Exercise, Immunity and Illness

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ABSTRACT

Identifying immune biomarkers in healthy humans that indicate an increased susceptibility to upper respiratory tract illness (URTI) is necessary to develop improved diagnostic and treatment strategies. URTI is associated with substantial socio-economic and personal cost. Small to moderate reductions in the severity and duration of illness could lead to substantial reductions in these costs. This thesis investigated the relationship between the immune system and URTI in healthy individuals utilising exercise as a model of stress. Chapter 2 (Section 2.2) reviews the effects of exercise on the immune system and URTI, with a particular focus on the way in which exercise can be used to better understand the role of the salivary antimicrobial proteins (AMPs) lactoferrin and lysozyme in host defence. Determining mucosal immune status, that is the condition of the immune system at body surfaces interfacing with the external environment, is necessary to understand the role of the immune system in host defence. Exercise-related disturbances in the immune system may increase susceptibility to URTI, particularly when prolonged intense exercise is undertaken frequently. The link between exercise-induced disturbances in immunity and URTI risk suggests that exercise may be a useful model by which to study the relationship between immunity and illness in healthy individuals.

The first study in this thesis (Chapter 3) was a prospective observational study comparing salivary lactoferrin and lysozyme concentrations over 5 months (chronic changes) in elite rowers with sedentary individuals (controls). The rowers also completed a graded exercise test to exhaustion to study acute changes in these salivary proteins. In the observational study lactoferrin, but not lysozyme, concentration was approximately 60% lower in rowers than control subjects at baseline and at the midpoint of the training season and 50% lower at the end of the season. In the graded exercise test to exhaustion there was a ~50% increase in the concentration of lactoferrin and lysozyme from pre-exercise to exhaustion. The practical relevance of the findings from this study is that chronic exercise associated reductions in resting salivary AMPs may increase the risk of URTI over a training season. In contrast, an increase in acute post-exercise salivary AMPs should reduce the risk of illness in the immediate period (minutes to hours) after exercise has finished.

Supplements that increase the number of beneficial bacteria in the gastrointestinal (GI) tract are popular as nutritional adjuncts to ameliorate the effects of stress on the immune system and to enhance health. Gut health, in which individuals free from acute and chronic disease have reduced symptoms of mild or moderate gut symptoms such as diarrhoea and constipation, flatulence, stomach cramps and other transient symptoms, is a major aim for the use of these supplements in healthy individuals. Chapter 2 (Section 2.3) reviews the potential benefits of modulating GI microbiota in physically active individuals to prevent illness compromising exercise training and athletic performance. Two double blind randomised active-control clinical trials and a double blind randomised placebo-controlled clinical trial were undertaken to examine the effects of modulating GI microbiota on immunity. The first active-control trial compared
a synbiotic supplement (Digestion 1-2-3-4™ – Chapter 4) with a prebiotic supplement, acacia gum, and the second examined a butyrylated high amylose maize starch supplement (HAMSB – Chapter 5) with a low amylose maize starch (LAMS) supplement. The placebo controlled trial examined a probiotic supplement (Lactobacillus fermentum – Chapter 6). The measures of immunity included a number of plasma cytokines (interleukin (IL)-1RA, IL-6, IL-8, IL-10, IL-12, IL-16, IL-18, tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and granulocyte macrophage colony stimulating factor (GM-CSF), gut permeability (lactulose to mannitol ratio), AMPs (lactoferrin and lysozyme) and salivary immunoglobulin A. The trial of L. fermentum also examined the effect of modulating GI microbiota on URTI and GI symptoms via a daily Web-based symptom questionnaire. Prior to the current research, there was little evidence of whether Digestion 1-2-3-4™ and HAMSB modified the composition of the commensal microbiome or enhanced immunity. Substantiating the effects of these interventions on gut bacteria and immunity were necessary, therefore, before a clinical trial incorporating illness was undertaken.

All three supplements had substantial effects on GI microbiota but only trivial effects on markers of immunity at rest. Digestion 1-2-3-4™ increased the faecal recovery of L. paracasei 40-fold relative to the active control acacia gum. HAMSB elicited an 81-fold increase in P. distasonis and a 5.1-fold increase in F. prausnitzii relative to LAMS supplementation. The probiotic L. fermentum had differing magnitudes of effects on GI microbiota between males and females, increasing the faecal recovery of total Lactobacillus species 8-fold in males but only 2-fold in females in comparison to those taking a placebo. In terms of immunity, supplementation elicited changes in a small number of serum cytokines only. Digestion 1-2-3-4™ was associated with a 50% smaller increase in the concentration of serum IL-16 in comparison to 4 weeks of supplementation with acacia gum, with the concentration of serum IL-16 increased 4-fold in those consuming the synbiotic and 8-fold in those on the prebiotic. In the HAMSB study, there was a relative 1.6-fold increase in serum IL-10 and a 2.5-fold increase in serum TNF-α. No substantial changes in other cytokines or in markers of mucosal immunity (salivary lactoferrin and gut barrier permeability) were evident. While there were no substantial effects on resting cytokine concentrations in the probiotic study there were substantial effects of supplementation on acute post-exercise cytokine perturbations. Examination of acute post-exercise cytokine responses was undertaken following evidence that illness prone athletes had a dysregulated cytokine response to exhaustive exercise. Individuals consuming L. fermentum had a substantial 20–60% reduction in acute post-exercise cytokine responses. The substantial moderation of cytokine perturbations suggests that the benefits of modulating GI microbiota in healthy individuals may be to make the immune system more robust to the stresses of intense exercise. Covariate analysis in the trials did not reveal any substantial association between changes in GI microbiota and changes in resting or acute post-exercise serum cytokines. Enhancing the number of beneficial bacteria in the GI tract suggests that these supplements may be useful nutritional adjuncts for gut health.

With regard to symptoms of illness, supplementation with the probiotic L. fermentum had beneficial clinical effects only in males. Lower respiratory illness symptoms were 70% lower in
males but 2-fold higher in females over the course of the study. There was no substantial difference in URTI between the probiotic and placebo groups. Furthermore, no clear trends between URTI and training load or between changes in GI microbiota and changes in illness patterns (incidence, duration or severity) were evident.

In conclusion, the finding that salivary lactoferrin was ~60% lower in elite rowers compared to healthy sedentary individuals indicates that prolonged intense exercise can induce chronic reductions in humoral factors of the innate mucosal immune system. Substantial increases in the number of beneficial bacteria in the GI tract with the selected supplements indicate that Digestion 1-2-3-4™, HAMSB, and *L. fermentum* may all be useful for promoting or maintaining GI health. The amelioration of acute post-exercise cytokine perturbations warrants further work to determine if immune responsiveness to stress may be a better predictor of immune competence than measures of resting immunity in healthy individuals. It would be pertinent to expand the measures of immunity in future exercise and nutrition immune intervention studies to include markers of immune activation and of immune cells implicated in maintaining airway homeostasis, such as CD4+ CD25+ FOXP3+ T-regulatory cells.
Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Signature: ……………………………………………………………

Date: …………………………………………………………………
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List of publications related to this thesis


List of abstracts related to this thesis


2. **West NP**, Pyne DB, Kyd JM, Renshaw GMC, Fricker PA, Cripps AW. Exercise induced changes in innate immunity of the airways. Griffith Institute for Health and Medical Research Conference, Gold Coast, December 2007 (Appendix G).

3. **West NP**, DB Pyne, WG Hopkins, PA Fricker, W McDonald, DC Eskesen, AW Cripps. Probiotic supplementation, illness and immunity in athletes; International Society of Exercise and Immunology, Tubingen, Germany, September 2009 (Appendix H).


List of abstracts not related to this thesis


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Abbreviations

AIS – Australian Institute of Sport
AMPs – antimicrobial proteins and peptides
*B. lactis – Bifidobacterium lactis*
BP/USP – British Pharmacopeia/United States Pharmacopeia
CD – cluster of differentiation
CFU – colony forming units
CMIS – common mucosal immune system
CSIRO – Commonwealth Scientific and Industrial Research Organisation
CV – coefficient of variation
CXCR – chemokine receptors
DGGE – denaturing gradient gel electrophoresis
DNA – DeoxyriboNucleic Acid
E. coli – Escherichia coli
EDTA – Ethylenediaminetetraacetic Acid
ELISA – enzyme linked immunosorbutant assay
F. prausnitzii – Faecalibacterium prausnitzii
FOS – fructooligosaccharide
FOX3+ – forkhead box P3
g – gravitational
GI – gastrointestinal
g – grams
GM-CSF – granulocyte macrophage colony stimulating factor
GOS – galactooligosaccharides
h.wk–1 – hours per week
HAMSB – High amylose maize starch
IFN-γ – interferon gamma
IL – interleukin
kg – kilogram
km.wk\(^{-1}\) – kilometres per week

*L. fermentum* – *Lactobacillus fermentum*

*L. paracasei* – *Lactobacillus fermentum*

*L. paracasei* – *Lactobacillus paracasei*

*L. rhamnosus* – *Lactobacillus rhamnosus*

LAMS – low amylose maize starch

mg – milligrams

ml – millilitre

ml.kg\(^{-1}\).min\(^{-1}\) – millilitres per kilogram per minute

mM – millimoles

n – number

ng.ml\(^{-1}\) – nanograms per millilitre

NK – natural killer

*P. distasonis* – *Parabacteroides distasonis*

PCA – Principal components analysis

PCR – polymerase chain reaction

Q-PCR – quantitative polymerase chain reaction

rRNA – ribosomal RNA

RS – resistant starch

SCFA – short chain fatty acid

SD – standard deviation

SIgA – secretory immunoglobulin A

TNF-\(\alpha\) – tumour necrosis factor – alpha

T\(_{\text{reg}}\) – T-regulatory

URTI – Upper respiratory tract illness

VO\(_2\)\(_{\text{max}}\) – maximal oxygen uptake

ww – wet weight

y – year
Chapter 1 – PREFACE
There is growing acknowledgement that progress in clinical immunology is being hampered by a limited understanding of what constitutes a healthy immune system (1, 2). Loosely defined as a vast array of molecules, cells and tissues that protect the body from environmental pathogens and endogenous cellular dysfunction, the immune system is universally acknowledged as essential to health. Along with traditionally being divided into the innate and adaptive immune system, a division is also made along the lines of mucosal and systemic immunity. The mucosal immune system comprises aspects of host defence located at surfaces that interface with the external environment, such as the skin, respiratory and genitourinary tracts. Additionally, mucosal lymphatic cells have markedly different circulatory pathways when compared to systemic lymphocytes. The systemic immune system refers to those aspects of host defence that are organ specific and in circulation. In an era where early diagnosis often predicts morbidity and mortality, identification of immune biomarkers indicative of common illness or disease risk has, in general, been elusive (3, 4). Basic immunology research has largely utilised animal (particularly rodent) and disease models due, in part, to the ethical constraints of utilising healthy human subjects (2). These animal and disease models have revealed many of the biological activities and interactions of individual immune parameters. They have, however, failed to shed light on the way in which normal variations in these parameters relate to illness and disease in a complex, integrated biological system. As a result, there is a recognition that greater focus on clinical human research in healthy individuals is required to further elucidate the role of the human immune system in health and disease (5).

The need for a better understanding of normal human immune function has taken on greater importance in recent years. With an increasingly health conscious, well-educated and technologically connected population, there is a growing interest in strategies that enhance the immune system and improve health (6, 7). Within healthy populations, a primary reason for enhancing the immune system is to reduce susceptibility to common infectious illness, in particular URTI. As the most common illness in the population (8, 9), URTI is associated with substantial socioeconomic burden (10, 11) and can have more deleterious consequences for sub-groups of the population (12). Recognition of the strong interest in preventing illness by enhancing the immune system has led to the production and marketing of a range of novel nutrition products and supplements. In response, regulators and other special interest groups have implemented policies requiring evidence of safety and efficacy to prevent misleading claims (13). Better understanding of normal immune function will assist in interpreting whether changes in immunity represent beneficial or detrimental outcomes.

Clinical investigation of the immune system in healthy individuals is limited by a lack of suitable human models that allow for an investigation of “cause to consequence” relationships (1). A model offering promise for undertaking clinical immunology research in healthy people is physical activity (14, 15). There is strong evidence that exercise elicits changes in immunity and alters susceptibility to illness, particularly upper respiratory tract illness (16–22). The effects of exercise on the immune system are well known, with moderate exercise having beneficial effects on innate
and adaptive components of immunity compared to prolonged, exhaustive exercise or no exercise (17, 23–27). While extensive investigation of the effects of exercise on the systemic immune system have been undertaken, less is understood of the way in which exercise affects the innate mucosal immune system, particularly humoral parameters such as antimicrobial peptides and proteins (Chapter 2, Section 2.2). As the portal of entry to the body, mucosal surfaces prevent infectious pathogens infecting the body. AMPs, such as salivary lactoferrin, are constituent components of mucosal secretions that act as part of the innate immune system to prevent pathogens infecting the host (28). A number of fundamental questions remain to be addressed. First, the acute and chronic effects of exercise on particular antimicrobial proteins need to be addressed (Chapter 3). Secondly, the issue of whether alteration in the concentration of AMPs in saliva relates to altered risk of URTI may shed insight into the higher incidence of illness in some athletes undertaking prolonged intense exercise. Investigation of the relationship between exercise, AMPs and illness will enhance our knowledge of the role of AMPs in mucosal immunity and whether they are useful as a diagnostic biomarker for altered susceptibility to illness.

Ameliorating the acute and chronic effects of prolonged exhaustive physical activity is a key theme in exercise immunology (22, 25). Investigation has focused on the use of nutrition supplements given the important role of macro and micro-nutrients in maintaining immune function. Examining the immunomodulatory and health promoting effects of GI microbiota in healthy active individuals in conjunction with the use of exercise (Chapter 2, Section 2.3) may also be a promising tool to gain insights into the relationship between immunity and illness (29). Enteric bacteria colonise the GI tract in increasing numbers from the stomach to the colon, where they reach numbers in the order of $10^{14}$ colony forming units (30). The relationship between the microbiota and their human hosts covers a continuum from synbiosis to dysbiosis, in which the microbiota has beneficial and detrimental effects on the host respectively. The GI microbiota is pivotal to the development of the immune system and its functioning throughout human life (31). Supplementation with the aim of enhancing the beneficial bacteria in the GI tract exploits the symbiotic relationship between the microbiota and their host. Supplements that alter GI microbiota have been shown to have potent immunomodulatory effects in-vitro and ex-vivo (32, 33). However, studies in healthy humans have had contradictory findings (34–37). There is a need for in-vivo research to elucidate the mechanistic and clinical effects of these supplements (Chapters 4, 5 and 6). Furthermore, examining the use of these supplements in individuals undertaking prolonged exhaustive exercise may improve understanding of the relationship between immunomodulation and susceptibility to illness.

**Thesis organisation**

This thesis is composed of eight chapters

- Chapter 2 comprises two literature reviews examining key issues in exercise immunology. The first review, published in *FEMS Immunology and Medical Microbiology*, examines the relationship between exercise and the innate mucosal immune system and the role of exercise...
in susceptibility to URTI. The second review, published in *Exercise Immunology Review*, examines the ways in which GI microbiota may alter immunity and susceptibility to illness. This review also addresses possible mechanisms by which the modulation of the GI microbiota can enhance immunity and reduce URTI in healthy physically active individuals. Both reviews identify key questions for further investigation and are presented in their published form inclusive of references.

- Chapter 3 details an observational study undertaken in elite rowers to quantify the acute and chronic effects of exercise on markers (lactoferrin and lysozyme) of innate mucosal immunity in saliva. The study was published in the *British Journal of Sports Medicine* and is included in its published format inclusive of references.

- Chapter 4 details a 21 day double blind active-controlled clinical trial in physically active individuals that examined whether a synbiotic supplement (Digestion 1-2-3-4™, Probiotech Pharma, Melbourne, Australia) modifies gut microflora and enhances markers of systemic and mucosal immunity. This Chapter is in publication format with references included at the end of the thesis.

- Chapter 5 details a 28 day double blind active-controlled study in healthy individuals undertaking moderate levels of physical activity that examined the effects of a high amylose maize starch (National Starch Food Innovation, Sydney, Australia) supplement on gut microflora and markers of systemic and mucosal immunity. This Chapter is in publication format with references included at the end of the thesis.

- Chapter 6 details a double blind placebo-controlled clinical trial to determine the effects of supplementation with *Lactobacillus fermentum (PCC*)* (Probiomics Ltd, Sydney, Australia) on URTI and GI symptoms in a cohort of physically active individuals over a 15-week winter period. A secondary aim was to establish the effect of supplementation with *L. fermentum (PCC*) on faecal microbiology and on key aspects of immunity at rest and in response to an exercise test to exhaustion. The study was published in the *Nutrition Journal* and is included in its published format inclusive of references.

- Chapter 7 discusses the overall outcomes of the studies presented in this thesis and the contribution of this research to understanding the relationship between the immune system and URTI in healthy individuals. The discussion includes consideration of the effects of a season of prolonged intense rowing training on innate humoral components of the mucosal immune system. Clinical and methodological aspects of the research program and the efficacy of supplements that modulate gut flora to enhance immunity and reduce susceptibility to illness are also considered.

- Chapter 8 provides a summary of key issues and future directions for clinical immunology research in healthy individuals utilising exercise in the future.
Chapter 2 – LITERATURE REVIEW
2.1 Introduction

Identifying markers of immunity that indicate altered susceptibility to URTI in healthy individuals is an active area of research (14, 38). Despite extensive investigation, however, identifying biomarkers with strong predictive value has proven difficult (3). This failure has led to calls for a greater emphasis on human-based research in healthy individuals. However, ethical constraints regarding the use of interventions and/or therapeutic agents to cause perturbations in the immune system and alter susceptibility to illness limit research utilising healthy individuals. Novel conditions that perturb the immune system are therefore required. This literature review comprises two published papers. The first paper examines whether the use of physical activity (Section 2.2) can shed insight into the role of AMPs in susceptibility to URTI. Given the accepted paradigm that prolonged intense exercise may increase susceptibility to URTI, study of exercise-induced modulation of AMPs may yield new opportunities for identifying individuals at increased risk of illness. The second paper explores whether the use of probiotics to modulate gut microbiota (Section 2.3) is an effective nutritional strategy to ameliorate exercise-induced perturbations in immunity. Using probiotic supplements in physically active individuals should shed light on the relationship between the immune system and URTI.

