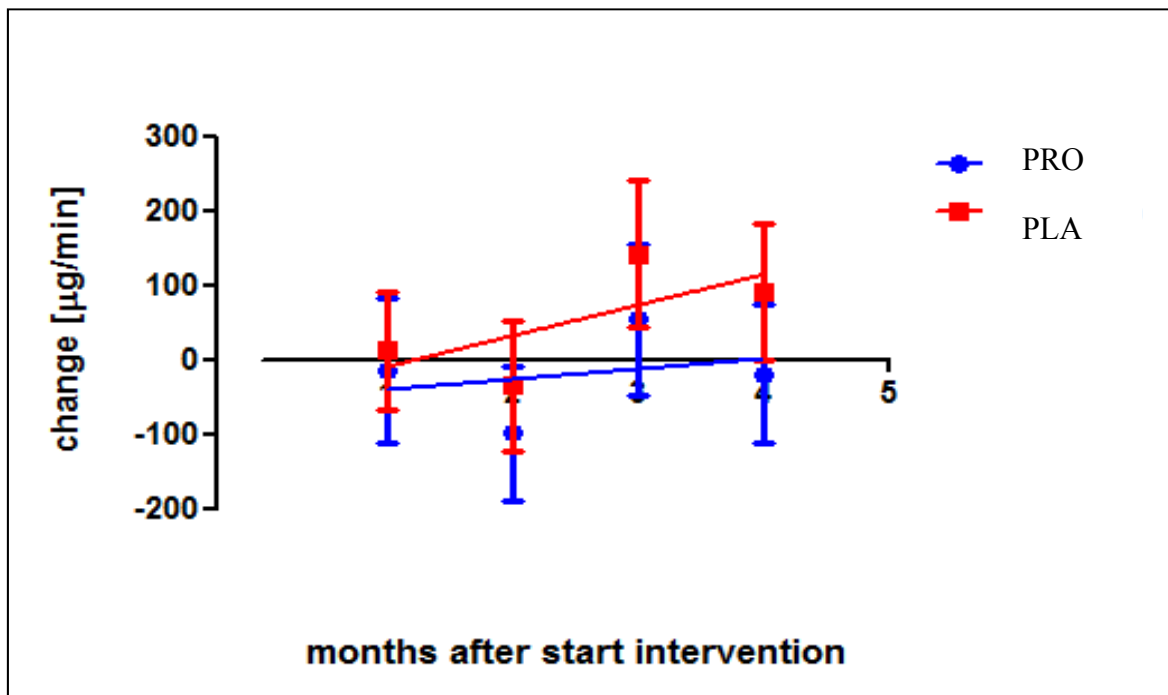
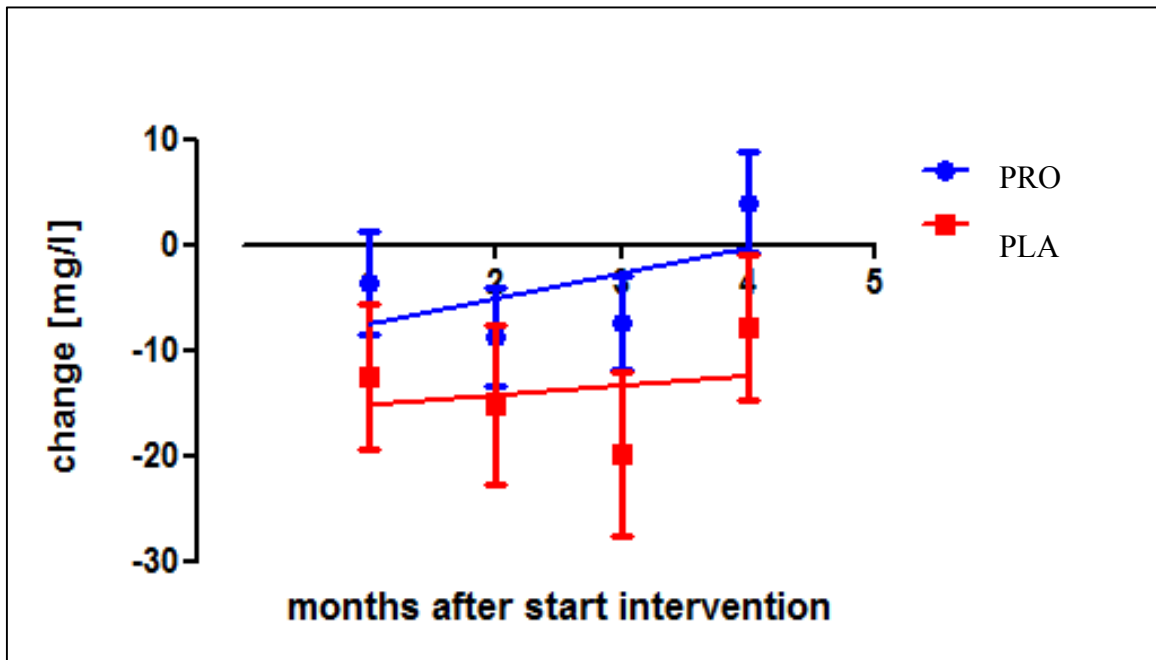


Figure 7.11: Change in salivary lysozyme concentration (A) and secretion rate (B) over the study period.