In addition to probiotics, two other nutritional supplements, a synbiotic (or multi-formulation) product and HAMSB, offer promise to enhance immunity and resistance to illness. Similar to probiotics, the mechanism by which synbiotic supplements and resistant starch purportedly enhance health is by modulation of GI microbiota (39–41). Both supplements may also increase the metabolic activities of the microbiota through the provision of non-digestible starches, otherwise known as prebiotics (41). Fermentation of these starches increases the concentration of short chain fatty acids (SCFAs) and enzymes and reduces colonic pH (42). Of the short chain fatty acids, butyrate has attracted most attention as it is a major metabolic fuel for colonocytes with several biological actions thought to promote a normal phenotype in these cells (43). The increasing evidence regarding synbiotic supplements and resistant starch in animal and in-vitro studies highlights their potential as nutrition adjuncts to ameliorate exercise-induced perturbations in immunity.
Antimicrobial peptides and proteins, exercise and innatemucosal immunity

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2.4 Summary of literature review

Interest in understanding the way in which normal variation in the immune system in healthy individuals alters susceptibility to illness has intensified due to the growing expectation that enhancing immune function will lead to better health. An increasing number of nutrition supplements for use by healthy individuals purportedly boost immunity and, by inference, reduce illness, particularly URTI and GI illness (44). Claims of enhanced immune function associated with these products has led to consumer, regulatory, academic and commercial interest in defining normal reference ranges of immune function (5). The human immune system consists of a vast array of molecules, cells and tissues that protect the body from environmental pathogens and from internal cellular aberrations. At the interface between the external environment and the body is the mucosal immune system, which protects the body from pathogenic microbes and maintains an anti-inflammatory profile in the mucosal milieu and normal physiological function. Interest in defining thresholds and metrics for enhanced immune function has led to calls for human-based studies that manipulate parameters of the immune system to investigate how changes alter susceptibility to illness (1). This focus may identify biomarkers that indicate increased susceptibility to illness and aid interpretation of data from immunology research.

Experimental models to study immunomodulation

Manipulation of physical activity may be a useful experimental model of stress to examine the relationship between the immune system and URTI. Stress elicits changes to the trafficking and activity of cells, secretion of cytokines, and induction of endogenous factors that regulate immune activity through the neuroendocrine system (45). Acute stress-induced changes are proposed to enhance immunity and defence against infection while chronic stress may suppress immunity if it is ongoing or occurs too regularly. Physical activity leads to transient acute and chronic changes in cellular, humoral and messenger molecules of the immune system (25, 46). The relationship between exercise and immunity is characterised as an inverse U-shape, with moderate exercise leading to enhanced immune function compared to no physical activity or prolonged intense exercise (47). Exercise-induced changes in immunity are similar to the changes that occur with other forms of lifestyle and environmental stress, and show similarity to age-related changes (Table 2). Physical activity is also known to alter susceptibility to URTI, with prolonged intense physical exercise increasing susceptibility to illness compared to moderate exercise which can reduce susceptibility to illness (48). The effects of physical activity on the immune system and susceptibility to illness make it a pertinent model to improve understanding between the immune system and illness.
Table 2. Summary of findings for stress induced effects on immune function

<table>
<thead>
<tr>
<th>Marker</th>
<th>Psychological stress</th>
<th>Exercise</th>
<th>Sleep deprivation</th>
<th>Ageing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Chronic</td>
<td>Acute</td>
<td>Chronic</td>
</tr>
<tr>
<td>URTI</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Salivary IgA</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>no data</td>
</tr>
<tr>
<td>Inflammatory cytokines</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>No data</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
</tbody>
</table>

(a) Conflicting data but most studies show a decrease in total IgA secreted with ageing (14).

Nutrition supplements that modulate GI microbiota may be a means to ameliorate the negative effects of prolonged intense exercise on the immune system. Enteric bacteria colonise the GI tract in increasing numbers from the stomach to the colon, where they are estimated at numbers in the order of 10^{14} CFU in the colon. Bacteria play an important role in the development and continuing function of the immune system throughout the lifespan. The mechanism or mechanisms of action by which the microbiota enhances immunity and contribute to host defence are unclear but are likely multi-factorial (49). Indigenous bacteria prevent antigens from physically colonising mucosal surfaces and restrict the availability of resources. Bacterial fermentation of non-digestible starches produces SCFA such as butyrate, propionate and acetate, which are utilised by host cells for normal functioning (50). The microbiota modulates immunity in the GI tract in-vitro by interacting with host immune and epithelial cells. Beyond the GI tract the microbiota are thought to enhance immunity via the common mucosal immune system (CMIS); an interconnected system that links inductive sites for antigen sampling and processing to diverse effector sites throughout the mucosa (that produce antibody, such as IgA, and regulate cellular immune activity (51, 52). There is a broad diversity of interaction between the microbiota and immune system. In conjunction with physical activity, utilising nutrition supplements to modulate enteric microbiota may be a useful means by which to investigate the relationship between the immune system and illness.

Current limitations and future directions
The prophylactic role of the innate immune system has, in recent years, received increased attention as the search continues for ways to reduce the burden of infectious illness. Research examining changes at mucosal surfaces of the respiratory tract have largely focused on the measurement of SIgA (53, 54). However, there is a diverse range of innate and adaptive mucosal immune parameters that limit the ability of antigens to infect the body. AMPs are constituent and inducible factors of secretions at mucosal surfaces that display activity against a broad range of pathogens. The most abundant AMPs in saliva are lactoferrin and lysozyme. There is a paucity of data regarding the effects of exercise on the concentration of salivary antimicrobial
peptides and proteins in athletes. Systematically examining the acute and chronic changes in AMP concentration in saliva after exercise will further elucidate the effects of physical stress at mucosal surfaces. Furthermore, measurement of AMPs may shed light on how moderate exercise reduces susceptibility to URTI and the increase in susceptibility to URTI suffered by some individuals undertaking prolonged intense exercise.

Should exercise alter the acute or chronic concentration of AMPs it would be pertinent to examine whether such changes, in conjunction with perturbations in other indices of immune function, are related to a change in susceptibility to illness. Considerable research has been conducted on the effect of physical stress on GI permeability and cytokines. Decreases in GI permeability are associated with increased infection and enteropathology (55). Strenuous and exhaustive exercise compromises gut barrier function (56). Alongside changes induced by exercise, the translocation of bacteria across the gut wall may induce the secretion of inflammatory cytokines and lead to a dysregulated immune response in the post-exercise period. Cytokines are messenger molecules of the immune system central to the regulation of homeostasis and inflammation. Research is now showing that athletes more susceptible to URTI may have a dysregulated cytokine response to exercise (19). Measuring a range of cytokines together with AMPs and GI permeability may provide a clearer picture of the effects of physical stress on host defence.

Modulating GI microbiota may be a way to ameliorate the acute and chronic effects of exercise on the immune system and to investigate the relationship between immunity and illness. In-vitro, animal and recently, human studies, indicate that modifying the composition of the GI microbiota has potent effects on various immune parameters and URTI (57–60). Three supplements offer promise as mechanisms to alter gut bacteria, modulate immunity and reduce susceptibility to URTI, including L. fermentum (a probiotic), Digestion 1-2-3-4™ (a synbiotic supplement) and HAMSB (resistant starch). The decision to utilise L fermentum was based on pilot research showing that supplementation in highly trained distance runners led to a reduction in the number of days and the severity of respiratory illness and the maintenance of IFN-γ (36). Further in-vivo and ex-vivo studies of the effects of L. fermentum on microbiology, immunity and URTI in a medium scale clinical trial utilising both genders is required. A medium sized clinical trial powered to detect substantial changes in URTI would facilitate an investigation of the relationship between immunity and illness. No previous research has examined the effect of supplementing with Digestion 1-2-3-4™ or resistant starch on GI microbiota, immunity or URTI in healthy physically active populations. Pilot studies in a human clinical trial setting are required for Digestion 1-2-3-4™ and HAMSB to determine the required dosage to modulate faecal microbiota and enhance immunity. Such evidence would justify a full scale clinical trial for Digestion 1-2-3-4™ and HAMSB, particularly if these supplements modulate the same aspects of immunity as L. fermentum. Furthermore, results from the smaller trials can be used to estimate the necessary sample size for a full scale clinical trial, which typically involves large participant numbers and considerable cost. The impact of these studies is to elucidate the dosage and duration of supplementation required to elicit clinically important changes in immunity and susceptibility

41
to illness. This information would provide evidence-based advice for the use of these supplements and enhance understanding of the relationship between immunity and illness.

2.5 Research Aims

Currently there is limited information on the effects of physical activity on the innate/epithelial mucosal immune system. Likewise, although extensive \textit{in-vitro}, \textit{ex-vivo} and animal model investigation supports the efficacy of modulating GI microbiota to elicit changes in immune activity and illness, there is a paucity of data from clinical human studies. Recent reviews highlight that the “jury is still out” on the use of the supplements (47). In the context of the highlighted issues in this literature review, the specific aims of the thesis are to:

- Characterise the acute and chronic effects of prolonged intense exercise on salivary antimicrobial proteins to gain further insight into the effects of high levels of physical stress on airway defences;
- Investigate the effectiveness of two novel supplements (synbiotic and resistant starch) in modulating GI microbiota and resting markers of mucosal and systemic immunity in healthy physically active individuals;
- Investigate the effects of supplementation with a probiotic (\textit{L. fermentum}) on GI microbiota, resting and acute post-exercise markers of systemic and mucosal immunity and URTI and GI illness in healthy physically active individuals; and
- Examine the relationship between changes in GI microbiota, immunity and illness.
Chapter 3 – THE EFFECT OF EXERCISE ON INNATE IMMUNITY
The Effect of Exercise on Innate Mucosal Immunity

Br J Sports Med 2010 44; 227-231 originally published online May 22, 2008
Chapter 4 – SYNBIOTIC SUPPLEMENTATION: EFFECTS ON FAECAL MICROBIOTA AND IMMUNITY IN HEALTHY PHYSICALLY ACTIVE INDIVIDUALS
Abstract

Synbiotic supplements are of interest to individuals undertaking endurance exercise to limit chronic exercise-associated disturbances to the immune system. The aim of this study was to compare a synbiotic supplement (Digestion 1-2-3-4™) containing four probiotic strains, two prebiotics, lactoferrin and bovine whey immunoglobulin-rich fraction with a prebiotic supplement (acacia gum) on gut microbiota, gut permeability and mucosal and systemic immunity in individuals undertaking moderate endurance exercise. Twenty two healthy physically active male subjects (mean age 33.9 ± 6.5y) were randomly allocated to either a prebiotic or synbiotic supplement for 21 days. Saliva, blood, urine and faecal samples were taken pre-, mid and post-intervention. Participants recorded patterns of physical activity on a self-reported questionnaire. There was a 9-fold (2-fold to 43-fold; 90% confidence intervals; \( P=0.03 \)) greater increase in faecal \( L. \) paracasei numbers over the course of the study in the synbiotic compared with the prebiotic group. Digestion 1-2-3-4™ was associated with a 50% (20% to 68%; \( P=0.02 \)) smaller increase in the concentration of serum IL-16 in comparison to acacia gum from pre- to post-study, eliciting a 4.3-fold (3- to 7-fold; \( P<0.01 \)) increase in comparison to an 8-fold (7- to 10-fold; \( P<0.01 \)) increase. No substantial effects of either supplement were evident in measures of mucosal immunity or GI permeability. Synbiotics may exert beneficial effects by increasing the number of beneficial bacteria in the gastrointestinal tract and augmenting the anti-inflammatory effects of regular exercise.
Introduction

Prebiotic and probiotic supplements are of interest to individuals undertaking endurance exercise to prevent exercise-related disturbances to the immune system (22). Supplementation with the prebiotics fructooligosaccharides (FOS or inulin) and galactooligosaccharides (GOS) (37, 61) and probiotics (62) can increase the number of Lactobacilli and Bifidobacterium in faeces, while prebiotics may increase the faecal concentration of short chain fatty acids (SCFA) (63). In-vivo research on prebiotics and probiotics on immunity has found beneficial effects on mucosal and systemic immune parameters (59, 64) although there are conflicting findings reported (65). Combining pre- and probiotics with other ingredients in a multi-component formulation or synbiotic may be more effective than use of the individual constituents alone. Recent technological advances have allowed the manufacture of nutrition supplements purported to enhance the immune system, such as bovine lactoferrin and immunoglobulins. These immune factors are present in humoral components of the body and act to prevent and limit infection.

Research with elite athletes indicates that those more prone to respiratory tract illness have a dysregulated cytokine response to exhaustive exercise (19). We chose to investigate the resting pro-inflammatory cytokines IL-16, IL-18, IFN-γ and IL-12 given their role in regulating CD4+ T cell status, innate immune cell trafficking and the activation of inflammatory mediators (66, 67). Furthermore, altered gut permeability from endurance exercise is proposed to increase mucosal and systemic inflammation via the translocation of bacterial products (68). As yet, there is no indication of whether a synbiotic supplement would have chronic beneficial effects in measures of mucosal or systemic immunity in individuals undertaking regular prolonged intense exercise.

The aim of this study was to examine the effects of a synbiotic supplement containing probiotics, prebiotics, lactoferrin and immunoglobulins with a prebiotic (acacia gum) on faecal microbiology, SCFA concentration, cytokines, salivary lactoferrin and gut permeability. Acacia gum was chosen as a positive control given evidence of its ability to modulate beneficial bacterial species in the gut (69). Employing a positive/active control is consistent with ethical approaches in the use of placebo interventions (70). New treatments must show greater efficacy than current practices and a traditional placebo treatment to justify production and manufacture of a new and novel nutrition supplement.

Material and Methods

Study design
The study employed a randomised, double blind, active-controlled parallel design consisting of a 14 day pre-intervention period where participants stopped eating yoghurt and supplements that modulate enteric microbiota, a 21 day treatment period and a 14 day post-intervention observation period. Subjects were paired on age and maximal oxygen uptake (VO2 max) and randomly allocated to either prebiotic (acacia gum) or synbiotic supplementation. VO2 max was used as a measure to identify subjects undertaking regular endurance activity and was performed
as previously described (71). Subjects consumed three capsules daily with or without food and in the morning or evening. The number of capsules consumed was recorded and all subjects returned their bottle following supplementation to verify compliance. The study was conducted according to the guidelines prescribed in the Declaration of Helsinki, and all procedures involving human subjects/patients were approved by the Human Research Ethics Committees of the Australian Institute of Sport and Griffith University. All subjects provided written informed consent.

**Subjects**

A total of 22 physically active healthy males aged 33.9 ± 6.5 y (mean ± SD) were recruited and completed the study. Subjects were required to declare their use of dietary and/or ergogenic aids that may influence underlying immune function and/or exercise performance. All subjects on immunomodulatory medications were excluded. Inclusion to the study was dependent upon the subjects not taking antibiotics and supplements or foods with probiotics for 14 days prior to and during the study period. At the start of the study subjects undertook an incremental exercise test to exhaustion to determine peak power output, VO₂ max and measure each subject’s fitness for inclusion to the study as physically active individuals. A VO₂ max of >45 ml.kg⁻¹.min⁻¹ is indicative of individuals undertaking regular physical activity. The test was performed on an electromagnetic cycle ergometer (Excalibur Sport, Lode NV Groningen, Netherlands).

**Product**

The synbiotic capsules (Digestion 1-2-3-4™, Probiotech Pharma, Melbourne, Australia) contained 200 mg Glycomax Immunoglobulin, 50mg Glycomax Lactoferrin, 100mg CBAR-Blend-100 (4.6 × 10⁸ *Lactobacillus paracasei*, 6 × 10⁸ *Bifidobacterium Lactis*, 4.6 × 10⁸ *Lactobacillus acidophilus*, 4.6 × 10⁸ *Lactobacillus rhamnosus*), 90 mg Raftiline, 10 mg Raftilose GR, and 10 mg magnesium stearate. The prebiotic supplement contained 116 mg acacia powder, 23 mg of microcrystalline cellulose, 8 mg of silica colloidal anhydrous British Pharmacopeia/United States Pharmacopeia (BP/USP), 31 mg chocolate flavour, 174 mg calcium hydrogen phosphate, 116.5 mg lecithin epikuron, and 5 mg magnesium stearate BP.

**Sample collection**

Faecal and urine samples were obtained at day 0 (after pre-intervention period) and day 21 (end of intervention period) while serum and saliva samples were collected at days 0, 12 (mid-intervention) and 21. Saliva was collected using an eye spear (Defries Industries Pty Ltd, Victoria, Australia). The eye spear was placed between the cheek and teeth for five min, centrifuged for 5 min at 778 g and frozen at −80°C until analysis. Albumin concentration was assessed to control for changes in salivary flow rate. All saliva samples were taken at the same time of the day to control for diurnal variation. Blood (5 ml) was drawn from an antecubital vein at rest and prior to the VO₂ max test. Blood samples were collected directly into K3EDTA tubes (Greiner Bio-one; Frickenhausen, Germany). Plasma was separated by centrifugation at 4974 g for 5 min and stored frozen at −80°C until analysis. Subjects were provided with a faecal sample collection kit and a portable −20°C freezer. A faecal sample was collected in a sealable plastic bag and frozen.
immediately at –20°C. Following collection of the freezers the samples were frozen at –80°C until analysis. Urine samples were collected using a commercial collection kit (ARL Pathology Pty Ltd). In brief, subjects were required to eliminate residual urine after an overnight fast and then consume an isomolar solution (120 ml) containing 6.0 g lactulose and 3.0 g mannitol. Participants then collected urine into a sealed flask over a period of 6 h. At the end of this period a 2.5 ml aliquot was taken from the flask and immediately frozen at –20°C.

**Measures of mucosal immunity**
Salivary lactoferrin concentration was measured spectrophotometrically by an enzyme linked immunosorbant assay (ELISA) using a commercial kit (EMD Chemicals, New Jersey, USA). Albumin concentration was measured by immunoturbidimetric assay on a Hitachi 911 Chemistry Analyser (Roche). The between-run coefficient of variation (CV). Urinary lactulose and mannitol were analysed by high-performance liquid chromatography as previously described (72) and expressed as the mean of two separate runs.

**Measures of systemic immunity**
The cytokines analysed were IL 16, IL-18, IL-12 (p70) and IFN-γ. The concentration of plasma cytokines were measured on a Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). The samples were analysed on custom manufactured Multiplex Cytokine Kits (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA) according to the manufacturer’s instructions and the plates were read using the Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA).

**Physical activity log and adverse symptoms**
Subjects recorded details of their exercise training during the study (Appendix A). For each session, training mileage (km.wk⁻¹), duration (h.wk⁻¹) and intensity (scored on a 1–5 scale: 1, easy; 5, maximal) were recorded. Subjects recorded daily information of symptoms of gastrointestinal illness (Appendix A) during the study as previously described to note any adverse or unusual effects or events during supplementation (73). Symptoms of GI illness included nausea, diarrhoea, bloating and pain. A classification of GI illness was made when two or more symptoms were recorded on consecutive days. The severity of symptoms were self-rated as mild, moderate or severe based on the impact of the symptoms on activities for that day, with mild symptoms associated with no change to normal activities, moderate symptoms resulting in a reduction in or modification to activities and severe symptoms requiring the total cessation of normal activities.

**Microbial analysis**
DNA was extracted according to Abell et al. (74), and quantified using Quant-iT™ Picogreen (Invitrogen). Microbial diversity was examined using a universal bacteria 16S rRNA primer set (907f – 1392rgc). The amplified product was analysed by denaturing gradient gel electrophoresis (DGGE). The PCR and DGGE gel conditions follow the protocol of Abell et al. (74) with the exception that a 35% – 70% denaturing gradient was used. Dominant DGGE bands were extracted
from the gels and sequenced for putative identification. DGGE banding patterns were analysed to estimate bacterial diversity for each specimen using GelCompar II version 6.0 (Applied Maths, Inc., Texas, USA) software package and the normalised banding patterns were further analysed with Primer6 version 6.1.12 and Permanova+ addition version 1.02 (PRIMER-E Ltd, Plymouth, UK) (75). SIMPER analysis was conducted as previous described (76). Total *Lactobacilli*, *L. paracasei*, *L. acidophilus*, *L. rhamnosus*, *B. lactis* and *E. coli* were quantified by real time PCR. The primer pairs, and their annealing temperature and concentration, used to detect groups and specific bacteria are outlined in Table 1.