### 7.3.8.3 Saliva S-IgA.

A significant ( $P < 0.01$ ) difference in concentration of S-IgA was observed at the start with higher values in the PLA group (Figure 7.12). During the interval of the study there was an initial decrease in S-IgA concentration in both groups, after which the PRO group increased significantly ( $P < 0.02$ ) faster than the control group, reaching the same level as at the start after 4 months in contrast to that in the PLA group (Figure 7.12A). The S-IgA secretion rate was not different between both groups at the start. Interestingly though, the S-IgA secretion rate increased significantly ( $P < 0.02$ ) during the interval of the study in both groups (Figure 7.12B).

A



B

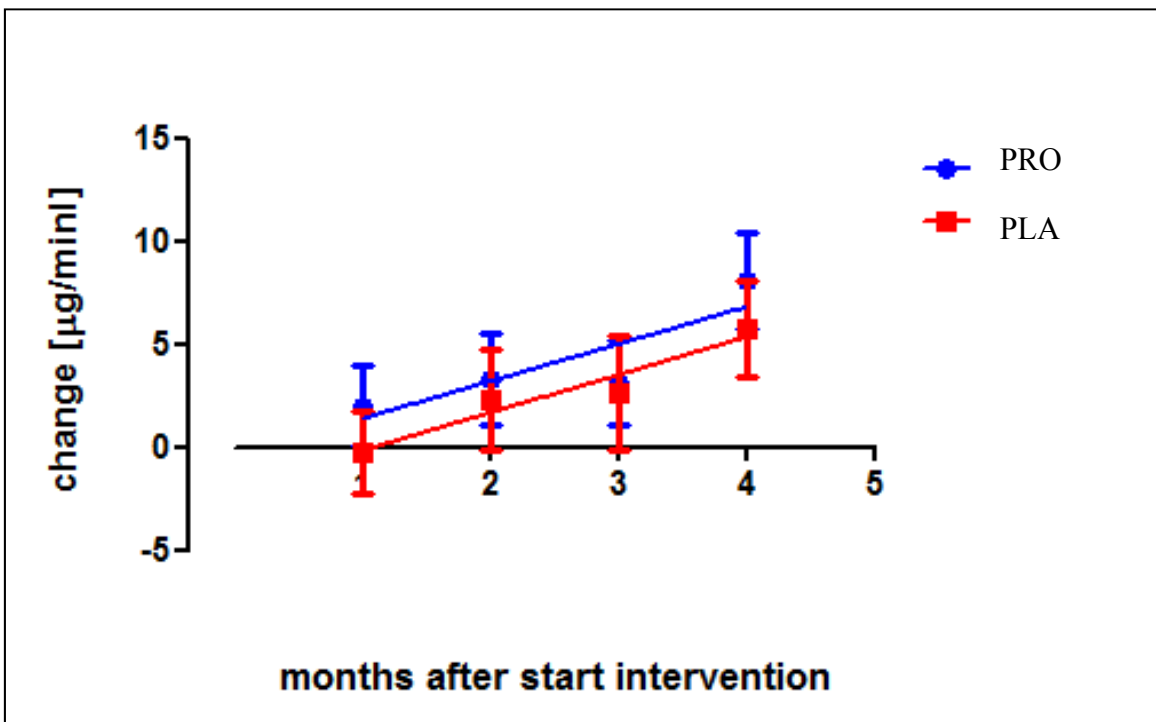


Figure 7.12: Change in S-IgA concentration (A) and secretion rate (B) over the study period.

#### 7.3.8.4 *Alpha-amylase.*

At the start of the intervention the absolute concentrations (measured as enzyme activities) were similar in both groups, during the intervention the concentration decreased in both groups at the same level. Similar observations were made for the amylase secretion rate (see Figure 7.9).

#### 7.3.9 Blood leukocyte counts.

There were no significant differences either before or after the 16-week supplementation period between PLA and PRO in blood total or differential leukocyte counts (Table 7.4).

#### 7.3.10 Blinding.

Of the subjects who expressed an opinion, 52% were correct and 48% were incorrect in their selection of treatment; hence, the study blinding was effective.



Table 7.4: Total and differential counts before and after 16 weeks of the intervention period.