**Short chain fatty acids**

Faecal samples were thawed at 4°C, pooled, homogenised and then sub-sampled for analysis. Weighed portions for the determination of free (unesterified) SCFA were diluted 1:3 w/w with deionised water containing 1.68mM heptanoic acid as an internal standard (Sigma Chemical Co, St Louis, MO). Unesterified SCFA were analysed as described previously (77). A three point linear standard curve containing acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acids was used for calibration at concentrations spanning the range of those measured in samples from this study.
<table>
<thead>
<tr>
<th>Target group</th>
<th>Primer name</th>
<th>Primer sequence (5’–3’)</th>
<th>Annealing (°C)</th>
<th>Primer conc. (nM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>1114f</td>
<td>CGGCAACGAGCGCAACCC</td>
<td>60</td>
<td>150</td>
<td>(78)</td>
</tr>
<tr>
<td></td>
<td>1275r</td>
<td>CCATTGTAGCACGTGTAGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus group</td>
<td>LactoF</td>
<td>AGCAGTAGGAATCTTCCA</td>
<td>58</td>
<td>500</td>
<td>(79)</td>
</tr>
<tr>
<td></td>
<td>LactoR</td>
<td>CACCGCTACACATGGAG</td>
<td></td>
<td></td>
<td>(80)</td>
</tr>
<tr>
<td>Lactobacillus paracasei</td>
<td>LcaseF</td>
<td>GCACCGAGATTCACATGG</td>
<td>60</td>
<td>500</td>
<td>(81)</td>
</tr>
<tr>
<td></td>
<td>LcaseR</td>
<td>GGTCTTTGGATYTATGCGGTATTAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus rhamnosus</td>
<td>LrhamF</td>
<td>TGCTTGATCTTGATTTATTTTG</td>
<td>62</td>
<td>500</td>
<td>(81)</td>
</tr>
<tr>
<td></td>
<td>LcaseR</td>
<td>GGTCTTTGGATYTATGCGGTATTAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium lactis</td>
<td>Bflact2F</td>
<td>GTGGAGACACGGTTTCCC</td>
<td>60</td>
<td>600</td>
<td>(82)</td>
</tr>
<tr>
<td></td>
<td>Bflact5R</td>
<td>CACACCACACAAATCCAAATAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Statistical analysis
The statistical approach to determine the effect of supplementation was based on clinical and statistical significance (83, 84). Utilising an approach focusing on clinical significance means that the effect of the supplement is determined in relation to the smallest clinically important values. In the absence of thresholds identified for positive and negative effects of nutrition supplementation on immunity and altered microbiota composition, the smallest clinical values were derived by standardisation; in this case 0.20 of the pooled between-subject standard deviation of the supplement groups. The differences between group means of outcome variables were assessed with a modification (85) of Cohen’s scale (86) for standardised effects (trivial 0.0–0.20; small, 0.20–0.60; moderate, 0.60–1.20; large, >1.20). The effect was deemed clear if it was very unlikely ($P<0.05$) to be substantially positive or very unlikely to be substantially negative, and the outcome was reported according to the magnitude (negative, trivial, positive) that had the highest probability. All other effects were deemed unclear.

Descriptive statistics of all measures are presented as mean ± standard deviation or mean ×/÷ factor standard deviation. To calculate the standard deviation as a factor the mean is multiplied by the SD for the upper level while the lower level is calculated by dividing the mean by the SD. DGGE banding patterns were analysed using the Bray-Curtis similarity matrix as previously described (74). Relationships between diet and DGGE banding patterns were examined using the analysis of similarity (ANOSIM) test (one-way). ANOSIM reflects the observed differences in DGGE banding patterns, between diets, contrasted with differences among the replicates within diets. The R statistic denotes the similarity between two groups, with a value of 0 if similarities within diets and between diets are the same on average, and a value of 1 only if all replicates within diets are more similar to each other than any replicates from different diets. Statistical analysis of measures of immunology and Q-PCR data involved a comparison of the difference in the mean change between the treatment groups from pre- to post-intervention. All measures were log-transformed before analysis to reduce non-uniformity of error, and permit the effect of the treatment to be analysed as a percent difference. Differences in the change in mean saliva and serum protein concentrations, and faecal numbers between the groups were analysed with a student’s t-test for independent samples (unequal variance) (87). All Q-PCR data were analysed as absolute numbers of bacteria in one g of wet weight faeces for comparison between treatments. Baseline values of the dependent variable were included as a covariate in these analyses to account for regression to the mean. The extent to which bacterial counts accounted for changes in outcome measures were investigated through covariate analysis. In these analyses the baseline log-transformed bacterial count or the pre-post change in the log-transformed count was the covariate, and the dependent variable was rank-transformed. The effects of supplementation are shown with 90% confidence limits. Significance was accepted when $P<0.05$. 
Results

Subjects
All 22 subjects completed the study. The groups were well-matched on all characteristics (Table 2). There was a large difference at baseline in the mean concentration of salivary lactoferrin between the groups (mean, prebiotic 1457 ×\(\div\) 2.9 versus synbiotic 5389 ×\(\div\) 3.0 ng.ml\(^{-1}\); Figure 4). There were five episodes of mild GI symptoms that included flatulence and stomach rumbles in both groups during supplementation. Both supplements were otherwise well tolerated.

Table 2. Differences in key measures between the groups at baseline.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Prebiotic</th>
<th>Synbiotic</th>
<th>Qualitative difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>31.4 ± 4.9</td>
<td>34.4 ± 3.5</td>
<td>Trivial</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>73.1 ± 4.9</td>
<td>79.1 ± 10.4</td>
<td>Trivial</td>
</tr>
<tr>
<td>VO(_2) max (ml.kg(^{-1}).min(^{-1}))</td>
<td>56.4 ± 4.9</td>
<td>57.9 ± 7.3</td>
<td>Trivial</td>
</tr>
<tr>
<td>Training load p/week (duration × intensity)</td>
<td>21.3 ± 18.5</td>
<td>21.4 ± 16.8</td>
<td>Trivial</td>
</tr>
</tbody>
</table>

VO\(_2\) max, maximal oxygen uptake.

Faecal microbiology and biochemistry
ANOSIM of the DGGE patterns indicated that synbiotic supplementation significantly altered the composition of the gut microflora compared to prebiotic supplementation (R = 0.27, \(P<0.001\)). SIMPER analysis of the DGGE patterns revealed that no one band contributed more than 5% to the dissimilarity between treatments (Figure 1). There were no substantial differences between the treatment groups in the diversity of dominant bacteria, total bacteria \(B.\) lactis, \(L.\) rhamnosus, \(L.\) acidophilus, \(E.\) coli or \(C.\) coccoides from pre- to post-supplementation. QPCR revealed that the detectible number of faecal \(L.\) paracasei increased 9-fold (4-fold to 24-fold; 90% confidence limits) in the synbiotic group and decreased 0.45-fold (–0.16-fold to 1.5-fold) in the prebiotic group, which was a relative 9-fold (2-fold to 43-fold; \(P=0.03\)) difference between the groups (Table 4). There were no substantial changes with Total \(Lactobacilli\), \(L.\) acidophilus, \(L.\) rhamnosus, \(B.\) lactis and \(E.\) coli in either group (Table 4). There were no substantial supplement-related differences between the groups in the concentrations of the individual short chain fatty acid concentrations (Table 4).
Figure 1. Denaturing gradient gel electrophoresis representing bacterial profiles from subjects in the prebiotic and synbiotic groups.

Individual samples from subjects in the prebiotic group are lanes one to nine (baseline) and 10 to 19 (post-supplementation). Samples from subjects in the synbiotic group are in lanes 20 to 28 (baseline) and 29 to 36 (post-supplementation). Identified bacterial species listed by row number can be found in Table 3. A normalised digitised version of this picture is in Appendix B.
Table 3. Identified bacterial species whose diversity changed as identified by DGGE. The percentage similarity to bacterial strains deposited in the National Centre for Biotechnology Information (NCBI).

<table>
<thead>
<tr>
<th>Row number</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Clostridiales clone (99%)</td>
</tr>
<tr>
<td>12</td>
<td>Clostridium clone (99%)</td>
</tr>
<tr>
<td>16</td>
<td>R. bromiiYE282 (97%)</td>
</tr>
<tr>
<td>18</td>
<td>R. bromii L2-63 (99%)</td>
</tr>
<tr>
<td>19</td>
<td>R. bomii L2-63</td>
</tr>
<tr>
<td>20</td>
<td>Lachnospiraceae (94%)</td>
</tr>
<tr>
<td>21</td>
<td>Eubacterium bioforme (99%)</td>
</tr>
<tr>
<td>22</td>
<td>Firmicutes (poss chimera)</td>
</tr>
<tr>
<td>23</td>
<td>Catenibacterium mitsuokai (99%)</td>
</tr>
<tr>
<td>26</td>
<td>F. prausnitzii (99%)</td>
</tr>
<tr>
<td>28</td>
<td>Eubacterium rectal (99%)</td>
</tr>
<tr>
<td>33</td>
<td>L. ruminis (100%)</td>
</tr>
<tr>
<td>36</td>
<td>Ruminococcus clone M3-27R (98%)</td>
</tr>
<tr>
<td>37</td>
<td>Ruminococcus clone M1-89 (98%)</td>
</tr>
<tr>
<td>38</td>
<td>Ruminococcus clone M6-75-R (98%)</td>
</tr>
<tr>
<td>39</td>
<td>Ruminococcus clone M3-27R (97%)</td>
</tr>
<tr>
<td>40</td>
<td>Rosebuiira sp. clone M2-35 (99%)</td>
</tr>
<tr>
<td>41</td>
<td>Rosebuiira sp. clone M2-35 (100%)</td>
</tr>
<tr>
<td>42</td>
<td>C. clostridioforme (99%)</td>
</tr>
<tr>
<td>47</td>
<td>Actinobacterium clone M2P2-71</td>
</tr>
<tr>
<td>51</td>
<td>Bacteroidales clone (93%)</td>
</tr>
<tr>
<td>53</td>
<td>Collinsella aerofaciens (99%)</td>
</tr>
<tr>
<td>55</td>
<td>Olsenella clone J26 (94%)</td>
</tr>
<tr>
<td>57</td>
<td>Collinsella sp. RCA56-80 (98%)</td>
</tr>
<tr>
<td>58</td>
<td>Clostridiales clone (99%)</td>
</tr>
<tr>
<td>61</td>
<td>Eggerthella sp. YY7918 (99%)</td>
</tr>
</tbody>
</table>
Table 4. The effect of supplementation on the concentration on faecal variables. Data are shown as a factor change, e.g. a mean difference in the change of means of 1.2 equates to a 20% difference.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Prebiotic Mean ± SD</th>
<th>Synbiotic Mean ± SD</th>
<th>Difference in change Mean (90% CI)</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
<td></td>
</tr>
<tr>
<td>Total bacteria (copies 16S rRNA gene per g ww)</td>
<td>$4 \times 10^8 \times \pm 4.0$</td>
<td>$6 \times 10^8 \times \pm 3.0$</td>
<td>$6 \times 10^8 \times \pm 3.0$</td>
<td>$-2% (-58 \text{ to } 128%)$</td>
</tr>
<tr>
<td>Total Lactobacillus (copies 16S rRNA gene per g ww)</td>
<td>$5 \times 10^3 \times \pm 12$</td>
<td>$2 \times 10^4 \times \pm 21$</td>
<td>$10 \times 10^3 \times \pm 26$</td>
<td>$-37% (-91 \text{ to } 364%)$</td>
</tr>
<tr>
<td>L. paracasei (copies 16S rRNA gene per g ww)</td>
<td>$4 \times 10^2 \times \pm 4.0$</td>
<td>$2 \times 10^2 \times \pm 5.0$</td>
<td>$2 \times 10^2 \times \pm 5.0$</td>
<td>9-fold (2.0- to 43-fold)</td>
</tr>
<tr>
<td>L. rhamnosus # samples with bacteria</td>
<td>2</td>
<td>8</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>B. lactis (copies 16S rRNA gene per g ww)</td>
<td>$2 \times 10^2 \times \pm 8.0$</td>
<td>$5 \times 10^3 \times \pm 12$</td>
<td>$3 \times 10^2 \times \pm 23$</td>
<td>$3 \times 10^4 \times \pm 12$</td>
</tr>
<tr>
<td>Acetate (μmol/g)</td>
<td>$58 \times \pm 1.3$</td>
<td>$52 \times \pm 1.3$</td>
<td>$54 \times \pm 1.5$</td>
<td>$52 \times \pm 1.4$</td>
</tr>
<tr>
<td>Propionate (μmol/g)</td>
<td>$17.5 \times \pm 1.4$</td>
<td>$16 \times \pm 1.4$</td>
<td>$16 \times \pm 1.6$</td>
<td>$14 \times \pm 1.4$</td>
</tr>
<tr>
<td>Butyrate (μmol/g)</td>
<td>$20 \times \pm 1.3$</td>
<td>$17 \times \pm 1.4$</td>
<td>$18 \times \pm 1.7$</td>
<td>$16 \times \pm 1.7$</td>
</tr>
</tbody>
</table>

SD: standard deviation expressed as a times/divide factor of the mean; CI, confidence interval; L. Paracasei, Lactobacillus paracasei; L. rhamnosus, Lactobacillus rhamnosus; B. Lactis, Bifidobacterium lactis. # Difference in raw units
**Systemic immunity**

The concentration of IL-16 over the course of the study is shown in Figure 2. Relative to the synbiotic group, there was a 50% (20–68%; 90% confidence interval; \( P=0.02 \)) greater increase in the concentration of IL-16 in the prebiotic group from pre- to post-supplementation. With respect to IL-18 (Figure 3), there was moderate increase in the prebiotic group (35%; 18% to 54%) and a small increase in the synbiotic group (23%; 12% to 23%) from pre- to post-supplementation. Covariate analysis did not find any associated between changes in microbiota and changes in resting cytokine changes. The concentration of both IL-16 and IL-18 was characterised by large between- and within-subject variability (~100–300%). No data is reported for IL-12 and IFN-\( \gamma \) as the concentration of both cytokines in the samples was below the detection limit of the assay.

**Figure 2. The effect of supplementation on the concentration of IL-16.**
The values presented are mean and standard deviation of the mean. *significantly different (\( P<0.02 \)) from placebo.

![Figure 2](image1)

**Sampling point**

**Figure 3. The effect of supplementation on the concentration of IL-18.**
The values presented are mean and standard deviation of the mean.

![Figure 3](image2)
**Mucosal immunity**

There was no substantial difference in the concentration of salivary lactoferrin (Figure 4) or the lactulose/mannitol ratio (Figure 5) between the groups from pre- to post-supplementation.

**Figure 4.** The effect of supplementation on salivary lactoferrin.
The values presented are mean and standard deviation of the mean.

![Figure 4](image1)

**Figure 5.** The effect of supplementation on the GI permeability (lactulose to mannitol ratio).
The values presented are mean and standard deviation of the mean.

![Figure 5](image2)
Discussion

The main finding from this study was that synbiotic supplementation elicits favourable changes in colonic microbiota in comparison to a prebiotic supplement. Synbiotic supplementation increased the faecal recovery of *L. paracasei* while supplementation with acacia gum, in contrast, was associated with a reduction in *L. paracasei* numbers. There were only trivial effects of supplementation on other species of faecal bacteria analysed. Both supplements had relatively little effect on the immune system, with the only substantial effect associated with supplementation being a 4-fold increase in the synbiotic group and 8-fold increase in the acacia gum group in resting IL-16 concentration. IL-16 is a pro-inflammatory cytokine that is a chemoattractant for immune cells. The amelioration of the increase in IL-16 concentration in the synbiotic group indicates synbiotic supplementation may have a beneficial anti-inflammatory effect given higher IL-16 expression has been associated with allergies (88). No substantial effects of supplementation on other cytokines or on parameters of mucosal immunity were evident. Increasing the number of beneficial bacteria in the GI tract indicates synbiotic supplementation may be useful for enhancing gastrointestinal health.

Supplementation with pre- and probiotics purportedly exert their positive effects on the immune system by increasing beneficial species of bacteria colonising the GI tract. In this study, only the synbiotic supplement fostered a substantial change in the microbiota, eliciting a 14-fold increase in the recovery of faecal *L. paracasei*. This finding is important given that GI microbiota may be disturbed as healthy individuals age and in response to stress, contributing to a heightened risk of disease in later life (89). Given that there were four strains of bacteria in the synbiotic, however, it was expected that a greater number of bacteria would be recovered following supplementation. Our findings regarding *L. paracasei* and *B. lactis* BB-12 are in contrast to previous research in which BB-12 was recoverable and *L. paracasei* was not (35, 62) while our inability to recover *L. acidophilus* is consistent with the findings of Shioya et al (35). The lack of recoverable BB-12 following supplementation with the synbiotic is also surprising given the bifidogenic effect reported for FOS and GOS (90). The results from the present research indicate that the dosage of probiotic bacteria and the dosage of the prebiotics in Digestion 1-2-3-4™ (90 mg Raftiline and 10 mg Raftilose GR per capsule) were not sufficient to elicit further changes in microbiota as evident from the bacterial diversity analysis conducted. That the dosage of prebiotics was too low was further confirmed by the lack of effect of supplementation on faecal SCFA. While this study shows for the first time that the concentration of SCFAs in endurance athletes are similar to the general population (42), our findings confirm previous research that dosages of 5 to 10 g/day of FOS and GOS are needed to induce changes in faecal bacteria and short chain fatty acid concentrations (91). The synbiotic formulation may have greater effects on faecal microbiota by removal of FOS and GOS and an increase in the other probiotic species to counts over one billion CFU.

Over the course of the present study there was an increase in resting IL-16 concentration in both treatment groups. However, synbiotic supplementation appears to have reduced the magnitude
of the increase in IL-16 in comparison to prebiotic supplementation. Examination of the training data from participants during the study indicated that the increase in cytokine concentration was not the result of altered physical activity patterns from pre- to post-supplementation. Furthermore, covariate analysis did not find any clear trends between supplement-induced changes in microbiota and the increase in IL-16 concentration. IL-16 is a pro-inflammatory cytokine that is chemotactic for immune cells, particularly T-cells (92). Higher IL-16 expression in the respiratory tract contributes to the inflammation observed in allergic rhinitis in healthy adults (88). Ageing is also associated with an increased inflammatory profile, known as inflammation-ageing, and immune senescence (93). Given the association between higher concentrations of IL-16 and allergies and the potential for IL-16 to contribute to chronic inflammation, moderation of the increase in plasma IL-16 concentration with synbiotic supplementation relative to prebiotic supplementation may have beneficial anti-inflammatory effects. Interpretation of the differences in IL-16 result must be approached with caution; whether changes in a single resting cytokine concentration in healthy individuals alone can be used as a marker for altered immunity is uncertain. No clinical thresholds for changes in cytokine concentrations associated with enhanced or suppressed immunity or with illness in healthy individuals have been determined. Expanding measures of immune function to ex-vivo or in-vitro testing may elucidate whether the changes in cytokine concentrations are associated with enhanced immune function. A number of human and animal studies utilising other supplements that alter gut microbiota indicate that these supplements alter immune cell activation and cytokine secretion following bacterial challenge (36, 65). Rather than altering the homeostatic activity of the immune system it appears that these supplements may alter the immune response to challenge in healthy individuals. Examining the effect of synbiotic supplementation on the immune response to challenge may provide further information on the mechanisms by which Digestion 1-2-3-4™ exerts its health benefits.

Neither synbiotic nor prebiotic supplementation had a substantial effect on gut permeability and the concentration of salivary lactoferrin. Probiotic administration enhances epithelial barrier integrity in-vitro and in animal models (94). There is less convincing evidence in-vivo, with studies of critically ill patients showing that probiotic supplementation may decrease gut permeability (95). There are few studies reporting effects on healthy individuals. Given that pre-, pro- and synbiotic supplements are marketed for reducing susceptibility to illness in healthy population groups, it is necessary to identify specific immune factors associated with particular types of illness. GI permeability was measured in the present study given evidence that individuals undertaking prolonged intense exercise may have higher barrier permeability (96). Baseline measures of the mean lactulose/mannitol ratio in each group were within normal values and it may be that improvements in GI permeability are more likely to occur only when permeability has previously been disturbed. Interest in examining the concentration of lactoferrin was based on previous research showing prolonged intense exercise reduces the concentration of lactoferrin by 60% (97). There is evidence that lactoferrin can modulate aspects of immunity (98). However, these benefits were in healthy children with recurrent respiratory infections. From this it may be concluded that lactoferrin may reverse aberrant but not immune-deficient parameters of
immunity. In this study the concentration of salivary lactoferrin at baseline was substantially
different between the groups, highlighting the need for the establishment of normal ranges to
determine the efficacy of immune-modulating supplements. Future studies examining lactoferrin
should focus on cellular activation to antigenic challenge given this may be where benefits are
most likely to occur.

There is evidence that supplementation with probiotics and lactoferrin have clinically beneficial
outcomes in healthy individuals. A synbiotic preparation that contained prebiotics, *L. rhamnosus*,
*B. lactis* and lactoferrin as part of its ingredients reduced the incidence and severity of respiratory
tract illness in healthy individuals (99). Furthermore, a meta-analysis indicates that use of
*Saccharomyces boulardii* and a mixture of *L. acidophilus* and *Bifidobacterium* can prevent traveller's
diarrhoea (100). This study provides evidence that a synbiotic preparation with probiotics and
lactoferrin increases the recovery of ingested beneficial bacteria, which provides a mechanism
for conferring benefits in the gastrointestinal and respiratory tracts via the common mucosal
immune system. As an interconnected system, the common mucosal immune system links
inductive sites in the GI tract with effector sites, such as the nasal associated lymphoid tissue.
Once primed through antigenic stimulation in the GI tract, lymphocytes home to effector sites
to prevent infection in these areas. The isolation of immunoglobulins from bovine colostrum
offers an opportunity to investigate a new and novel protein on illness. Milk immunoglobulins
are considered an essential immune mediator for protection of infants from illness (54). There are
no studies the author is aware of examining the clinical efficacy of orally consumed whey-derived
immunoglobulins, either alone or in combination with other supplements, on common illnesses.

In conclusion, supplementation with a synbiotic supplement (Digestion 1-2-3-4™) in healthy
physically active individuals elicited a substantial increase in the recovery of *L. paracasei*. There
were minor effects of supplementation on immunity overall, with synbiotic supplementation
attenuating the increase in resting IL-16 concentration to half that of the prebiotic group. No
effects were evident for other resting cytokines or measures of mucosal immunity. Further
research focusing on cellular markers of activity and on conditions associated with aberrant
immune responses, particularly inflammatory disorders, should provide further evidence on the
usefulness of synbiotics in healthy active adults. Dietary intervention with a synbiotic supplement
may be a nutritional adjunct for gastrointestinal health given its effect on commensal microbiota.
Chapter 5 – BUTYRYLATED HIGH AMYLOSE MAIZE STARCH AND IMMUNITY
Abstract

The relationship between dietary supplementation, commensal microbiota and immune function in physically active, healthy individuals is uncertain. The aim of this study was to determine the effect of supplementation with butyrylated high amylose resistant starch (HAMSB) and low amylose resistance starch (LAMS) on faecal chemistry, faecal microbiota, salivary and serum immune markers. Twenty-three male and eighteen female healthy physically active volunteers were divided randomly into two groups. Participants consumed supplements containing either 20 g HAMSB (n=23; age 37.9 ± 7.8 y; mean ± SD) or low amylose maize starch (LAMS) (n=18 age 36.9 ± 9.5 y) twice daily for 28 d. Saliva, blood and faecal samples were collected on days 0, 10 and 28. Predominant bacterial groups, faecal short chain fatty acids, pH, ammonia, serum cytokines and salivary IgA, lactoferrin and lysozyme were measured. HAMSB consumption elicited a 42% increase in faecal butyrate (8% to 80%; \( P=0.03 \)) and propionate (12% to 83%; \( P=0.02 \)), and lowered pH by 3% (5% to 0.5%; \( P=0.05 \)) relative to LAMS. HAMSB consumption also led to an 81-fold (28- to 237-fold; 90% confidence interval, \( P<0.01 \)) and 5.1-fold (2.1- to 12-fold; \( P<0.01 \)) relative increase in faecal \( P. distasonis \) and \( F. prausnitzii \) respectively compared to LAMS. HAMSB was associated with a relative 1.6-fold (1.2- to 2.0-fold; \( P<0.01 \)) and 2.5-fold (1.4- to 4.4-fold; \( P=0.01 \)) increase in serum IL-10 and TNF-\( \alpha \) compared to LAMS. There were no substantial differences for other cytokines, measures of mucosal immunity or DNA damage between the groups. The increases in beneficial bacteria and faecal biochemistry indicate HAMSB may enhance gastrointestinal health. In contrast, the effects of LAMS may have deleterious effects on GI health.
Introduction

Prolonged physical and psychological stress, such as experienced by elite athletes (97, 101), nurses (102) and students undertaking examinations (103) can increase susceptibility to illness via perturbations in immune function (17). Such stresses can lower the salivary concentrations of IgA, lactoferrin and lysozyme, humoral factors of the immune system that provide defence at mucosal surfaces. Lowering of salivary IgA is correlated moderately with an increased risk of URTI (104). A recent study showed dysregulated cytokine responses to metabolic stress were associated with a higher rate of URTI in active individuals (19). Clearly, there is scope to modulate the activity of the immune system through dietary and lifestyle changes to reduce the risk of stress-induced illnesses.

The GI tract is a key factor in mucosal and systemic immune response through the gut-associated lymphoid tissue (GALT). The commensal microbiome of the large bowel has a strong influence on immune and inflammatory processes, especially through its interaction with GALT (105, 106). Furthermore, microbial fermentation of dietary fibre carbohydrates produces short chain fatty acids (SCFA), principally acetate, propionate and butyrate, which play a key role in epithelial cell and mucosal barrier function (43). Of these, butyrate has attracted considerable attention. It is a major metabolic fuel for colonocytes and has a number of actions thought to promote a normal phenotype in these cells (107).

The concentrations and relative proportions of the major SCFA are affected strongly by dietary intakes, especially fibre. One of the key influences on SCFA production is the entry of undigested starches into the large bowel, particularly resistant starch (RS). Animal and human studies have shown that RS fermentation, in general, favours butyrate production and that in animals this SCFA modulates various indices of cellular activity and immunity (108). However, human studies investigating the latter have produced contradictory results (34), possibly due to inter-individual variation in the composition of the microflora in humans. The composition of the bacteria inhabiting the GI tract can vary greatly due to the influences of genetic, environmental, dietary and age factors and such differences may be too great to detect alterations in immune status. One means of overcoming this variability is to deliver SCFA to the colon using starches acylated with specific SCFA. These starches resist small intestinal digestion while the esterified acids are digested by bacterial enzymes in the large bowel (77). They have proven effective at promoting sustained increases in colonic SCFA. This technology has been used to determine the effect of butyrylated starch on faecal SCFA and microbiology and on biomarkers of mucosal and systemic immunity in healthy, physically active individuals.
Material and Methods

Study design
The study was a randomised, double blind, controlled parallel trial to compare the effects of HAMSB with LAMS. LAMS was used as a control to ensure both groups had equivalent levels of dietary resistant starch. Animal studies indicate that LAMS has only trivial effects on faecal bacteria and the production of SCFA (109). The study consisted of a 14 day pre-intervention period where participants were asked to refrain from eating yoghurt and any supplements that could modulate the enteric microbiota. This was followed by a 28 day period when participants consumed the supplements and a 14 day post-intervention observation period. Participants were paired on age and maximal oxygen uptake (VO2max), and randomly allocated to either experimental (HAMSB) or LAMS supplementation. Participants consumed two specially formulated beverages daily; one in the morning and one in the evening. The study was conducted according to the guidelines prescribed in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Human Research Ethics Committees of the AIS, CSIRO and Griffith University. All participants provided written informed consent.

Subjects
A total of 41 healthy active male (n=23; age 37.9 ± 7.8 y (mean ± SD)) and female (n=18; age 36.9 ± 9.5 y) cyclists were recruited from the general community. Subjects were required to declare their use of dietary and/or ergogenic aids that may influence underlying immune function and/or exercise performance. All subjects consuming immunomodulatory medications were excluded. Inclusion to the study was dependent upon the subjects not taking antibiotics and supplements or foods with probiotics for at least one month prior to and during the study period.

Product
The drinks comprised 60 g of Protein Plus Protein powder (Powerbar Oceania, Rhodes, Australia) with either 20 g HAMSB (National Starch Food Innovation, Sydney, Australia) or low amylose maize starch (LAMS) (National Starch Food Innovation, Sydney, Australia) in 200 ml of water or milk. Participants were asked to consume one beverage in the morning and one in the evening. The beverages could be consumed with or without food.

Sample collection
Faecal, saliva and blood samples were obtained pre-, mid (day 14) and post-supplementation (day 28) from all subjects. Saliva was collected using an oral eyespear swab (Defries Industries Pty Ltd, Victoria, Australia). The eyespear was placed between the cheek and teeth for 5 min, removed and immediately centrifuged for 5 min at 778 g and frozen at ~80°C until analysis. Albumin concentration was assessed to control for changes in salivary flow rate. All saliva samples were taken at the same time of the day to control for diurnal variation. A blood sample (9 ml) was drawn from the antecubital vein to quantify resting serum cytokine concentrations. Each sample was collected directly into a K3EDTA tube (Greiner Bio-one; Frickenhausen, Germany)
and frozen at –80°C until analysis. Participants provided a faecal sample within 48 hours of the blood and saliva sampling. Faeces was collected in a sealable plastic bag and frozen immediately at –20°C in a portable freezer until transfer to laboratory storage at –80°C.

**Dietary control**

All participants maintained a four day food diary (Appendix C) during the study that incorporated two week days and a weekend. Subjects were asked to maintain a normal diet beyond the instruction to refrain from eating yoghurt and other probiotic or prebiotic-enriched foods. Participants were also required to complete a food frequency questionnaire (Appendix D) prior to providing a faecal sample. The baseline pre-faecal dietary intake was repeated before the end of study faecal sample to reduce the effects of short-term variation in starch consumption modifying GI microbial flora and the concentration of SCFA.

**Measures of mucosal immunity**

Lactoferrin, lysozyme and SIgA concentrations were measured spectrophotometrically by enzyme linked immunosorbent assay (ELISA) using commercial kits (lactoferrin – EMD Chemicals, New Jersey, USA, lysozyme – Sapphire Bioscience Redfern Australia, SIgA – Salimetrics, IgA – Salimetrics, and Philadelphia, USA). Albumin concentration was measured by immunoturbidimetric assay on a Hitachi 911 Chemistry Analyser (Roche). Osmolality was measured on a Model 3320 Osmometer (Advanced Instruments Inc.) as per the manufacturer’s instructions. Variability was acceptable at <10% for the low and high positive controls.

**Measures of systemic immunity**

Granulocyte macrophage-colony stimulating factor, (GM-CSF), interleukin (IL)-1RA, IL-6, IL-8, IL-10, tumour necrosis factor (TNF-α) and interferon gamma (IFN-γ) cytokines were measured on a Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). The samples were analysed on custom manufactured Multiplex Cytokine Kits (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). Plates were read using the Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). A full blood count including white cell count and differential was performed on a haematology analyser (Advia, GMI, Michigan, USA). Results from each assay were accepted if the positive controls were within two standard deviations of their established mean concentration. Each plate included a control.

**Illness, training and performance measures**

Symptoms of upper respiratory, chest and gastrointestinal illness were recorded daily (Appendix A) by subjects as described previously (73). Subjects also completed a well-being questionnaire pre- and post-study and a gastrointestinal health-specific questionnaire post-supplementation. Details of exercise or physical training undertaken during the study were also recorded. For each session, training mileage (km.wk⁻¹), duration (h.wk⁻¹) and intensity (scored on a 1–5 scale: 1, easy; 5, maximal) were recorded. At the start of the study subjects undertook an incremental exercise test to exhaustion to determine peak power output and maximal oxygen uptake (VO₂max). The test
was performed on an electromagnetic cycle ergometer (Excalibur Sport, Lode NV Groningen, Netherlands) as previously described (71).

Faecal samples

**Microbial analysis**

DNA was extracted from stool samples according to the method of Yu & Morrison (110). Briefly, the protocol combines a mechanical (bead beating) and enzymatic lysis of bacterial cells followed by a clean up to eliminate contamination from other cell debris.

**Phylogenetic profile using a custom microarray**

A custom phylogenetic microarray developed and validated for gut bacteria was used to analyse the microbiota (111). Briefly, extracted DNA from faecal samples obtained pre- and post- intervention were amplified using the prokaryote 16S rRNA gene primer sets 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and T7/1492R (5′-TCTAATACGACTCACTATAGGGGGYTACCTTGTTACGACTT-3′) (the underlined region is modified to include a T7 promoter sequence). Detailed methods of cRNA synthesis and labelling, hybridization, image capture and analysis have been previously described (111). Filtered and normalized data were analysed by the multivariate analysis tool, principal component analysis (PCA), using Genespring 7.3 software (Agilent Technologies, Santa Clara, CA, USA), which automatically determined the number of components in the PCA models. Volcano plot analysis was also performed on data with a two-fold or greater difference and a significant \( P \)-value \( (P=0.05) \) between the supplement groups (HAMSB and LAMS) and time points (Pre and Post) using the Genespring program. The selected probes filtered by volcano plot analysis were compared by box plot using the same software and differences were confirmed by Q-PCR analysis.

**Quantitative real time PCR (Q-PCR)**

Q-PCR was performed to confirm the findings of the microbial changes identified with the human gut microarray. Total bacteria, *Faecalibacterium prausnitzii* and *Parabacteroides distasonis* were quantified using QPCR, which were performed in reaction volumes of 10 or 20 μl containing 1X SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA) and 0.2 mg/ml BSA. Primers (concentration): Total bacteria (150 nM), 1114f (5′-CGGCAACGAGCGCAACCC-3′) and 1275r (5′-CCATTGTAGCAGTGTGATCC-3′) (78) *F. prausnitzii* (500 nM), FPR-1 (5′-AGATGGCCTCGCGATCCGA-3′) and FPR-2 (5′-CCGAAGACCTTCTTCCGCTCC-3′) (112), and *P. distasonis* (500 nM) BdisF (5′-TGATCCCTTGTGCTGCT-3′) and (5′-ATCCCCCTCATTCCGA-3′) (113). For quantification a total of 10 ng of template DNA was used and PCR cycling was performed in a Chromo-4 thermocycler (Bio-Rad Laboratories, Hercules, CA). The PCR cycling conditions were as follows: 4 min at 98°C followed by 35 cycles of 98°C for 20 s, 60–62°C for 20 s (total bacteria: 60°C; *F. prausnitzii* and *P. distasonis*: 62°C) and 72°C for 30 s with fluorescent acquisition after each cycle. A final melt curve analysis was performed after completion of all cycles with fluorescence acquired at 0.5°C intervals between
55 and 95°C to verify that only the expected fragment was amplified. PCR product was also visualised on a 1.5% agarose gel. Non-template controls were included and assays were performed in technical triplicate by analysing the same DNA sample in 3 independent reactions. An 8-series of 10-fold dilutions of a sample derived plasmid construct (Topo chemical competent cells, Invitrogen) containing the target amplicon were analysed in parallel with DNA samples for estimation of absolute abundance and PCR efficiency for all assays. Results were analysed with the Opticon Monitor 3 software (ver. 3.1) (Bio-Rad Laboratories, Hercules, CA). All Q-PCR data were analysed as absolute numbers of bacteria in one g of wet weight faeces.

Short chain fatty acids

Faecal samples were thawed at 4°C, pooled, homogenised and then sub-sampled for analysis. Weighed portions for the determination of free (unesterified) SCFA were diluted 1:3 w/w with deionised water containing 1.68mM heptanoic acid as an internal standard (Sigma Chemical Co, St Louis, MO). Unesterified SCFA were analysed as described previously (77). A three point linear standard curve containing acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acids was used for calibration at concentrations spanning the range of those measured in samples from this study. Total faecal ammonia concentration and pH were determined using a previously described method (114).

Statistical analysis

All data is presented as mean ± standard deviation. Statistical analysis of measures of immunology and enteric microbiota (Q-PCR data) evaluated the magnitude of the difference in the mean change between the treatment groups from pre- to post-intervention. The measures of training hours, training load, salivary and serum proteins and faecal microbiology were log-transformed before analysis to reduce non-uniformity of error, and permit the effect of the treatment to be analysed as a percent. Differences in the change in mean saliva and serum protein concentrations, and faecal numbers, between the groups were analysed with a student's t-test for independent samples (unequal variance) (87). Baseline values of the dependent variable were included as a covariate in these analyses to account for regression to the mean. The extent to which changes in bacterial counts accounted for changes in other outcome measures was examined through covariate analysis.

Standardised mean changes were used to characterise differences between groups. A modification of Cohen's effect size (ES) classification system (trivial: 0.0–0.2; small: 0.2–0.6; moderate: 0.6–1.2; large: 1.2–2.0) was used to interpret the magnitude of observed changes (85). An effect was inferred to be unclear if its confidence interval spanned substantial positive and substantial negative values. The effects of supplementation are shown with 90% confidence limits. Statistical significance was accepted at $P<0.05$
Results

Subjects
Eighteen women and 23 men entered the study. One male subject withdrew from the study indicating a dislike for the supplement while three female and two male volunteers reduced their intake of supplementation from 40 g of supplement to 20 g in the first seven days due to a feeling of fullness. Differences between the groups in baseline characteristics at group allocation are detailed in Table 1. There were no significant differences between the HAMSB and LAMS groups in any of the parameters measured at baseline.