	Before 16 weeks	After 16 weeks	<i>P</i> value
<b>Leukocytes (cells x 10<sup>9</sup>/L)</b>			
<b>PLA</b>	6.30 ± 0.14	5.82 ± 0.14	0.619
<b>PRO</b>	6.18 ± 0.13	5.85 ± 0.13	0.258
<b>Neutrophils (cells x 10<sup>9</sup>/L)</b>			
<b>PLA</b>	3.36 ± 0.12	2.93 ± 0.11	0.388
<b>PRO</b>	3.23 ± 0.12	2.96 ± 0.10	0.597
<b>Monocytes (cells x 10<sup>9</sup>/L)</b>			
<b>PLA</b>	0.64 ± 0.03	0.55 ± 0.02	0.374
<b>PRO</b>	0.60 ± 0.02	0.55 ± 0.01	0.492
<b>Lymphocytes (cells x 10<sup>9</sup>/L)</b>			
<b>PLA</b>	2.07 ± 0.05	2.05 ± 0.05	0.349
<b>PRO</b>	2.10 ± 0.05	2.11 ± 0.05	0.282

Values are expressed as mean ± SEM; n = 113 PRO; n = 119 PLA.

## 7.4 Discussion.

The present study investigated the effects of regular probiotic intake on self-reported URTI (i.e. common cold infections) symptoms in a group of highly active individuals engaged in their normal level of training and competition during the period November 2011 to March 2012. The main findings of the present study were that 4 months of daily probiotic supplementation resulted in improved maintenance of training volume when URTI episodes were present, but did not reduce incidence of URTI or the number of days with a symptom score  $\geq 5$ . There was also no difference between PRO and PLA in the proportion of subjects who experienced at least one URTI episode. Similarly, in those who experienced one or more URTI episodes, the duration and severity of the episodes were not different between PRO and PLA. An increase in S-IgA concentration over time was found in PRO but not in PLA.

Overall, men ( $n = 169$ ) and women ( $n = 70$ ) had similar URTI incidence ( $0.59 \pm 0.06$  vs.  $0.76 \pm 0.11$ , respectively;  $P = 0.185$ ) but women tended to have longer URTI episodes ( $15.1 \pm 1.1$  vs.  $11.8 \pm 0.06$ , respectively;  $P = 0.066$ ) and more days with URTI symptom score 5 ( $6.5 \pm 0.8$  vs.  $4.9 \pm 0.4$ , respectively;  $P = 0.071$ ). Women had lower saliva flow rates ( $P = 0.013$ ) which was expected given that their body mass was 15 kg lower than the men. However, the saliva concentrations of lactoferrin, lysozyme and amylase were  $\sim 15\%$  lower in women than men (all  $P < 0.05$ ), though S-IgA concentration was not different. The secretion rates for lactoferrin, lysozyme, amylase and S-IgA were all significantly lower in women than men (all  $P < 0.03$ ).

These results suggest that regular *Lactobacillus* probiotic intake allows highly-trained athletes to maintain their training loads when a respiratory infection is present. Considering the fact that reductions in training load and interruptions of training regimens are the main undesirable side effects of infection for athletes (Gleeson, 2005; Bishop and Gleeson, 2009),

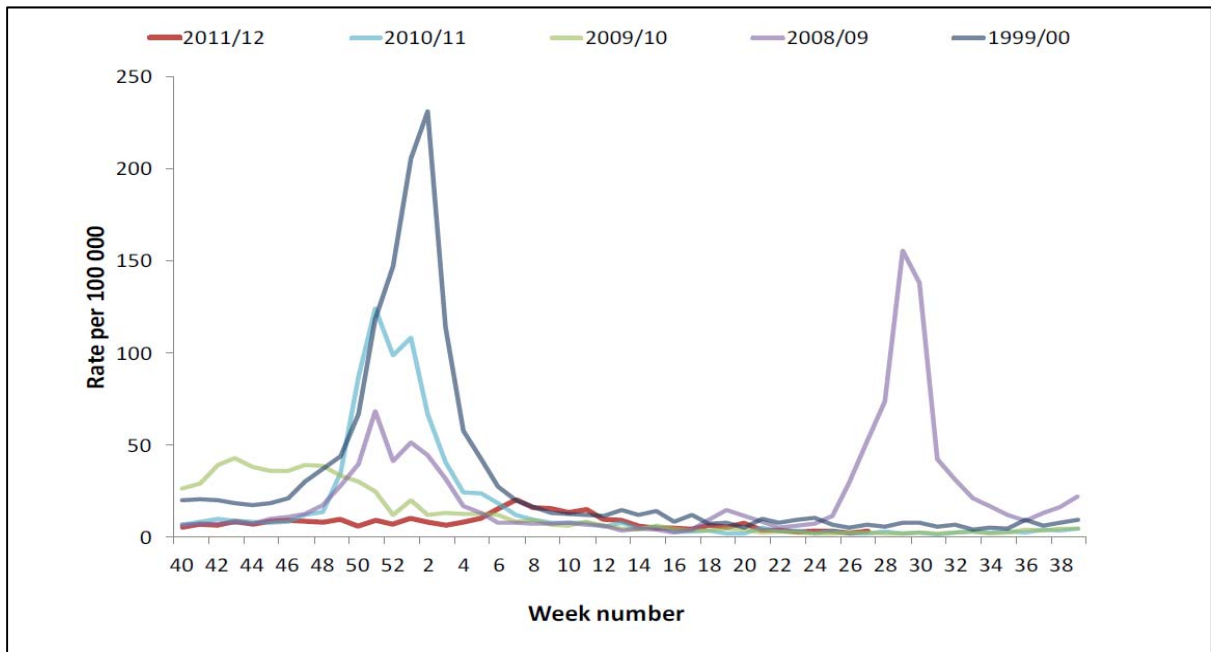
these better preserved training loads are of crucial importance. Additionally, regular probiotic intake increased both S-IgA concentration and its secretion rate which are known to be inversely correlated with URTI incidence in athletes (Fahlman and Engels, 2005; Gleeson et al., 1999, 2011b; Neville et al., 2008; Walsh et al., 2011; West et al., 2006). These data imply that probiotics may provide better protection from common colds in physically active individuals. However, regular probiotic intake in the present study did not appear to be beneficial in reducing the number of URTI episodes in a highly-trained athletic population in contrast with previous reports demonstrating reduced incidence and/or duration and severity of URTI episodes following chronic probiotic intake in endurance athletes (Cox et al., 2010; Gleeson et al., 2011a; West et al., 2011).