Table 1. Differences between the LAMS and HAMSB groups at baseline

<table>
<thead>
<tr>
<th></th>
<th>LAMS</th>
<th>HAMSB</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>23</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>37.2 ± 8.5</td>
<td>37.6 ± 8.3</td>
<td>0.87</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>69.0 ± 14.2</td>
<td>66.0 ± 11.2</td>
<td>0.45</td>
</tr>
<tr>
<td>VO2max (ml.kg⁻¹)</td>
<td>57.5 ± 5.0</td>
<td>54.9 ± 7.2</td>
<td>0.18</td>
</tr>
<tr>
<td>Total energy (Kj)</td>
<td>8954 ± 2870</td>
<td>8069 ± 1850</td>
<td>0.26</td>
</tr>
<tr>
<td>Fibre (g)</td>
<td>19 ± 11.8</td>
<td>16 ± 6.8</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation of the mean, VO2max, maximal oxygen uptake.

Faecal biomarkers
Faecal pH was a relative 4.6% (7.7% to 1.4%; 90% confidence interval; P=0.02) and 3.0% (5.4% to –0.5%; P=0.05) lower in the HAMSB than LAMS group at the mid- and end-points of the study respectively (Table 2). There was a 33% reduction (50% to 12%) in the concentration of ammonia from pre- to mid-study in the HAMSB group and 17% increase (0% to 36%) in the LAMS group, which is a relative reduction of 36% (53% to 12%; P=0.01) with HAMSB supplementation. The difference between the groups in the concentration of faecal ammonia from pre- to post-supplementation was unclear.

Changes in the concentration of total SCFA, acetate, propionate, butyrate are presented in Table 2. There was a relative 45% (14% to 84%; P=0.01) difference in total SCFA concentration between the groups from pre- to mid-study, with HAMSB increasing total SCFA concentration by 14% (–4 to 36%) and LAMS reducing total SCFA concentration by 20% (33% to 5%). No substantial effects were evident in total SCFA concentration between the groups from pre- to post supplementation or from mid- to post-supplementation. There was a moderate difference between the groups in the concentration of acetate (28%; 1.5% to 62%; P=0.08) from pre- to mid-supplement (mean change, HAMSB –1.3% ± 18% versus LAMS –21% ± 20%) but there was no substantial difference between the groups in acetate concentration from pre- to post-supplementation. The concentration of propionate increased by 25% (7% to 47%) in the HAMSB group and decreased by 11% (26% to –9%) in the LAMS group from pre- to post-supplementation.
There was a 42% (8% to 88%; \(P=0.04\)) difference between the groups in butyrate, with HAMSB eliciting a 13% (−5% to 36%) increase and LAMS resulting in a 21% (37% to 2%) reduction in butyrate concentration from pre- to post-supplementation.

**Faecal microbiology**

Microarray analysis comparing pre- to post-supplementation microbial profiles showed clear differences between post-HAMSB and post-LAMS treatments. Principal components analysis (PCA) showed differences in the microbial profiles between supplement groups (Figure 1 A and B). The analysis indicated that HAMSB supplementation elicited a higher abundance of *P. distasonis* than other groups. Furthermore, some individuals in the HAMSB group showed a significant increase in the signal intensity of *F. prausnitzii* probes but this was not consistent for the whole treatment group.

The microarray results were confirmed by Q-PCR using primers specific for *P. distasonis* and *F. prausnitzii*. The effect of supplementation based on Q-PCR analysis of *P. distasonis* and *F. prausnitzii* is shown in Figure 2. HAMSB supplementation elicited an 81-fold relative increase in *P. distasonis* compared to LAMS (mean change, HAMSB 31-fold ×/÷ 2.6-fold versus LAMS −0.3-fold ×/÷ 1.7-fold) and a 5.1-fold relative increase in the abundance of *F. prausnitzii* (mean change, HAMSB 1.6-fold ×/÷ 1.9-fold versus LAMS −0.3-fold ×/÷ 1.8-fold). Differences in total bacteria between the supplement groups were not significant.

Covariate analysis did not reveal any clear trends between changes in *P. distasonis* and *F. prausnitzii* and changes in SCFA. Furthermore, no clear relationships between changes in SCFA, individually or in total, were evident with changes in faecal pH or ammonia.
Table 2. Effects of supplementation with HAMSB and LAMS on faecal output, pH, and the concentration of ammonia and short chain fatty acids. Data are shown as a factor change, e.g. a mean difference in the change of means of 1.2 equates to a 20% difference.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LAMS</th>
<th>HAMSB</th>
<th>Difference in Change (Pre to Post)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Mean ± SD</td>
<td>Mid Mean ± SD</td>
<td>Post Mean ± SD</td>
</tr>
<tr>
<td>Faecal output (g)</td>
<td>128 ± 2.0</td>
<td>166 ± 1.7</td>
<td>103 ± 2.2</td>
</tr>
<tr>
<td>pH</td>
<td>6.9 ± 1.1</td>
<td>7.1 ± 1.1</td>
<td>7.1 ± 1.06</td>
</tr>
<tr>
<td>Ammonia μmol/g</td>
<td>18 ± 2.0</td>
<td>21 ± 1.5</td>
<td>16 ± 1.6</td>
</tr>
<tr>
<td>Total SCFA μmol/g</td>
<td>80 ± 1.3</td>
<td>64 ± 1.6</td>
<td>67 ± 1.8</td>
</tr>
<tr>
<td>Acetate μmol/g</td>
<td>50 ± 1.4</td>
<td>39 ± 1.6</td>
<td>51 ± 1.4</td>
</tr>
<tr>
<td>Propionate μmol/g</td>
<td>15 ± 1.4</td>
<td>12 ± 1.6</td>
<td>13 ± 1.8</td>
</tr>
<tr>
<td>Butyrate μmol/g</td>
<td>15 ± 1.4</td>
<td>11 ± 1.3</td>
<td>11 ± 1.9</td>
</tr>
</tbody>
</table>

HAMSB: butyrylated high amylose maize starch; LAMS: high amylose maize starch; SD: standard deviation expressed as a times/divide factor of the mean. 90%CL: 90% confidence limits expressed as a times/divide factor of the mean effect.
Figure 1. Principal component analysis (PCA) of variation between the bacterial communities present in all individual faecal samples. Each data point represents an individual sample. Black squares are HAMSB diet samples, clear squares are LAMS diet samples and A) and B) represent different time points PRE and POST. Figure was calculated using selected 305 probes passed by one way ANOVA test.

A) PCA Component 1 (A, 18.1% and B, 15.7% variance)

B) PCA Component 1 (A, 18.1% and B, 15.7% variance)
Figure 2. The effect of supplementation with HAMSB and LAMS on *P. distasonis* and *F. prausnitzii*. Values presented are mean and standard deviation of the mean. *significant difference (P<0.01) between the treatment groups.

Systemic immunity

The effect of supplementation on resting IL-1RA, IL-10 and TNF-α is shown in Figure 3. IL-1RA concentration was 1.9-fold higher in the HAMSB group compared with the LAMS group from pre- to mid-study but there was only a trivial difference between the groups from pre- to post-supplementation. The differences between the groups from pre- to mid-study in IL-1RA were a result of a reduction in IL-1RA concentration in the LAMS group (∼43%; ∼55 to ∼28; 90% confidence interval). From pre- to post supplementation there was a greater decline in IL-10 in the LAMS group than the HAMSB group (by a factor of 1.6-fold; 1.2- to 2.0-fold; P<0.01) while TNF-α concentration was 2.5-fold (1.4- to 4.4-fold; P=0.01) higher in the HAMSB group.
compared with the LAMS group. No other substantial effects between the groups were evident in the concentrations IL-6, IL-8 or GM-CSF from pre- to post-supplementation.

**Figure 3.** The effect of supplementation on the concentration of IL-1RA, IL-10 and TNF-α from pre- to post-intervention. Values presented are mean and standard deviation of the mean. *significant difference between the treatment groups.
Mucosal immunity
There were no substantial differences between treatment groups in any of the salivary proteins measured (Figure 4).

Figure 4. The effect of supplementation on salivary proteins from pre to post study. Values presented are mean and standard deviation of the mean.
Discussion

The major findings from this study were that HAMSB supplementation raised faecal SCFA concentrations and the numbers of two beneficial species of bacteria in faeces in healthy adults while LAMS, unexpectedly, had the opposite effects. Of the SCFAs, butyrate and propionate concentration showed the greatest proportional rise in response to HAMSB supplementation. This result is consistent with the documented ability of acylated starches to deliver SCFA to the human large bowel and of the ability of the microbiota to ferment dietary fibre. Butyrate is known to promote cell turnover and protect against epithelial damage. Supplementation with HAMSB substantially increased the numbers of faecal *P. distasonis* and maintained the numbers of faecal *F. prausnitzii*. In contrast to HAMSB, consumption with LAMS substantially reduced faecal recovery of *P. distasonis* and *F. prausnitzii*, and the concentration of SCFA. Collectively these data provide evidence for the beneficial effects of HAMSB supplementation for GI health in physically active adults.

Animal experiments have confirmed that acylated starches raise large bowel SCFA through the bacterial hydrolysis of the starch-SCFA bond and the subsequent fermentation of the residual starch (77). This combined action helps to explain the higher faecal concentrations of the other major SCFA (propionate) in addition to the esterified acid as well as the lower pH values in the present study. The latter effect is thought to occur via direct acidification of gut contents by SCFA as well as the lower NH₃ levels. However, covariate analysis found no clear trend between the increasing SCFA and lower NH₃ levels with pH, which is unsurprising as a much larger sample size would be required to investigate the relationship between changes in SCFA and NH₃ with a secondary outcome such as pH. Animal studies have shown also that the ileal digestibility of LAMS is ~100% and it had been anticipated its consumption would not alter any of the faecal biomarkers relative to baseline (115). However, this was not the case as HAMSB and LAMS had divergent effects on faecal SCFA and pH. While butyrate concentrations increased with HAMSB supplementation, this effect was only evident to the mid-point of the study. The significant difference between the groups from pre- to post-supplementation was instead related to a moderate decrease in the concentration of butyrate in the LAMS group (43). The explanation for the reduction in butyrate concentration in the HAMSB group from mid- to post-supplementation is uncertain given there were no substantial changes in dietary practices and compliance throughout the study. Whether the reduction in butyrate concentration was related to increased uptake by colonocytes is an open question. The reason for the difference in total SCFA concentration and acetate between the groups to the mid-point of the study was also related to a fall in total SCFA and acetate concentration in the LAMS group rather than a substantial increase in the HAMSB group. The substantial falls in total SCFA, acetate and butyrate concentration, and the increases in faecal pH and ammonia in the LAMS group, have important implications for dietary practice as it had been expected that this highly digestible starch would have no impact on faecal biomarkers.
The commensal microbiota plays an important role in gut and immune development, homeostasis and prevention of disease (116). Estimates of faecal abundance of *P. distasonis* and *F. prausnitzii* showed no differences between the two groups at entry into the trial. The dose of the HAMSB supplement (40 g/person/d) was sufficient to promote an increase in faecal recovery of *P. distasonis* and maintain the level of faecal *F. prausnitzii*. In contrast, consumption of LAMS lowered the faecal recovery of both bacterial species substantially. Increased faecal numbers of *P. distasonis* have been observed in feeding trials with acylated starches suggesting that it may be involved in their degradation (76). Animal models indicate that increasing the abundance of *P. distasonis* may have clinical benefits. Oral administration of *P. distasonis* in a mouse model of colitis reduced the severity of intestinal inflammation (106). *F. prausnitzii* has been reported to favour butyrate production, which is of considerable importance in light of the putative role of this SCFA in bowel health. Numbers of this bacterium are low in Crohn's disease and irritable bowel syndrome patients compared to healthy controls (111, 117). However, a recent report of clinical improvement in Crohn's disease correlated with a substantial decrease in *F. prausnitzii* abundance (112), highlighting the complexity in understanding the role of the microbiota in health and disease. This study provides evidence that HAMSB promotes selective growth of potentially beneficial bacteria in healthy active individuals within 28 days of supplementation while consumption of LAMS reduced the number of those organisms.

There were indications of an effect of supplementation on some (but not all of those measured) serum cytokines, while salivary indices were unchanged. Cytokines have an essential role in regulating and co-ordinating immune activity. There was a substantial difference from pre- to post-supplementation in the concentration of TNF-α between the groups. This difference was due largely to a substantial increase in TNF-α concentration in the HAMSB group from mid- to post-study. Furthermore, the concentration of the anti-inflammatory cytokine IL-10 was maintained in the HAMSB group in comparison to the LAMS group, which had a moderate reduction in IL-10 from pre- to post-study. Other inflammatory markers were unchanged. An important health benefit of exercise is that it promotes an anti-inflammatory influence on the immune system (118). However, a possible consequence of this down-regulation of inflammatory mediators may be an increased susceptibility to common infections, particularly upper respiratory tract illness, in people undertaking prolonged intense exercise. In the context of this sample of healthy active adults, an increase in TNF-α may promote a Th-1 cell mediated immune profile, which would confer protection against viral infection. There is evidence from colitis animal models and *in-vitro* studies of colitis that *P. distasonis* and *F. prausnitzii* modulates cytokine production from resident tissue immune cells, the gut and peripheral blood mononuclear cells (119, 120), which may explain why changes occurred in cytokine concentrations in both groups. The changes in cytokine concentration in this study are in contrast to a previous report which indicated little change in serum cytokines in twenty healthy adults who consumed HAMS at 25 grams per day for four weeks (34). It is necessary to determine whether the higher dosage of starch and / or the addition of butyric acid to the starch mediated this difference. Furthermore, elucidating whether HAMSB
promotes upregulation of functional markers of the immune system, such as natural killer cell activity, is necessary to determine whether supplementation enhances host defence capability.

In conclusion, this study confirms the beneficial effects of supplementation with HAMSB on faecal bacteria and chemistry and markers of inflammation in healthy physically active adults. RS (as HAMSB) increased the abundance of *F. prausnitzii* and *P. distasonis*, the concentrations of colonic propionate and butyrate and maintained the concentration of IL-1RA, TNF-α and IL-10. Furthermore, the results also highlight the detrimental effects of LAMS on these parameters with substantial reductions in both species of bacteria, and in the concentrations of SCFA and cytokines. These deleterious physiological effects warrant further investigation as do the potential use of HAMSB to reduce susceptibility to common illnesses of the GI and respiratory tract in healthy individuals.
Chapter 6 – *LACTOBACILLUS FERMENTUM* (PCC®) SUPPLEMENTATION AND GASTROINTESTINAL AND RESPIRATORY TRACT ILLNESS SYMPTOMS: A RANDOMISED CONTROL TRIAL IN ATHLETES
Lactobacillus fermentum (PCC®) supplementation and gastrointestinal and respiratory-tract illness symptoms: a randomised control trial in athletes

Nicholas P West1,2, David B Pyne1,3, Allan W Cripps4, William G Hopkins, Dorte C Eskesens, Ashok Jairath7, Claus T Christophersen, Michael A Conlon5 and Peter A Fricker9
Chapter 7 – DISCUSSION
This thesis investigated the acute and chronic effects of exercise on salivary markers of immunity in elite rowers and the effects of modulating microbial flora on immunity and URTI in physically active healthy individuals. Over a five month training season, elite rowers had a substantially lower concentration of salivary lactoferrin (~60%) at the start and mid-point of training, but not at the end, in comparison to sedentary individuals (Chapter 3). As intrinsic immune factors in saliva, chronic reductions in lactoferrin may compromise the defensive capabilities of the body to external stimuli. The major finding from the supplement studies (Chapters 4, 5 and 6) was that each product substantially increased the number of beneficial bacteria in faeces, indicating that these supplements may promote GI health. Supplementation with L. fermentum also substantially reduced acute post-exercise perturbations in cytokines. It appears that the benefits of modulating GI microbiota in healthy physically active individuals could be mediated through the amelioration of systemic exercise-induced immune perturbations. Supplementation with L. fermentum also had positive clinical benefits in males, indicating that it may be a useful nutritional adjunct to reduce lower respiratory illness in this cohort. No clear trends between illness and changes in GI microbiota or variations in cytokines were evident. Collectively, the data from these studies indicate the potential health benefits of nutrition interventions that modulate GI microbiota. Examining whether altering GI microbiota enhances functional markers of the immune system may shed further light on the immune mechanisms underpinning the clinical benefits of these supplements.

The chronic reduction in salivary lactoferrin concentration during a season of elite rowing training indicates that prolonged intense exercise has negative effects on mucosal immunity. Saliva contains a range of immune factors that act to protect the respiratory tract from infection (121). Salivary AMPs work synergistically to protect the airways from infection (122). A decrease in salivary AMPs has been associated with increased oral infection (123). Furthermore, AMPs can inhibit respiratory viruses in-vitro (121, 124, 125), which indicates they have an important role in mucosal immunity. Elite athletes undertaking prolonged intense exercise may suffer from a higher rate of URTIs during heavy training and competition (12). Chronically low concentrations of lactoferrin may contribute to the higher rate of respiratory infection during these periods of exercise training and competition. However, the reduction of salivary lactoferrin but not lysozyme complicates understanding the clinical relevance of exercise-induced reductions in AMP concentration. Redundancy is a recognised feature of the immune system to ensure continued host defence in the event that any individual factor is compromised, such as, for example, the compensatory effect of increased salivary immunoglobulin M when SIgA is low or missing (126). Synergistic and additive activity between AMPs may further reduce the clinical implications of any one AMP having a low concentration. The concentration of salivary AMPs in-vivo is below the minimum inhibitory concentration to be individually microbicidal, indicating that several AMPs are required simultaneously to act against microbes under normal physiological conditions. Given that there are a number of AMPs in saliva it may be that low concentrations of one individually has little meaning for susceptibility to illness. Identifying clinically important thresholds of salivary AMP concentration below which infection risk increases is now important.
The chronic reduction of salivary lactoferrin over a training season may be useful as a diagnostic marker to determine susceptibility to illness in elite athletes when used in conjunction with SIgA. Research examining the effects of exercise on the mucosal immune system of the upper respiratory tract has traditionally focused on SIgA (17, 53). This research has found that low SIgA levels, particularly IgA1, are associated with an increased risk of respiratory illness (127). In elite athletes, the concentration of SIgA1 at the commencement of a training season was reported to have moderate predictive value for determining illness risk during a training season (128). Despite recording episodes of illness during the observational rowing study there were too few episodes reported for any meaningful covariate analysis to be undertaken, which highlights the need for large sample sizes to obtain reliable illness data. Combining analysis of SIgA and salivary lactoferrin with illness risk in an elite athlete setting is required to determine the relevance of the findings of chronic reductions in salivary lactoferrin from the work in this thesis.

In contrast to the chronic effects of exercise, exhaustive exercise led to acute increases in salivary lactoferrin and lysozyme. Following a progressive exercise test to exhaustion in rowers, both salivary lactoferrin and lysozyme increased by ~100%. Increased in salivary lysozyme in the acute post-exercise period is consistent with other research also showing immediate post-exercise increases in salivary lysozyme at 75% of VO2max and at maximal exertion, which returned to baseline levels within an hour of exercise cessation (18). Increased concentrations of lactoferrin and lysozyme in the acute post-exercise period contrasts with findings from research into other mucosal immune parameters after exercise. Cilia beat frequency and mucociliary transit time are impaired and phagocytic activity reduced for up to 24 hours after exercise in marathon runners (129–131). Upregulation of lactoferrin and lysozyme, innate factors of the mucosal immune system, in the acute post-exercise period also conflicts with the accepted paradigm that following prolonged intense exercise there is a window whereby the immune system is suppressed or perturbed (132). Whether or not the increased concentration of AMPs reflects enhanced immunity may depend on the structure of the protein at the time of secretion, particularly in relation to lactoferrin. AMPs have a broad number of immunoregulatory roles (133). More recent studies indicate that for lactoferrin to be directly microbicidal it must be expressed in the form of the apoprotein (134). Given that the stimulus for increased secretion following exhaustive exercise may be a combination of localized epithelial inflammation of the upper airways from hyperventilation, unrecognized or poorly controlled asthma (135) and stimulation of the sympathetic nervous system rather than being pathogen-related, it may be that increased AMP secretion may not be microbicidal. *Ex-vivo* investigation of the microbicidal ability of lactoferrin secreted in the post-exercise period will elucidate whether increased concentration in this time period will protect against infection.