These surprisingly opposite findings may be accounted for by several factors. The individuals recruited to this study were highly-trained, competitive athletes undertaking daily exercise and similar cohorts engaged in similar volumes of training have been shown to be at a higher risk of URTI when exposed to pathogens (Gleeson, 2005; Nieman, 1994). However, the mean number of URTI episodes in the PLA group (0.6) in the present study was far lower than that expected based on the results of our previous study (Gleeson et al., 2011a) where it was 2.1 in the PLA group. Data from the Health Protection Agency National Influenza Report (2012) suggest that the 2011 – 2012 winter period over which our present probiotic intervention study took place had an unusually low rate of common cold and flu incidence among the general population in the UK compared with previous years (Figure 7.13). The weather during the 2011 – 2012 winter was one of the driest on record and the much lower rate of common cold incidence than anticipated, may have prevented us from detecting a significant effect of probiotic supplementation on respiratory infection incidence.

Furthermore, despite using a fully randomized, double-blind study design, participants in the PRO group had, by chance, a significantly lower S-IgA concentration at baseline and tended to have lower S-IgA concentration at other time points (Figure 7.5; treatment main effect,  $P = 0.048$ ). Saliva concentrations of lactoferrin and lysozyme also tended to be lower in the PRO group than in the PLA group (treatment main effect  $P$  values of 0.062 and 0.059, respectively). Considering that S-IgA concentration and/or secretion rate have been shown in numerous studies, involving endurance athletes, to be inversely correlated with numbers of URTI episodes (Fahlman and Engels, 2005; Gleeson et al., 1999; Gleeson and Pyne, 2000; Neville et al., 2008), such a chance effect of lower baseline S-IgA in the PRO group could place them in a disadvantaged position with regard to URTI risk. To put a positive spin on things, it could be argued that the probiotic supplementation may have prevented a higher incidence of URTI among this group of athletes with relatively lower levels of S-IgA and other AMPs. Moreover, despite lower baseline S-IgA the probiotic supplementation increased S-IgA concentration and secretion rate over time, illustrating its potential immune-boosting effects.

The findings of the present study also suggest that other saliva AMPs did not compensate for reduced concentration of S-IgA, further underlining the importance of this salivary immunoglobulin. The concentrations of lactoferrin ( $r = 0.270$ ;  $P < 0.05$ ) and lysozyme ( $r = 0.265$ ;  $P < 0.05$ ) were significantly correlated with the S-IgA concentration in saliva in the whole subject cohort and the correlations for the secretion rates of these saliva AMPs were even stronger (lactoferrin vs S-IgA:  $r = 0.424$ ;  $P < 0.01$ ; lysozyme vs S-IgA:  $r = 0.393$ ;  $P < 0.01$ ). The present study demonstrated that probiotic supplementation increases both concentration and secretion rates of S-IgA but not any other AMPs, which is in line with the previous studies showing that *Lactobacillus* probiotics may help maintain saliva S-IgA during periods of physical stress (Tiollier et al., 2007; Gleeson et al., 2011a).

A



B

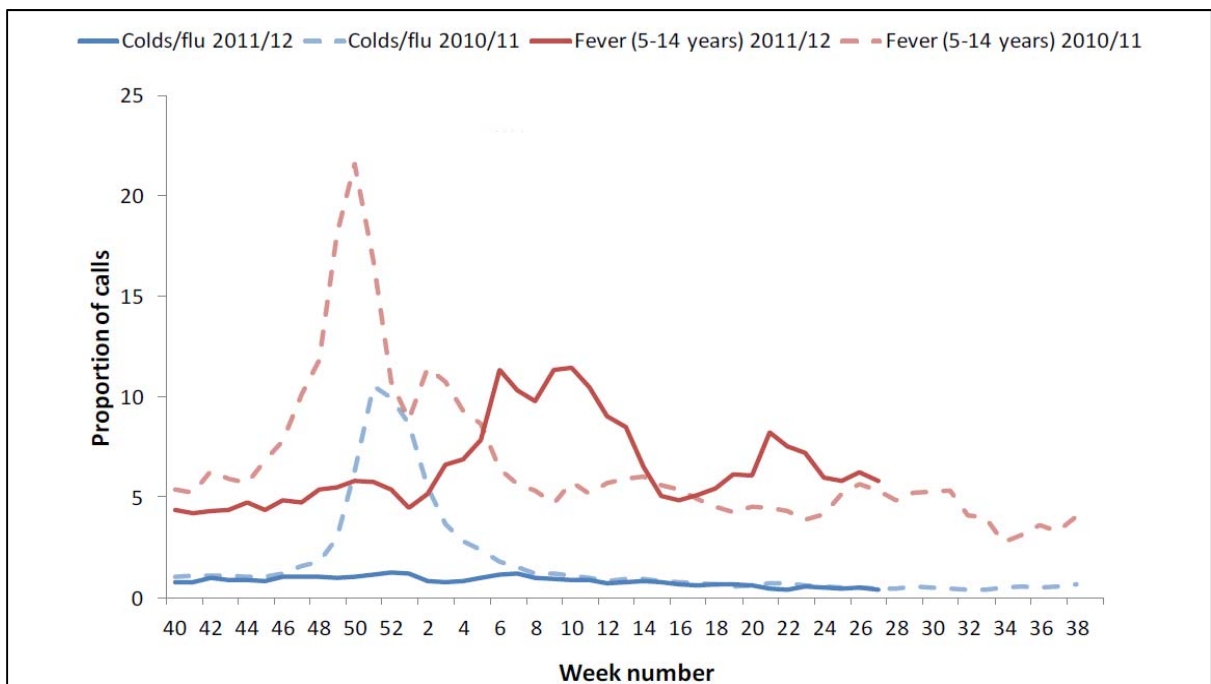


Figure 7.13: UK Health Protection Agency Influenza Surveillance data (2012) demonstrating incidence of influenza-like illness in current and recent seasons (A) and proportion of calls for cold/flu symptoms in current and recent seasons (B). Week 42 (2011) to week 12 (2012) represents the present study period.

## **7.5 Conclusion.**

In summary, regular ingestion of *Lactobacillus* probiotic reduced the extent to which training was negatively affected in endurance athletes when infection was present, and increased both saliva S-IgA concentration and secretion rate over time but did not appear to reduce URTI incidence or the duration and severity of URTI episodes. Two major confounding factors, namely the unexpectedly low incidence of URTI during the winter period and the lower baseline S-IgA in the probiotic group may have prevented potential beneficial effects of probiotic supplementation from being identified.

## CHAPTER 8

### GENERAL DISCUSSION

The aim of this thesis was to examine the effects of several nutritional supplements on athletes' immune function. This section presents a summary of the main findings of studies reported in this thesis.

#### **8.1 Effective stimulant (100 x dilution Pediacel vaccine) dose and Evidence Investigator.**

The first study (Chapter 3) was carried out in order to determine which vaccine dose should be used to stimulate whole blood culture. Seven different doses were introduced to the whole blood culture (dose 1: no vaccine, dose 2: 20,000 dilution factor, dose 3: 10,000 dilution factor, dose 4: 4,000 dilution factor, dose 5: 2,000 dilution factor, dose 6: 1,000 dilution factor, dose 7: 200 dilution factor) and the production of several cytokines (IL-2, IL-4, IL-6, IL-8, IL-10, TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\alpha$ , and IL-1 $\beta$ ) were analysed with an Evidence Investigator biochip array after 20 h incubation. From the graphs plotted (Figure 3.1 – 3.9), it appeared that the effective dose was somehow between dose 4 and 6 (corresponding to a 1,000 – 4,000 fold dilution). This was determined based on idea that the effective dose should induce at least a 3-fold increase above the unstimulated (dose 1) cytokine production but less than 50% of the maximal response. However, different cytokines give different a range of dose active response; hence, an effective dose for some cytokines might not be effective for other cytokines. Based on the findings, dose 5 was decided to be used in the next 3 studies that followed.

The Evidence Investigator (Randox, UK) was used in this study to analyse multiple cytokine production instead of the traditional method of ELISA. This is because Evidence Investigator can analyse multiple cytokines from a small sample (0.1 ml plasma), and it only

took 3 – 4 h to be carried out. This machine is quite new and not yet as widely used as ELISA methods. Thus, a simple correlation (Spearman's correlation coefficient) was carried out to determine the relationship between results from the Evidence Investigator and ELISA data for IL-10. There was a moderately strong, positive correlation between the two variables;  $r = 0.828$ ,  $n = 38$ ,  $P < 0.001$ . Thus, using the Evidence Investigator for analysis is an acceptable alternative to ELISA.