Supplements targeting the GI microbiota may be useful to enhance immune function and reduce susceptibility to illness in healthy physically active individuals (49). Changes in GI microbiota are quantified by examining whether the composition of bacterial species in faeces are altered. All three supplements examined in this thesis altered the composition of the GI microbiota to varying
degrees. The synbiotic (Chapter 4) and probiotic (Chapter 6) supplements increased the recovery of the individual species of probiotic bacteria in each supplement. The synbiotic supplement increased the recovery of *L. paracasei* 40-fold. However, with four strains of probiotic bacteria and two types of prebiotic, the synbiotic supplement would have been expected to increase the count of more than one species of bacteria. The inability to recover a greater number of probiotic bacteria most likely reflects an inadequate dosage of probiotics and prebiotics in the supplement. The dosage of each bacterial strain was approximately half a billion. It is generally recognised that dosages of one billion CFU per g are necessary to elicit changes in faecal recovery of ingested strains (136). Furthermore, dosages in excess of 5 g per day are generally accepted as necessary for prebiotics to stimulate the growth of indigenous bacteria (136, 137). *L. fermentum* increased the count of total *Lactobacillus* species 7.7-fold in males compared with males on the placebo. While there was a substantial 6-fold increase in the recovery of total *Lactobacillus* species in females the effect was obscured by a 3-fold increase in females on the placebo. Dietary analysis and further scrutiny of compliance did not reveal any particular explanation for the increase in the recovery of total *Lactobacillus* in females in the placebo group. It is possible that higher doses of probiotics are required in females. The contrasting ability of the probiotic strains to be recovered in faeces highlights the differences between probiotic bacteria in their ability to colonise the GI tract as well as the need for dose response studies to establish the minimum required dose of pre- and probiotics to effect substantial change in the microbiota.

Interest in the use of HAMSB (Chapter 5) as a preventive and therapeutic supplement for GI health is based on evidence that resistant starch promotes an increase in indigenous bacterial species and their metabolic activity. Supplementation with resistant starch increased the count of *P. distasonis* 81-fold and *F. prausnitzii* 5.1-fold. Reductions in the faecal recovery of these two species of bacteria have consistently been observed in human and animal models of Crohn's disease and intestinal inflammation (111). However, the finding of an increase in these two species with HAMSB supplementation contrasts with a previous study in healthy adults utilising a non-butyrylated high amylose maize starch (HAMS), where only one species of faecal bacteria, *Lachnospiraceae* (34), increased in concentration. A recent study in rats showed that HAMS and HAMSB had differential effects on faecal flora (109). While butyric acid has not previously been shown to modulate gut flora it may have had unanticipated positive effects on the strains of bacteria that benefit from fermentation of the resistant starch. HAMSB supplementation was also associated with increased concentration of faecal short chain fatty acids and decreases in faecal pH and the concentration of ammonia. These additional beneficial changes enhance epithelial integrity and colonocyte function, which protect against GI infection and carcinogenesis (107, 138). Evidence that *L. fermentum*, Digestion 1-2-3 and HAMSB modulate faecal microbiota indicates that they may be associated with improved gastrointestinal health.

Overall the three supplements had only trivial effects on resting immune parameters, including salivary antimicrobial proteins and IgA, serum cytokines and gastrointestinal permeability. Resting, rather than post-exercise aspects of immunity were chosen to determine whether
changes in GI microbiota enhanced the immune system during homeostasis. Enhanced
immunity at rest should, theoretically, reduce susceptibility to illness. Supplementation with
the synbiotic and HAMSB modified a select number of resting plasma cytokines. In contrast,
probiotic supplementation substantially ameliorated acute exercise-induced perturbations in
cytokines. Cytokines are messenger molecules that play an important role in regulating immune
homeostasis and inflammation. Synbiotic supplementation ameliorated the increase in resting
IL-16 by 50% compared to the control group. IL-16 is an inflammatory cytokine that polarises
T-cells toward cell-mediated activation. Consumption of resistant starch prevented the fall in two
resting anti-inflammatory cytokines (IL-1RA and IL-10) and increased the concentration of a
resting inflammatory cytokine (TNF-α) in comparison to consumption of a low amylose maize
starch. These findings indicate that, in general, supplementation with synbiotics and resistant
starch may alter the cytokine milieu. Given that the source of circulating cytokine secretion is
unknown and that there is a lack of established thresholds for normal ranges of resting cytokine
concentrations, more research to examine functional markers of immune activity is required
before the consequence of these changes in healthy adults can be fully ascertained.

The finding that probiotic supplementation reduced the magnitude of acute exercise-induced
changes in plasma cytokines implies that the immune response to stress, rather than basal
immune status, may underpin the health benefits from changes in GI microbiota. The impetus for
investigating acute exercise-induced changes in cytokines arose from a previous study that found
runners prone to URTI showed evidence of impaired inflammatory regulation post-exercise (19).
Immune response to stress has been proposed as a mechanism to predict vulnerability to illness
(139). The measures of immunity were chosen on the basis of evidence that they contributed
to defence of mucosal surfaces at rest (salivary factors, gastrointestinal permeability and faecal
microbiology) and/or regulation of the immune system (serum cytokines). Stress response and
coping are, however, linked to changes in illness (45). The benefits of modulating GI microbiota
in healthy physically active individuals may be through the amelioration of systemic immune
perturbations caused by prolonged intense exercise.

Despite selection of a broad range of mucosal immune markers it is apparent that probiotics have
only trivial effects on basal mucosal immune status. The lack of a substantial effect of L. fermentum
on SIgA confirms previous research with this probiotic (36). Other research investigating the
efficacy of supplements that modulate GI microbiota to enhance immunity in-vivo and ex-vivo is
conflicting. A recent study reported that supplementation with $6.5 \times 10^9$ CFU of L. casei shirota
for 16 weeks in highly active individuals prevented a decline in SIgA (35). Other studies report
increased phagocytosis, NK cell activity, plasma levels of immunoglobulins and resting plasma
cytokines with prebiotic and probiotic administration (37, 140–143). Many of these studies
examining prebiotic and synbiotic administration on immunity are in models of disease, or
populations with greater vulnerability to illness, such as the elderly or infants, making it difficult
to determine their efficacy in healthy physically active adults. Focusing on functional markers of
defence (cell activity and circulation and immunoglobulin subclasses), immune regulation (in
particular CD4+, CD25+, FOXP3+ T-regulatory (Treg) cells) and immune response to stress and challenge, should yield further information about the benefits of supplements that modify gut flora on the immune system in healthy individuals.

Given previous evidence that \textit{L. fermentum} reduced the duration of URTI in elite runners and enhanced the secretion of IFN-\(\gamma\) (36), it was an ideal choice of probiotic to examine whether modification of gut flora altered the risk of URTI and GI illness in healthy, physically active individuals. In the current research males taking \textit{L. fermentum} had a 70% reduction in the severity of symptoms and illness load of lower respiratory illness. In contrast, females had a substantial increase in symptoms but a decrease in the severity of lower respiratory symptoms. The effect of probiotics on URTI was unclear in both genders. The reduction in the severity of lower respiratory symptoms in females is difficult to reconcile given the increase in duration and frequency of symptoms. However, the decrease in the severity of symptoms in females was consistent with their use of cold and flu medication usage. While the number of medications and total days of medications was higher in females taking \textit{L. fermentum} the mean number of medications per episode was lower, which suggests less symptom relief was required for each illness. Clinical and immunological differences between the genders is recognised (144). Furthermore, differences in environmental exposure between the sexes may also explain the contrasting effects of probiotic supplementation. Environmental factors, such as social interaction, may alter susceptibility to illness. One possibility is that males engaged in activities that increased their exposure to infectious agents compared to females. As such, infection risk in females may not have been high enough to benefit from probiotic supplementation. Further research to clarify sex differences in physiological and clinical outcomes to probiotic supplementation is necessary, particularly in relation to \textit{L. fermentum}.

There was an increase in mild GI symptoms during supplementation in males and females on the probiotic but a reduction in the severity of GI symptoms at high training loads in males. An increase in mild GI symptoms in individuals taking the probiotic is consistent with other research examining probiotic supplementation in healthy individuals. Mild GI symptoms in this study were rated as conditions that did not affect an individual’s ability to undertake exercise training and as such were of little functional consequence. The finding that probiotic supplementation was associated with a reduction in GI symptom intensity at higher training loads indicates that \textit{L. fermentum} may be effective in athletes undertaking high levels of exercise in routine training, particularly in sports such as rowing, cycling, swimming and triathlon and/or in prolonged and exhaustive competitive events that occur over multi-day time frames, such as cycling tours. Training in these sports and events is characterised by high volume, high intensity exercise and often an increase in GI symptoms in some individuals. Reducing the severity of symptoms may prevent illness from negatively affecting competitive performance.

Few studies have directly examined the effect of probiotic supplementation on URTI in healthy, physically active individuals. Given that probiotics are increasing in popularity in healthy active groups (7), the finding that probiotics had only trivial effects on symptoms of upper respiratory tract
illness is, therefore, of importance. Investigation of the efficacy of probiotics in active individuals has largely been conducted in elite athletes or in the military. While evidence is conflicting, it appears that highly trained (elite) endurance-based athletes appear to benefit most from probiotic supplementation, with two studies now reporting reductions in either the frequency (59) or duration (36) of illness. In contrast, there was no substantial effect of probiotic supplementation in the two week period after a marathon in community (non-elite) runners (145) or in commando cadets during a training and combat course (146). In comparison to the active individuals that participated in the current research, elite cyclists and triathletes typically expend up to four times more energy (147). The current paradigm in exercise immunology literature depicts some elite athletes as more vulnerable to URTI in comparison to moderately active individuals. It may be, therefore, that only athletes undertaking high training loads are likely to experience probiotic-induced reductions in URTI and that no further substantial reduction in URTI is possible in those undertaking moderate levels of physical activity, such as the individuals in this study. Given strain-specific differences between probiotic bacteria on immune activity, however, the provision of evidence-based advice to physically active healthy individuals on probiotics will require considerably more research to quantify efficacy and determine strain-specific dosing regimens to reduce illness.

Correlation analyses did not reveal any clear relationships between the changes in microbiota and cytokines with illness symptoms. The low prevalence of illness and the uncertainty in the precision of the estimates indicate that larger sample sizes are required than had been initially determined. It would be premature on the basis of this analysis to conclude that there is no substantial relationship between changes in the immune markers examined and URTI or GI illness. The inability to find a substantial relationship between perturbations in the immune system and illness may relate to the level of physical activity undertaken by participants, and the low underlying prevalence of the illnesses examined. It appears that only prolonged intense exercise causes perturbations in immunity, while moderate exercise enhances immunity. While recruitment for the probiotic study targeted well-trained individuals, retrospective analysis of the training data indicates that the levels of training could largely be defined as moderate. It may be that the level of exercise, therefore, may not have been sufficient to induce large enough disturbances in the immune system, and coupled with the low prevalence of both URTI and GI illnesses, no substantial relationships could be identified. The chronic reduction in salivary lactoferrin in the rowers compared with sedentary individuals offers some evidence of the importance of high training loads in studies using exercise as a model of stress. Rowers in this study were undertaking four to six hours of exercise daily compared with a mean of seven hours a week being undertaken by participants in the intervention studies. Future immunonutrition research needs to consider this issue along with diligent selection of functional markers of immune activation.

It is possible that probiotic supplementation may have led to an improvement in other indices of immunity not measured. Examination of intervention studies with successful reductions in URTI illness, such as OM-85, an orally administered immunostimulator containing lyophilised bacterial fractions, offers promise for the identification of relevant immune biomarkers (148). Given that
many of the symptoms of respiratory infection are inflammatory processes of the body, a further promising avenue to explore may be the use of anti-inflammatory drugs to dampen symptoms while the immune response runs its course. Given a third of rhinovirus infections do not produce symptoms it appears that the inflammatory processes underpinning cold symptoms are not necessary for elimination of URTI viruses (149). Studies examining airway hyper-responsiveness have found that T\textsubscript{reg} cells in the respiratory tract play an essential role in regulating mucosal immunology. T\textsubscript{reg} cells suppress inflammation by the expression of IL-10 and TGF-β. Animal studies show that depletion of T\textsubscript{reg} cells following respiratory viral infection leads to increased severity and that increasing respiratory virus specific levels of T\textsubscript{reg} cells reduces the severity of respiratory illness without affecting viral clearance (150, 151). Examining whether probiotics induce an active T\textsubscript{reg} phenotype is necessary to determine whether this regulator of immune activity can reduce the symptoms of respiratory illnesses.

A model outlining a possible mechanism by which supplement-associated changes in GI microbiota can enhance immunity in the respiratory tract is presented in Figure 1 (page 104). Given the central role of T\textsubscript{reg} cells in maintaining homeostasis at mucosal surfaces, future research should investigate whether supplements that modulate the composition and activity of the microbiota promote T\textsubscript{reg} phenotypes in T-cells at inductive sites of the GI tract and in lymph nodes. Use of \textit{in-vitro} cell models of respiratory epithelia may elucidate whether probiotic bacteria induce T\textsubscript{reg} cells as the mechanism to enhance immunity. Further to this, examining the expression of T\textsubscript{reg} cell surface markers may provide further evidence for these lymphocytes in mucosal immunity. Integrin \(\alpha_4\beta_7\), the chemokine receptors 9\textsuperscript{–} and 5\textsuperscript{–}, lymphocyte function-associated antigen 1 and CXCR3 allow the migration of lymphocytes to mucosal sites. Upregulation of these surface markers would provide further evidence to support a role for inducible T\textsubscript{reg} as a key mechanism by which GI microbiota influence immunity beyond the GI tract.

In conclusion, prolonged intense exercise chronically reduces the concentration of salivary lactoferrin and lysozyme, which are humoral factors of the innate immune system at mucosal surfaces. Together with other research, these findings strengthen the evidence that regular high levels of exercise can lead to a chronic reduction in humoral immune parameters at mucosal surfaces. In addition, synbiotic, probiotic and resistant starch nutritional supplements substantially alter GI microbiota, although differences between the genders in the dosing regimens of probiotics to elicit such effects require further research. The ability of these supplements to increase the number of beneficial bacteria in the GI tract underlines the promise of these supplements in improving gut health. All three supplements had trivial effects on basal immune parameters of mucosal and systemic immunity. Probiotic consumption did, however, substantially reduce the magnitude of acute post-exercise cytokine perturbations. This outcome suggests that the benefit of taking probiotics by healthy active individuals may be mediated through the amelioration of exercise-induced disturbances in the immune system, thus increasing immune resilience to stress and pathogenic exposure. Probiotic supplementation reduced symptoms of lower respiratory illness in males but not females, mirroring the effects of supplementation with the probiotic on
GI microbiota. Covariate analysis of changes in microbiota, immunity and illness yielded no clear relationships. Future research examining functional markers of the immune system and T$_{reg}$ cells may provide insights on how the changes in GI microbiota enhance immunity and reduce susceptibility to illness.

**Figure 1. Microbiota and immunity.**
The gut microbiota influences the immune system in several ways. Macrophages (M) or dendritic cells (DC) below M cells interact with probiotic bacteria and trigger immune responses (suppressive or inflammatory). DCs extend their dendrites between intestinal epithelial cells and sample probiotics in the gut lumen to regulate immunity. Probiotic interaction with intestinal epithelial cells induces secretion of antimicrobial factors and cytokines that regulate the activity of DCs, T cells and B cells in the gut-associated lymphoid tissue. Beyond the GI tract the microbiota influences immunity by inducing CD4+ CD25+ Foxp3+ T-regulatory cells that travel through the MSN to MALT that subsequently exert anti-inflammatory influence. Abbreviations: TLR – Toll like receptor; NF-xb, nuclear factor – kappa B; IFN-$\gamma$ – interferon gamma; IL- interleukin; TGF-$\beta$ – transforming growth factor- beta; SIgA – secretory immunoglobulin A; MSN – mesenteric lymph nodes; MALT – mucosal associated lymphoid tissue.
Chapter 8 – FUTURE DIRECTIONS
This thesis has characterised the acute and chronic effects of exercise on salivary immune markers and found that acute post exercise-induced disturbances in immunity can be moderated by probiotic treatment. Supplementation with a synbiotic, HAMSB and a probiotic increased the beneficial bacteria in the GI tract but had relatively little effect on resting immunity. Furthermore, probiotic supplementation was found to have clinically beneficial effects on lower respiratory tract illness.

From these studies a number of key directions for future research have been identified.

1) The clinical consequences of chronically low concentrations of antimicrobial proteins for URTI in elite athletes should be investigated. Such studies will provide information on whether URTI symptoms correlate with the concentration of AMPs. Clinically based research that includes a more comprehensive laboratory investigation of URTI symptoms is suggested. Determining the infectious or non-infectious nature of URTI symptoms and their relationship with the concentration of AMPs may shed light on the role of AMPs in the maintenance of airway homeostasis.

2) Further exploration of acute post-exercise changes in the concentration of salivary AMPs is warranted to determine if the increased AMP concentration reported in this thesis have microbicidal capacity. This research should include ex-vivo examination of saliva collected in the acute post-exercise period to act against respiratory related pathogens.

3) Undertaking a dose response study to determine the optimum dosage of *L. fermentum* supplementation in healthy active females is required. Further work to determine the clinical benefit of supplementation once an optimum dosage in females is identified would be also be warranted.

4) There is a need to examine the make-up of the synbiotic supplement. Clinical research is required to determine whether combining ingredients at dosages below levels found to be optimal at an individual ingredient level has additional microbiological, immunological or clinical effects.

5) Investigation of the uptake of butyric acid by colonocytes in healthy individuals is required. HAMSB supplementation substantially increases the concentration of butyrate and propionate in the colon. Furthermore, clinical investigation of colonocyte uptake of butyric acid should also examine whether there is a related improvement in gastrointestinal barrier function.