## **8.2 Kaloba (*Pelargonium sidoides*) supplementation and immune response.**

In Chapter 4, several immunomodulatory nutritional compounds were used *in vitro* to determine their effects on antigen-stimulated whole blood culture cytokine production. Despite no noticeable effects of alcohol, curcumin, and echinacea on cytokine production, caffeine and quercetin showed a tendency towards decreased cytokine production as the doses were increased. On the other hand, an upward trend was evident with Kaloba, where a high dose of Kaloba seemed to increase the cytokine production. This might suggest Kaloba has a potential as an immunostimulant. Therefore, in Chapter 5, Kaloba was used as a supplement to investigate its effects on the immune response to prolonged exercise in healthy male recreational athletes.

To date, effects of Kaloba on athletes' immune function have not been extensively studied. Nevertheless, previous studies suggest its efficacy in treating patients with acute bronchitis (Kamin et al., 2010a; Kamin et al., 2010b) and common cold (Lizogub et al., 2007). Furthermore, studies also reported that Kaloba was successfully employed for the treatment of ear, nose, and throat disorders as well as respiratory tract infections (Kolodziej and Kiderlen, 2007; Matthys and Funk, 2008; Thale et al., 2008; Matthys et al., 2010). Kaloba has been reported to have a multifactorial mode of action; antiviral and cytoprotective properties, antibacterial properties, secretomotor properties and inhibition of 'sickness behaviour'.



Several *in vitro* studies found that Kaloba triggers stimulation of IFN synthesis (Trun et al., 2006; Kayser et al., 2007) and improves phagocyte function (Conrad et al., 2007). It was also shown to increase the ciliary beat frequency, thus increasing the transportation of mucous and pathogens from the respiratory tract. This in turn reduced pathogen count, removed the nutrient medium for their subsequent proliferation and substantially improved respiration (Neugebauer et al., 2005).

However, the present study (Chapter 5) found no effects of Kaloba on cytokine production, leukocyte counts, S-IgA response, and UTI symptoms assessed using the Wisconsin Upper Respiratory Symptom Survey-21 (WURSS-21). A previous study (Luna et al., 2010) reported that 28 days of Kaloba supplementation (3 x 30 drops/day of a solution of 80 g of extract/100 ml in solution) significantly increased S-IgA concentration in response to a high intensity running session. However, the post-exercise sample was collected 48 h after the exercise was completed. The authors did not justify the selection of this sampling time point and the study reported in this thesis indicated that the IgA response to exercise after Kaloba supplementation for 7 days was unaltered in the immediate post-exercise recovery period.

Despite several clinical studies regarding the effectiveness of Kaloba (Lizogub et al., 2007; Matthys and Funk, 2008; Bachert et al., 2009; Kamin et al, 2010a and 2010b; Matthys et al., 2010), this study failed to prove its effects on immune response in athletes. Thus, further study is warranted with a higher dose and a longer supplementation period with Kaloba.

### **8.3 A 14-strain probiotic supplementation and salivary antimicrobial proteins (AMPs).**

To date, there is no published study regarding effects of multi-strain probiotic (probiotic mixtures) on immune response in athletes. Although, there were a few studies regarding effects of a single strain of probiotic on immune response in athletes (Pujol et al., 2000; Clancy et al., 2006; Kekkonen et al., 2007; Gleeson, 2008; Cox et al., 2010; Gleeson et al., 2011a; Gleeson et al., 2012). Some have reported that probiotic mixtures are more effective in modifying immune function and preventing respiratory tract infection (Chapman et al., 2011). It has been reported that probiotic mixtures reduced the number of days with fever, duration of cold episodes, total symptom score, and reduced incidence of respiratory tract and gastrointestinal infections (Winkler et al., 2005; de Vrese et al., 2006; Lin et al., 2009). However, it is unknown if this is a result of synergistic interactions between strains or a consequence of the higher probiotic dose used in some of these studies. Most of multi-strain probiotic studies did not directly compare between a mixture and its component strains. This makes us unable to make a definite conclusion about the efficacy of the probiotic mixtures or their component strains. Potential modes of action of probiotics include (1) modulation of the intestinal immune system, (2) displacement of potential pathogens through competitive exclusion and (3) production of antimicrobial agents (Rowland et al., 2010), so intuitively, one might expect a combination of bacterial strains to be more effective than a single strain.

The present study (Chapter 6) found that 30 days supplementation of the 14-strain probiotic did not alter salivary antimicrobial proteins (S-IgA, lactoferrin, and lysozyme concentrations, and  $\alpha$ -amylase activity) at rest and in response to an acute bout of prolonged exercise. It was speculated that 30 days supplementation period and the dose/concentration of the 14-strain probiotic used in this study was not enough to induce any beneficial effects on athletes' salivary antimicrobial proteins at rest and in response to an acute bout of prolonged

exercise. This was based on the previous studies with longer supplementation period, 16 weeks (Gleeson et al., 2011a) and 12 weeks (Kotani et al., 2010), where they found positive effects of probiotics on S-IgA. Thus, further study is warranted with a higher dose and longer supplementation period of this 14-strain probiotic to see if it has any positive effects on an athlete's immune function.

#### **8.4 Single strain *Lactobacillus* probiotic supplementation, common cold infection, and mucosal immunity in endurance athletes.**

There is now a reasonable body of evidence that regular consumption of probiotics can modify the population of the gut microbiota and influence immune function (Matsuzaki, 1998; Gill and Cross, 2002; Gill and Prasad, 2008; Mengheri, 2008; Borchers et al., 2009; Minocha, 2009). However, such effects are strain specific. Even though there were numerous studies of probiotics carried out on patients, studies regarding single strain probiotic effects on athletes' immune function are scarce in literature. Gleeson and colleagues (2011a) found that a commercially available and popular probiotic (*L. casei* Shirota) appeared to be beneficial in reducing the frequency of URTI in athletes during a winter period (16 weeks) of training and competition, which may be related to better maintenance of S-IgA levels. However, during 16 weeks of spring training period, they found no effects of another strain of *Lactobacillus* probiotic (*L. salivarius*) on URTI and S-IgA level (Gleeson et al., 2012). It seems that cold weather which is associated with higher rate of common cold in the winter months helps in revealing potential beneficial effects of probiotics supplementation or that this different strain had a different (non-efficacious) effect on human immune function.

Other study has also found no difference in incidence of respiratory infections with probiotic intervention (Kekkonen et al., 2007). Similarly, the present study (Chapter 7), despite using a larger population of athletes than most previous studies, also found that

regular ingestion of a *Lactobacillus* probiotic did not reduce URTI incidence or the duration and severity of URTI episodes. Two major confounding factors, namely the unexpectedly low incidence of URTI during the winter period of 2010-11 and the lower baseline S-IgA in the probiotic group compared with placebo may have prevented potential beneficial effects of probiotic supplementation from being identified. Nevertheless, the probiotic appeared to reduce the extent to which training was negatively affected in endurance athletes when infection was present, and increased both saliva S-IgA concentration and secretion rate over time.

### **8.5 Prolonged exercise and immune response.**

Many studies have reported that prolonged and strenuous exercise temporarily impairs both innate and adaptive immune function (Pyne, 1994; Pedersen and Bruunsgaard, 1995; Ronsen et al., 2001), and hence increases infection risk. According to Gleeson (2006a), post-exercise depression of immune function is most pronounced when the exercise is continuous, prolonged (> 1.5 h), of moderate to high intensity (55 – 75% of aerobic capacity), and performed without food intake. It was suggested that bouts of intense prolonged exercise induce decreased numbers and functions of leukocytes as a result of increased stress hormone secretion, thus resulting in the entry of less mature leukocytes into circulation, particularly NK cells (Gleeson, 2005). In addition, it was found that an intensive exercise at intensity of 90% and 120% VO<sub>2</sub>max caused a significant decrease in plasma glutamine concentration, which in turn may lead to immunodepression (Keast et al., 1995) since it has been shown previously that amino acid glutamine is important for the function of some immune cells (macrophages and lymphocytes) (Newsholme et al., 1987).

However, a review by Hiscock and Pedersen (2002) concluded that plasma glutamine is not likely to play a role in exercise-induced immunodepression because it was speculated

that the magnitude of the decrease in plasma glutamine after an exercise is not sufficient to compromise the function of immune cells. For example, a previous study (compare the effects of exercise at 80%  $\dot{V}O_2$ max to fatigue with more prolonged exercise at 50%  $\dot{V}O_2$ max for up to 3 h) concluded that reductions in neutrophil function after exercise at 80%  $\dot{V}O_2$ max were not related to changes in plasma glutamine concentration, although both plasma glutamine and neutrophil function were decreased at 1 and 2.5 h post-exercise in the long duration exercise trial (Robson et al., 1999).

In Chapter 5, participants cycled for 90 min at 60%  $\dot{V}O_2$ max. This study found that prolonged moderate intensity exercise significantly decreased S-IgA concentration and secretion rate post-exercise with the values returned to baseline by 1 h post-exercise. However, total leukocyte count was significantly increased post-exercise, and was further increased 1 h post-exercise. This was mainly due to a rise in the neutrophil count. In Chapter 6, participants cycled for 2 h at 60%  $\dot{V}O_2$ max. However, no change was evident in S-IgA and lysozyme concentrations. But, lactoferrin concentration and  $\alpha$ -amylase activity was significantly increased post-exercise. Findings about S-IgA in both studies were inconsistent. However, several studies were also reported inconsistent finding about effects of exercise on S-IgA concentration. Several studies have reported that S-IgA concentration is reduced after intense exercise (Steerenberg et al., 1997; Fahlman et al., 2001), increased after moderate or lower intensity exercise (Williams et al., 2001; Dorrington et al., 2003; Li and Gleeson, 2004) or not affected by exercise intensity (McDowell et al., 1991; Blannin et al., 1998).