6) Further clinical and mechanistic investigation of the immune response to metabolic stress in physically active individuals is necessary to determine if it may be useful as a marker of immune competence. This work could be undertaken in individuals or athletes who self-report more frequent URTI or retrospectively by identifying individuals in research projects who experience higher rates of illness.
7) Expanding markers of immunity to include measures of immune activation and cells that play a role in airway homeostasis may yield information of the way in which modulating GI microbiota reduces the duration and severity of illness. *In-vitro* evidence indicates cells, in particular T<sub>reg</sub> cells, primed by GI microbiota home to lymphoid tissues in the mucosa. Clinical research that examines the activation status of these cells and the expression of specific cellular adhesion molecules that home to lymphoid tissue is suggested.
REFERENCES


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APPENDICES
Appendix A – Physical activity and illness questionnaire

APPENDICES
Appendix A - Physical activity and illness questionnaire

Instructions: Complete each night prior to sleeping
Step 1: Use the drop down lists on days where you have trained.
Step 2: Detail up to 4 x daily training sessions including session type, intensity and duration.
Step 3: Use the drop down list to indicate if you have an injury (that will affect your ability to train).
Step 4: Indicate using (Y/N) days where illness was experienced.
Step 5: If you are experiencing any illness, use the drop down lists to detail if you have visited the Doctor, completed any blood testing or taken any medications.
Step 6: Indicate severity of symptom to the extent to which it affected training.
Step 7: Use the space provided below to add any ‘Additional Comments’ that would give us greater insight to your training and illness information that can not be detailed by the drop-down lists.

| Date       | T | W | T | F | S | M | T | W | F | S | M | T | W | F | S | M | T | W | F | S | M | T | W |
| Training   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Did you train (Y/N)? |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Intensity  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| (1 low : 5 high) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Exercise duration (min) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| SESSION ONE |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Intensity  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| (1 low : 5 high) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Exercise duration (min) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| SESSION TWO |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Intensity  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| (1 low : 5 high) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Exercise duration (min) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| SESSION THREE |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Intensity  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| (1 low : 5 high) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Exercise duration (min) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Are you injured (Y/N)? |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

Illness (Y/N)
Doctor’s visits
Blood Tests
Medication (Any)
antibiotics
anti-inflammatory
pain killers
dehcongestant
anti-histamine
other (specify)

Symptom and effect on training
Upper Respiratory - blocked or runny nose, sore throat, sneezing
Severity
Chest Infection - coughing, sputum, chest congestion, wheezing, high temperature (give measurement)
Severity
Muscles and joints - aching or swollen (not related to injury)
Severity
Gastrointestinal problems - nausea, vomiting, diarrhoea, abdominal pain, bloating, painful swallowing, loss of appetite
Severity
Head - headache, migraine, dizziness, vision impairment, vertigo, glare/light intolerance
Severity
Eye irritation - itchiness, redness, sticky discharge, watery eyes
Severity
General fatigue - lethargy, tiredness
Severity
Other (please specify)
Please specify
Severity
Additional Comment
Appendix B – Normalised DGGE picture

Attachment B: Normalised DGGE picture representing bacterial profiles from subjects in the prebiotic and synbiotic groups. Individual samples from subjects by treatment group and sample collection time are on the X-axis. Bacterial strains and their percentage similarity to the strains in the NCBI database are on the Y-axis. The digitised version is at Figure 1, Chapter 4 (page 59).
Appendix C – Food diary

Keeping a food diary helps to

- Make you aware of your real eating patterns (not the patterns you think you follow) and how well you are following the study requirements
- Motivate you to eat better while you are keeping the diary
- Allow us to provide you with some dietary feedback about good strategies that can be consolidated, and poor strategies that can be changed

Tips for making this a valuable exercise

- Carry the diary around with you and record EVERYTHING you eat and drink AT THE TIME THAT YOU DO IT
  - you will be able to record everything accurately
  - you will be continually reminded to evaluate how you are eating, and to give yourself good feedback about the changes you are making
- Provide accurate detail about the amount and type of foods and drinks consumed
- Record comments about what was happening over your day and at the time of eating (training, environment, experiences, feelings of well-being or problems) so that your eating behaviour is put into context
  - it helps to know these factors when changes need to be made
  - it helps to identify eating behaviours that are causing problems

THE MORE YOU PUT IN TO THIS EXERCISE, THE GREATER VALUE YOU WILL GET BACK!

Return to Nic West (Nicholas.west@ausport.gov.au)
Australian Institute of Sport
TIPS FOR RECORDING FOOD AND DRINK ACCURATELY

1. Record all foods and drinks at the time you consume them, allowing for anything you didn't finish

2. Note the weight or volume of things that come in packages (e.g. 60 g Mars Bar)

3. Describe the number of portions of foods that come in standard units (e.g. 2 sandwich slices of white bread, 4 weetbix)

4. Provide individual detail of foods/drinks that come in portion sizes
   a. Describe “solid food” items in terms of cm dimensions, using grid below. (e.g. piece of grilled steak, fat trimmed, 20 cm × 8 cm × 4 cm thick, or green apple 10 cm diameter)
   b. Use household measures to describe the serving size that you choose of other foods and drinks
      i. Level teaspoon (e.g. 1.5 tsp margarine on toast)
      ii. Level tablespoon (e.g. 3 Tablespoons syrup on pancakes)
      iii. Cups (250 ml) (e.g. 1.5 cups steamed broccoli or 2.25 cups Nutragrain)

5. Describe the type of food, using brand names of well-known products, and including details of white/wholemeal or low fat/reduced fat/full cream or no added sugar/added sugar when foods come in different styles

6. Describe a mixed dish listing major ingredients and cooking methods
   a. if you made it yourself, provide an estimate of the recipe proportions (e.g. 2 cups of risotto – ¼ of recipe made with 1.5 cups uncooked rice, 400 g chicken, 500 g peas, 440 g can corn and 1 litre chicken stock, minimal oil added in cooking)
   b. If you are eating out, provide a guess at the ingredients (e.g. 2 cups of risotto – looked a bit creamy/oily, small amount of chicken in it, and a few peas and corn. Mostly rice)

7. Remember all the things added to foods – marg to bread, milk on cereal, sugar in tea/coffee. If it isn't described, I will assume you didn't add it

Name ...........................................................................................................

Mobile number ..............................................................................................

Email address .................................................................................................
<table>
<thead>
<tr>
<th>TIME</th>
<th>FOOD</th>
<th>QUANTITIES</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNACKS</td>
<td>Rush chocolate milk (99% fat free)</td>
<td>500 ml bottle</td>
<td>Was still hungry after lunch but didn’t want to eat solid food before hard training session</td>
</tr>
<tr>
<td>TIME: 2.20 pm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAINING</td>
<td>Gatorade during ride</td>
<td>750 ml bottle</td>
<td>Tried to hydrate better during session. Kept burping it up – is this from drinking too much? Couldn’t face more Gatorade after the session – went for the water. Starving but only had a banana with me. Should have brought something with me. What do I need?</td>
</tr>
<tr>
<td>TIME: 3.00</td>
<td>Banana immediately after session + water</td>
<td>Large – 16 cm long 2 × 300 ml cups</td>
<td></td>
</tr>
<tr>
<td>(TYPE AND DURATION )</td>
<td>2 hrs speed/endurance ride</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DINNER</td>
<td>Tim tams Pasta</td>
<td>4 ½ 500 g packet</td>
<td>Got home stuffed and ravenous and couldn’t resist TTs.</td>
</tr>
<tr>
<td>TIME 6.30 pm</td>
<td>Pasta Bolognaiise sauce</td>
<td>fresh Latino fettucine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bread roll Cantaloupe water</td>
<td>Latino brand – whole of 500g tub from supermarket fridge section 70 g Half – 12 cm diameter 500 ml</td>
<td></td>
</tr>
<tr>
<td>SNACKS</td>
<td>Wholemeal bread Toasted Scrape Marg Nutella Coffee, low fat milk, no sugar</td>
<td>2 thick toast slices teaspoon on each slice</td>
<td>Munchies before bed. Felt like something sweet. Thought toast and nutella would be better than finishing off the Tim tams. Quite filling</td>
</tr>
<tr>
<td>TIME 9.30 pm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUPPLEMENTS</td>
<td>nil</td>
<td></td>
<td>Heard that carnitine helps you lose weight.</td>
</tr>
<tr>
<td>(NOTING BRAND AND DOSE )</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix D – Food frequency questionnaire

Dietary Questionnaire

QUESTIONS ABOUT WHAT YOU USUALLY EAT AND DRINK

INSTRUCTIONS:
This questionnaire is about your usual eating habits over the past 12 months. Where possible give only one answer per question for the type of food you eat most often.

1. How many pieces of fresh fruit do you usually eat per day? (Count 1/2 cup of diced fruit, berries or grapes as one piece.)
   - I didn't eat fruit
   - less than 1 piece of fruit per day
   - 1 piece of fruit per day
   - 2 pieces of fruit per day
   - 3 pieces of fruit per day
   - 4 or more pieces of fruit per day

2. How many different vegetables do you usually eat per day? (Count all types, fresh, frozen or tinned.)
   - less than 1 vegetable per day
   - 1 vegetable per day
   - 2 vegetables per day
   - 3 vegetables per day
   - 4 vegetables per day
   - 5 vegetables per day
   - 6 or more vegetables per day

3. What type of milk do you usually use?
   - none
   - full cream milk
   - reduced fat milk
   - skim milk
   - soya milk

4. How much milk do you usually use per day? (Include flavoured milk and milk added to tea, coffee, cereal, etc.)
   - none
   - less than 250 ml (1 large cup or mug)
   - between 250 and 500 ml (1-2 cups)
   - between 500 and 750 ml (2-3 cups)
   - 750 ml (3 cups) or more

5. What type of bread do you usually eat?
   - I don't eat bread
   - high fibre white bread
   - white bread
   - wholemeal bread
   - rye bread
   - multi-grain bread

6. How many slices of bread do you usually eat per day? (Include all types, fresh or toasted and count one bread roll as 2 slices.)
   - less than 1 slice per day
   - 1 slice per day
   - 2 slices per day
   - 3 slices per day
   - 4 slices per day
   - 5-7 slices per day
   - 8 or more slices per day

7. Which spread do you usually put on bread?
   - I don't usually use any fat spread
   - margarine of any kind
   - polyunsaturated margarine
   - monounsaturated margarine
   - butter and margarine blends
   - butter

8. On average, how many teaspoons of sugar do you usually use per day? (Include sugar taken with tea and coffee and on breakfast cereal, etc.)
   - none
   - 1 to 4 teaspoons per day
   - 5 to 8 teaspoons per day
   - 9 to 12 teaspoons per day
   - more than 12 teaspoons per day

9. On average, how many eggs do you usually eat per week?
   - I don't eat eggs
   - less than 1 egg per week
   - 1 to 2 eggs per week
   - 3 to 5 eggs per week
   - 6 or more eggs per week

10. What types of cheese do you usually eat?
    - I don't eat cheese
    - hard cheeses, e.g. parmesan, romano
    - firm cheeses, e.g. cheddar, edam
    - soft cheeses, e.g. camembert, brie
    - ricotta or cottage cheese
    - cream cheese
    - low fat cheese

Please fill in the date you completed this questionnaire:

DAY | MTH | YEAR
--- | --- | ---
| JAN | 2004 |
| FEB | 2005 |
| MAR | 2006 |
| APR | 2007 |
| MAY | 2008 |
| JUN | 2009 |
| JUL | 2010 |
| AUG | 2011 |
| SEP | 2012 |
| OCT | 2013 |
| NOV | 2014 |
| DEC | 2015 |

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00 01 02 03 04 05

Appendix D – Food frequency questionnaire

Please mark like this:

[ ] [ ] [ ]
For each food shown on this page, indicate how much on average you would usually have eaten at main meals during the past 12 months. When answering each question, think of the amount of that food you usually ate, even though you may rarely have eaten the food on its own.

If you usually ate more than one helping, fill in the oval for the serving size closest to the total amount you ate.

11. When you ate potato, did you usually eat:
   - ○ I never ate potato
   - ○ Less than A
   - ○ A
   - ○ Between A & B
   - ○ B
   - ○ Between B & C
   - ○ C
   - ○ More than C

12. When you ate vegetables, did you usually eat:
   - ○ I never ate vegetables
   - ○ Less than A
   - ○ A
   - ○ Between A & B
   - ○ B
   - ○ Between B & C
   - ○ C
   - ○ More than C

13. When you ate steak, did you usually eat:
   - ○ I never ate steak
   - ○ Less than A
   - ○ A
   - ○ Between A & B
   - ○ B
   - ○ Between B & C
   - ○ C
   - ○ More than C

14. When you ate meat or vegetable casserole, did you usually eat:
   - ○ I never ate casserole
   - ○ Less than A
   - ○ A
   - ○ Between A & B
   - ○ B
   - ○ Between B & C
   - ○ C
   - ○ More than C
### Cereal Foods, Sweets & Snacks

<table>
<thead>
<tr>
<th></th>
<th>Never</th>
<th>1 to 3 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Bran™</strong></td>
<td>A1</td>
<td></td>
<td></td>
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<tr>
<td><strong>Sultana Bran™, FibrePlus™, Branflakes™</strong></td>
<td>A2</td>
<td></td>
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<tr>
<td><strong>Weet Bix™, Vita Brits™, Weeties™</strong></td>
<td>A3</td>
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<tr>
<td><strong>Cornflakes, Nutigrain™, Special K™</strong></td>
<td>A4</td>
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<tr>
<td><strong>Porridge</strong></td>
<td>A5</td>
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<tr>
<td><strong>Muesli</strong></td>
<td>A6</td>
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<tr>
<td><strong>Rice</strong></td>
<td>A7</td>
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<tr>
<td><strong>Pasta or noodles (include lasagne)</strong></td>
<td>A8</td>
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<td></td>
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<tr>
<td>** Crackers, crispbreads, dry biscuits**</td>
<td>A9</td>
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<tr>
<td><strong>Sweet biscuits</strong></td>
<td>A10</td>
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<td></td>
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</tr>
<tr>
<td><strong>Cakes, sweet pies, tarts and other sweet pastries</strong></td>
<td>A11</td>
<td></td>
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<tr>
<td><strong>Meat pies, pasties, quiche and other savoury pastries</strong></td>
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<td><strong>Pizza</strong></td>
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<tr>
<td><strong>Hamburger with a bun</strong></td>
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<td><strong>Chocolate</strong></td>
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<td><strong>Flavoured milk drink (cocoa, Milo™, etc.)</strong></td>
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<td><strong>Nuts</strong></td>
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<td><strong>Peanut butter or peanut paste</strong></td>
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<tr>
<td><strong>Corn chips, potato crisps, Twisties™, etc.</strong></td>
<td>A19</td>
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<tr>
<td><strong>Jam, marmalade, honey or syrups</strong></td>
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<tr>
<td><strong>Vegemite™, Marmite™ or Promite™</strong></td>
<td>A21</td>
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### Dairy Products, Meat & Fish

<table>
<thead>
<tr>
<th></th>
<th>Never</th>
<th>1 to 3 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
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<th>2 times</th>
<th>3 or more times</th>
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<tbody>
<tr>
<td><strong>Cheese</strong></td>
<td>B1</td>
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<tr>
<td><strong>Ice-cream</strong></td>
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<td><strong>Yoghurt</strong></td>
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<td><strong>Beef</strong></td>
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<td><strong>Veal</strong></td>
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<td><strong>Lamb</strong></td>
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<td><strong>Pork</strong></td>
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<td><strong>Bacon</strong></td>
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<td><strong>Ham</strong></td>
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<tr>
<td><strong>Corned beef, luncheon meats or salami</strong></td>
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<tr>
<td><strong>Sausages or frankfurters</strong></td>
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<td><strong>Fish, steamed, grilled or baked</strong></td>
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<tr>
<td><strong>Fish, fried (include take-away)</strong></td>
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<tr>
<td><strong>Fish, tinned (salmon, tuna, sardines, etc.)</strong></td>
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### Fruit

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<tr>
<th></th>
<th>Never</th>
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<th>2 times</th>
<th>3 to 4 times</th>
<th>5 to 6 times</th>
<th>1 time</th>
<th>2 times</th>
<th>3 or more times</th>
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<tbody>
<tr>
<td><strong>Tinned or frozen fruit (any kind)</strong></td>
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<td><strong>Fruit juice</strong></td>
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<td><strong>Oranges or other citrus fruit</strong></td>
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<td><strong>Apples</strong></td>
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<td><strong>Pears</strong></td>
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<td><strong>Bananas</strong></td>
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<tr>
<td><strong>Watermelon, rockmelon (cantaloupe), honeydew, etc.</strong></td>
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<td><strong>Pineapple</strong></td>
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<td><strong>Strawberries</strong></td>
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<td><strong>Apricots</strong></td>
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<tr>
<td><strong>Peaches or nectarines</strong></td>
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<tr>
<td><strong>Mango or paw paw</strong></td>
<td>C12</td>
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<td><strong>Avocado</strong></td>
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</table>
Over the last 12 months, how often did you drink beer, wine and/or spirits?

16. Over the last 12 months, how often did you drink beer, wine and/or spirits?

### Times You Have Eaten

<table>
<thead>
<tr>
<th>Vegetables (including fresh, frozen and tinned)</th>
<th>Never</th>
<th>Less than once per month</th>
<th>1 to 3 times per month</th>
<th>1 time per week</th>
<th>2 times per week</th>
<th>3 to 4 times per week</th>
<th>5 to 6 times per week</th>
<th>1 time per month</th>
<th>2 times per month</th>
<th>3 or more times per month</th>
</tr>
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<tbody>
<tr>
<td>Potatoes, roasted or fried (include hot chips)</td>
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<td>Potatoes cooked without fat</td>
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<tr>
<td>Tomato sauce, tomato paste or dried tomatoes</td>
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<tr>
<td>Fresh or tinned tomatoes</td>
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<tr>
<td>Peppers (capsicum)</td>
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<tr>
<td>Lettuce, endive, or other salad greens</td>
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<td>Cucumber</td>
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<td>Celery</td>
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<td>Beetroot</td>
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<td>Carrots</td>
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<tr>
<td>Cabbage or Brussels sprouts</td>
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<tr>
<td>Cauliflower</td>
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<td>Broccoli</td>
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<td>Silverbeet or spinach</td>
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<td>Peas</td>
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<td>Green beans</td>
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<td>Bean sprouts or alfalfa sprouts</td>
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<td>Baked beans</td>
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<td>Soy beans, soy bean curd or tofu</td>
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<tr>
<td>Other beans (include chick peas, lentils, etc.)</td>
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<tr>
<td>Pumpkin</td>
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<td>Onion or leeks</td>
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<td>Garlic (not garlic tablets)</td>
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<td>Mushrooms</td>
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<td>Zucchini</td>
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### Times That You Drank

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<th>Less than once a month</th>
<th>1-3 days per month</th>
<th>1 day per week</th>
<th>2 days per week</th>
<th>3 days per week</th>
<th>4 days per week</th>
<th>5 days per week</th>
<th>6 days per week</th>
<th>everyday</th>
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<tbody>
<tr>
<td>Beer (low alcohol)</td>
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<tr>
<td>Beer (full strength)</td>
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<tr>
<td>Red wine</td>
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<td>White wine (include sparkling wines)</td>
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<tr>
<td>Fortified wines, port, sherry, etc.</td>
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<tr>
<td>Spirits, liqueurs, etc.</td>
<td>6</td>
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</tbody>
</table>

When answering the next two questions, please convert the amounts you drank into glasses using the examples given below. For spirits, liqueurs, and mixed drinks containing spirits, please count each nip (30 ml) as one glass.

1 can or stubby of beer = 2 glasses
1 bottle of wine (750 ml) = 6 glasses
1 large bottle of beer (750 ml) = 4 glasses
1 bottle of port or sherry (750 ml) = 12 glasses

17. Over the last 12 months, on days when you were drinking, how many glasses of beer, wine and/or spirits altogether did you usually drink?