Exercise-induced increases in the concentration of AMPs are most likely related to sympathetic nervous system activity (Allgrove et al., 2008). Damage to epithelial cells through hyperventilation and subsequent exposure to environmental irritants, and the activation of neutrophils that follows may have led to increased secretion of these AMPs.

Following physical damage (Dorschner et al., 2001) and contact with microbes (Duits et al., 2003), epithelial cells increase their expression of AMPs. However, investigation on effects of exercise on AMPs is still limited in literature currently.

## **8.6 Summary and future direction.**

The general finding of this thesis is that nutritional supplements (Kaloba, a 14-strain probiotic, and single strain probiotic) may or may not (indecisive) affect immune function of athletes. Further investigation need to be carried out.

- Following findings from study 2 (Chapter 4), further investigation needs to be carried out for some of the cytokines since their response was unable to be measured due to vaccine dose used being beyond their effective dose range (thus they were exhibiting a maximal response). Some of the cytokine responses were inconclusive either they were leading to immunostimulant or immunosuppressant.
- Following findings from study 3 (Chapter 5), further investigations with a higher dose of Kaloba and with a longer supplementation period is warranted. Numerous studies reported beneficial effects of Kaloba *in vitro*, thus this supplement might have potential effects on athletes' immune function.
- Following findings from study 4 (Chapter 6), further investigations is also recommended with longer supplementation period and higher dose. The results will be very valuable since to date there was no other study investigating multi-strain probiotics on athletes' immune function.
- Following findings from study 5 (Chapter 7), further investigations is encouraged with the hope that the two major confounding factors that may have prevented potential beneficial effects of probiotic supplementation from being identified are removed.

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[ title of the study]

**INFORMED CONSENT FORM**  
**(to be completed after Participant Information Sheet has been read)**

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethical Advisory Committee.

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.

I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others.

I agree to participate in this study.

Your name

---

Your signature

---

Email

---

Phone number

---

Date

---

Signature of investigator

---

Name/Number .....

### Health Screen Questionnaire for Study Volunteers

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research.

**Please complete this brief questionnaire to confirm your fitness to participate:**

**1. At present,** do you have any health problem for which you are:

(a) on medication, prescribed or otherwise .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(b) attending your general practitioner .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(c) on a hospital waiting list.....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>

**2. In the past two years,** have you had any illness which required you to:

(a) consult your GP .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(b) attend a hospital outpatient department .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(c) be admitted to hospital .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>

**3. Have you ever** had any of the following:

(a) Convulsions/epilepsy .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(b) Asthma .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(c) Eczema .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(d) Diabetes .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(e) A blood disorder .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(f) Head injury .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(g) Digestive problems .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(h) Heart problems .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(i) Problems with bones or joints .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(j) Disturbance of balance/coordination .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(k) Numbness in hands or feet .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(l) Disturbance of vision .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(m) Ear / hearing problems .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(n) Thyroid problems .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(o) Kidney or liver problems .....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>

(p) Allergy to nuts ..... Yes  No

4. **Has any**, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? ..... Yes  No

**If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)**

.....  
.....

**5. Allergy Information**

(a) are you allergic to any food products? Yes  No   
(b) are you allergic to any medicines? Yes  No   
(c) are you allergic to plasters? Yes  No

**If YES to any of the above, please provide additional information on the allergy**

.....  
.....

**6. Additional questions for female participants**

(a) are your periods normal/regular? ..... Yes  No   
(b) are you on "the pill"? ..... Yes  No   
(c) could you be pregnant? ..... Yes  No   
(d) are you taking hormone replacement therapy (HRT)? ..... Yes  No

**7. Are you currently involved in any other research studies at the University?**

Yes  No

If yes, please provide details of the study

.....

**8. Do you smoke?**

Yes  No

**Subject characteristics:**

Gender: Male / Female

Body Mass: \_\_\_\_\_ kg

Height: \_\_\_\_\_ cm

BMI: \_\_\_\_\_ kg/m<sup>2</sup>

Age: \_\_\_\_\_ years

What is your main sport? \_\_\_\_\_

How many times per week do you train and/or compete (all sports)? \_\_\_\_\_

How many hours in total per week do you train/compete (all sports)? \_\_\_\_\_

Have you suffered any illness in the last 3 months? Y / N if yes, when? \_\_\_\_\_

Have you donated blood (>500 ml) in the last 3 months? Y / N