### Total number of glasses per day

<table>
<thead>
<tr>
<th>Days per week</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10 or more</th>
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<tbody>
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</table>

18. Over the last 12 months, what was the maximum number of glasses of beer, wine and/or spirits that you drank in 24 hours?

### Maximum number of glasses per 24 hours

<table>
<thead>
<tr>
<th>Glasses per 24 hours</th>
<th>1-2</th>
<th>3-4</th>
<th>5-6</th>
<th>7-8</th>
<th>9-10</th>
<th>11-12</th>
<th>13-14</th>
<th>15-16</th>
<th>17-18</th>
<th>19 or more</th>
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</table>

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# CONSORT 2010 checklist of information to include when reporting a randomised trial

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<thead>
<tr>
<th>Section/Topic</th>
<th>Item No</th>
<th>Checklist item</th>
<th>Reported on page No</th>
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<tbody>
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<tr>
<td>1a</td>
<td></td>
<td>Identification as a randomised trial in the title</td>
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</tr>
<tr>
<td>1b</td>
<td></td>
<td>Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)</td>
<td>2</td>
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<tr>
<td><strong>Introduction</strong></td>
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<tr>
<td>Background and objectives</td>
<td>2a</td>
<td>Scientific background and explanation of rationale</td>
<td>3</td>
</tr>
<tr>
<td>2b</td>
<td></td>
<td>Specific objectives or hypotheses</td>
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<td><strong>Methods</strong></td>
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<td>Trial design</td>
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<td>Description of trial design (such as parallel, factorial) including allocation ratio</td>
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<tr>
<td>3b</td>
<td></td>
<td>Important changes to methods after trial commencement (such as eligibility criteria), with reasons</td>
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<td>4a</td>
<td>Eligibility criteria for participants</td>
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<tr>
<td>4b</td>
<td></td>
<td>Settings and locations where the data were collected</td>
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<tr>
<td>Interventions</td>
<td>5</td>
<td>The interventions for each group with sufficient details to allow replication, including how and when they were actually administered</td>
<td>4</td>
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<tr>
<td>Outcomes</td>
<td>6a</td>
<td>Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed</td>
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<tr>
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<td>Checklist item</td>
<td>Reported on page No</td>
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<td>How sample size was determined</td>
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<td>7b</td>
<td>When applicable, explanation of any interim analyses and stopping guidelines</td>
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<td>Sequence generation</td>
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<td>Method used to generate the random allocation sequence</td>
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<td>8b</td>
<td>Type of randomisation; details of any restriction (such as blocking and block size)</td>
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<tr>
<td>Allocation concealment mechanism</td>
<td>9</td>
<td>Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned</td>
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<td>Implementation</td>
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<td>Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions</td>
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<td>Blinding</td>
<td>11a</td>
<td>If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how</td>
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<td></td>
<td>11b</td>
<td>If relevant, description of the similarity of interventions</td>
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<td>Statistical methods</td>
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<td>Statistical methods used to compare groups for primary and secondary outcomes</td>
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<td>12b</td>
<td>Methods for additional analyses, such as subgroup analyses and adjusted analyses</td>
<td>7–8</td>
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<tr>
<td>Results</td>
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<td>Participant flow (a diagram is strongly recommended)</td>
<td>13a</td>
<td>For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome</td>
<td>4</td>
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<tr>
<td></td>
<td>13b</td>
<td>For each group, losses and exclusions after randomisation, together with reasons</td>
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<td>Recruitment</td>
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<td>Baseline data</td>
<td>15</td>
<td>A table showing baseline demographic and clinical characteristics for each group</td>
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<td>Numbers analysed</td>
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<td>For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups</td>
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<tr>
<td>Outcomes and estimation</td>
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<td>For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)</td>
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<td></td>
<td>17b</td>
<td>For binary outcomes, presentation of both absolute and relative effect sizes is recommended</td>
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<td>Ancillary analyses</td>
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<td>Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory</td>
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<td>Harms</td>
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<td>All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)</td>
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<td>Discussion</td>
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<td>Limitations</td>
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<td>Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses</td>
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<td>Generalisability</td>
<td>21</td>
<td>Generalisability (external validity, applicability) of the trial findings</td>
<td>10–11</td>
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<td>22</td>
<td>Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence</td>
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<td>Other information</td>
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<td>Registration</td>
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<td>Registration number and name of trial registry</td>
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<td>Protocol</td>
<td>24</td>
<td>Where the full trial protocol can be accessed, if available</td>
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<tr>
<td>Funding</td>
<td>25</td>
<td>Sources of funding and other support (such as supply of drugs), role of funders</td>
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</table>
Analytical approach

Our approach to an inference about the true (large-sample) effect of the supplement on a given symptom was based on the uncertainty in the effect in relation to the smallest clinically important values rather than in relation to the null (85, 152). Specifically, we used the same assumptions underlying null-hypothesis testing to derive probabilities that the true effect of the treatment was a substantial increase or decrease in symptoms. An effect with a sufficiently low probability (p) of increasing symptoms ($P<0.005$ or 0.5%) and a sufficiently high probability of reducing symptoms ($P>0.25$ or 25%) was deemed to be a clear outcome and reported with the probability of a positive effect interpreted according to the following scale: 0.25–0.75, possible; 0.75–0.95, likely; 0.95–0.995, very likely; >0.995, most likely. A reduction in symptoms with a low probability of occurrence ($P<0.25$) was deemed to be trivial or negative, depending on the relative probabilities of these two outcomes. An effect that was possibly positive ($P>0.25$) but with an unacceptable risk of increasing symptoms ($P>0.005$) was deemed to be unclear and would require more research with a larger sample before the outcome could be decided. Uncertainty in the effect on a symptom is also shown as the 99% confidence interval, because any overlap of this interval with the smallest clinical value for a substantial increase in symptoms implies that the treatment should not be used. Quantifying the likelihood of both a substantial decrease and/or increase in symptoms in this way is a useful analytical approach in clinically-based research (8) to assist professionals and consumers in making evidence-based choices.

An effect of the probiotic on a bacterial count or on a physiological variable is a mechanistic outcome, so our approach to inference here was based on giving equal importance to substantially positive and negative values. The effect was deemed clear if it was very unlikely ($P<0.05$) to be substantially positive or very unlikely to be substantially negative, and the outcome was reported according to which magnitude (negative, trivial, positive) had the highest probability. All other effects were deemed unclear. For consistency with this approach, uncertainty in a mechanistic effect is shown as the 90% confidence interval. A given sample size has equal power for clinical and mechanistic inferences (153).

All symptoms were analysed per 100 days. To account for effects of “shoulder” periods following the start and end of supplementation, a proportion of each subject’s symptom scores in the shoulder periods was assigned to the subject’s accumulated scores for the baseline and supplementation periods. The shoulder periods were assumed to be two weeks, and the proportions of a symptom score in the onset and offset shoulder assigned to the treatment period were $d/14$ and $(14-d)/14$ respectively, where $d$ was the count of the days from the start of the shoulder period. The proportions of the score assigned to the baseline and post supplementation period were respectively $(14-d)/14$ and $d/14$.

We undertook two kinds of analysis to estimate the effect of the probiotic on symptoms: a comparison of the probiotic- and control-group means only for the period of supplementation (a “post-only” analysis) and a comparison of the group mean changes between baseline and supplementation
periods (a “pre-post” analysis). The effects of supplementation on illness symptoms were more precise for the post-only analysis and are the only effects reported here. The number of symptom episodes of a given symptom per 100 days, total number of days of the symptom per 100 days, and total load of the symptom per 100 days (sum of the product of symptom intensity and number of days of the symptom per 100 days) were analysed as ratios: the mean of the probiotic group divided by the mean of the placebo group during treatment. Ratios of 1.20 and 1/1.20 (≈ 0.83) were chosen as smallest clinically important differences. Effects on symptom intensity and on all training-related measures were analysed as differences rather than ratios of means. The smallest important effects for these measures were derived by standardisation: 0.20 of the pooled between-subject standard deviation in the control group and probiotic group (86). Partly for this reason, descriptive statistics of all measures are presented as mean ± standard deviation. The smallest important effects for these measures were derived by standardisation: 0.20 of the pooled between-subject standard deviation in the control group and probiotic group (86). Descriptive statistics of all measures are presented as mean ± standard deviation; differences between group means of subject characteristics were assessed with a modification (85) of Cohen’s scale (86) for standardised effects (small, 0.20–0.60; moderate, 0.60–1.20; large, >1.20). Confidence limits for the effects on symptom scores and training measures were obtained with bootstrapping. These analyses were performed with the Statistical Analysis System (Version 9.1, SAS Institute, Cary, NC).

Effects of the probiotic on measures of immunology and enteric microflora (Q-PCR data) are presented for pre-post analyses, which were found to give more precision than post-only analyses. These measures were log-transformed before analysis to permit the effect of the treatment to be properly analyzed as factors or percents, and magnitudes of effects were determined by standardisation of the log-transformed variable. The analyses were performed with a spreadsheet (38) that was based on the t-statistic for independent samples with unequal variances. Baseline values of the dependent variable were included as a covariate in these analyses to account for regression to the mean. The spreadsheet was also used to investigate the extent to which bacterial counts accounted for symptom scores in this subsample of subjects. In these analyses the log-transformed bacterial count or the pre-post change in the log-transformed count was the covariate, and the dependent variable was rank-transformed.

**Systemic immunity**
The effects of supplementation on serum cytokine concentrations at rest are shown in Table S1 (male) and Table S2 (female). Supplementing with *L. fermentum* attenuated reductions in the concentration of several resting serum cytokine concentrations in both males and females.

Table S1 shows that supplementing with *L. fermentum* for 11 weeks attenuated the fall in resting TNF-α concentration in males. TNF-α is a T-helper 1 (Th1) pro-inflammatory cytokine whose primary role is to regulate immune cells and activate inflammatory pathways. Exercise is typically associated with an anti-inflammatory T-helper 2 (Th2) profile characterised by reductions in Th1 cytokines (IL-6, IFN-γ, GM-CSF and TNF-α). An enhanced Th2 profile has been
Table S1. Changes in resting cytokine concentrations pre and post supplementation in males. Data are shown as a factor change, e.g. a mean difference in the change of means of 1.2 equates to a 20% difference. Units: pg.ml⁻¹.

|                               | Probiotic                  |              | Placebo                  |              | % change | % change | Mean;  | Inference               |
|-------------------------------|-----------------------------|--------------|--------------------------|--------------|----------|----------|------------------------|
|                               | Pre-intervention            | Post-intervention |              |              |          |          | Mean;     |                        |
|                               | Mean x/± SD                 | Mean x/± SD  | Mean ± SD                | Mean ± SD    | x/± 90%CL|          | x/± 1.41 |                        |
| Anti-inflammatory cytokines    |                             |              |                          |              |          |          |            |                        |
| IL-1ra                        | 114 x/± 1.8                 | 112 x/± 2.2  | -7 ± 113                 | 119 x/± 1.8  | 93 x/± 2.1| -22 ± 73 | 1.19 x/± 1.41 | Inconclusive            |
| IL-10                         | 5.8 x/± 2.8                 | 5.9 x/± 3.9  | -4 ± 62                  | 5.7 x/± 2.4  | 5.0 x/± 2.6| -12 ± 52 | 1.09 x/± 1.26 | Trivial                |
| Immuno-regulatory cytokines    |                             |              |                          |              |          |          |            |                        |
| IL-6                          | 5 x/± 1.8                   | 4 x/± 2.5    | -16 ± 117                | 5 x/± 1.8    | 4.0 x/± 2.5| -16 ± 50 | 0.99 x/± 1.39 | Inconclusive            |
| Pro-inflammatory cytokines     |                             |              |                          |              |          |          |            |                        |
| IL-8                          | 2.6 x/± 2.5                 | 3.2 x/± 1.9  | 10 ± 87                  | 2.9 x/± 2.1  | 2.9 x/± 1.8| 3.8 ± 47 | 1.06 x/± 1.32 | Inconclusive            |
| GM-CSF                        | 29.6 x/± 2.9                | 27.2 x/± 4.1 | -17 ± 166                | 41.8 x/± 3.2 | 27.9 x/± 3.8| -33 ± 101| 1.24 x/± 1.56 | Inconclusive            |
| IFN-γ                         | 86 x/± 1.6                  | 64 x/± 2.3   | -27 ± 113                | 87 x/± 2.0   | 55 x/± 2.5| -36 ± 78 | 1.14 x/± 1.42 | Inconclusive            |
| TNF-α                         | 24.0 x/± 1.8                | 17.8 x/± 2.5 | -30 ± 80                 | 23.0 x/± 2.3 | 13.2 x/± 2.4| -43 ± 58 | 1.27 x/± 1.37 | Smaller reduction       |

SD: standard deviation expressed as a times/divide factor of the mean.
90%CL: 90% confidence limits expressed as a times/divide factor of the mean effect
proposed to increase susceptibility to respiratory infection. There were no substantial differences in the response of the anti-inflammatory or immunoregulatory cytokines to the probiotic treatment in males.

There were substantial differences between females in the probiotic and placebo groups in the change in concentration of IL-8 and GM-CSF cytokines at rest from pre- to post-intervention (Table S2). IL-8 and GM-CSF are pro-inflammatory chemokines. The concentration of both cytokines increased in females taking *L. fermentum*, but decreased in those taking the placebo. This pattern of response is suggestive of an enhanced Th1 profile in females in the probiotic group. There were trivial differences between the two groups in all other cytokines for the female subjects.
Table S2. Changes in resting cytokine concentrations pre and post supplementation in females. Data are shown as a factor change, e.g. a mean difference in the change of means of 1.2 equates to a 20% difference. (Units: pg.ml).

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Pre Mean</th>
<th>Post Mean</th>
<th>% change</th>
<th>Pre Mean</th>
<th>Post Mean</th>
<th>% change</th>
<th>Mean ± SD</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>×/÷ SD</td>
<td>×/÷ SD</td>
<td>Mean ± SD</td>
<td>×/÷ SD</td>
<td>×/÷ SD</td>
<td>Mean ± SD</td>
<td>×/÷ 90%CL</td>
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</tr>
<tr>
<td><strong>Anti-inflammatory cytokines</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1ra</td>
<td>91.7 ×/÷ 2.2</td>
<td>90.3 ×/÷ 2.0</td>
<td>−2 ± 68</td>
<td>102.3 ×/÷ 2.7</td>
<td>68.7 ×/÷ 1.8</td>
<td>−21 ± 100</td>
<td>1.25 ×/÷ 1.53</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.2 ×/÷ 7.3</td>
<td>6.6 ×/÷ 6.9</td>
<td>−8 ± 55</td>
<td>4.9 ×/÷ 3.1</td>
<td>2.9 ×/÷ 6.7</td>
<td>−23 ± 63</td>
<td>1.19 ×/÷ 1.38</td>
<td>Trivial</td>
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<tr>
<td><strong>Immuno-regulatory cytokine</strong></td>
<td></td>
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</tr>
<tr>
<td>IL-6</td>
<td>3.1 ×/÷ 2.0</td>
<td>4.0 ×/÷ 1.8</td>
<td>6 ± 102.5</td>
<td>4.1 ×/÷ 3.8</td>
<td>2.8 ×/÷ 2.5</td>
<td>−13 ± 165</td>
<td>1.21 ×/÷ 1.81</td>
<td>Inconclusive</td>
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<td><strong>Pro-inflammatory cytokines</strong></td>
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<tr>
<td>IL-8</td>
<td>2.1 ×/÷ 2.6</td>
<td>2.8 ×/÷ 2.3</td>
<td>23 ± 53</td>
<td>2.7 ×/÷ 2.3</td>
<td>2.9 ×/÷ 2.3</td>
<td>−3 ± 69</td>
<td>1.26 ×/÷ 1.40</td>
<td>Small</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>14.7 ×/÷ 2.7</td>
<td>15.0 ×/÷ 3.3</td>
<td>2 ± 210</td>
<td>32.7 ×/÷ 4.8</td>
<td>16.0 ×/÷ 6.0</td>
<td>−36 ± 162</td>
<td>1.59 ×/÷ 2.09</td>
<td>Small</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>70.7 ×/÷ 1.6</td>
<td>61.3 ×/÷ 2.0</td>
<td>−13 ± 107</td>
<td>78.9 ×/÷ 3.0</td>
<td>47.6 ×/÷ 2.5</td>
<td>−30 ± 128</td>
<td>1.23 ×/÷ 2.10</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>TNF-α</td>
<td>17.2 ×/÷ 2.9</td>
<td>14.6 ×/÷ 3.5</td>
<td>−15 ± 95</td>
<td>23.2 ×/÷ 2.4</td>
<td>19.0 ×/÷ 2.5</td>
<td>−13 ± 86</td>
<td>0.98 ×/÷ 1.6</td>
<td>Inconclusive</td>
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</tbody>
</table>

SD: standard deviation expressed as a times/divide factor of the mean.
90%CL: 90% confidence limits expressed as a times/divide factor of the mean effect
Table S3. Raw cytokine concentrations pre and post VO\textsubscript{2} max at baseline and at the end of supplementation in females. Data are shown as a factor change, e.g. a mean difference in the change of means of 1.2 equates to a 20% difference. (Units: pg.ml).

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Pre baseline VO\textsubscript{2} Mean x/± SD</th>
<th>Post baseline VO\textsubscript{2} Mean x/± SD</th>
<th>Pre End study VO\textsubscript{2} Mean x/± SD</th>
<th>Post End study VO\textsubscript{2} Mean x/± SD</th>
<th>Pre baseline VO\textsubscript{2} Mean x/± SD</th>
<th>Post baseline VO\textsubscript{2} Mean x/± SD</th>
<th>Pre End study VO\textsubscript{2} Mean x/± SD</th>
<th>Post End study VO\textsubscript{2} Mean x/± SD</th>
<th>Inference*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-inflammatory cytokines</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IL-1ra</td>
<td>91.7 x/± 2.2</td>
<td>120.0 x/± 2.0</td>
<td>90.3 x/± 1.7</td>
<td>91.3 x/± 2.7</td>
<td>102.3 x/± 2.7</td>
<td>101 x/± 3.0</td>
<td>68.7 x/± 1.8</td>
<td>97.9 x/± 1.7</td>
<td>Very Likely ↓</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.2 x/± 7.3</td>
<td>7.7 x/± 7.2</td>
<td>6.6 x/± 6.9</td>
<td>6.1 x/± 6.9</td>
<td>4.9 x/± 3.1</td>
<td>5.5 x/± 3.3</td>
<td>2.9 x/± 1.8</td>
<td>3.9 x/± 1.8</td>
<td>Possible ↓</td>
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<td><strong>Immuno-regulatory cytokine</strong></td>
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</tr>
<tr>
<td>IL-6</td>
<td>3.0 x/± 2.0</td>
<td>4.4 x/± 1.7</td>
<td>3.2 x/± 2.5</td>
<td>3.1 x/± 1.9</td>
<td>4.1 x/± 3.8</td>
<td>4.1 x/± 4.5</td>
<td>2.8 x/± 2.5</td>
<td>4.0 x/± 2.1</td>
<td>Likely ↓</td>
</tr>
<tr>
<td><strong>Pro-inflammatory cytokines</strong></td>
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<tr>
<td>IL-8</td>
<td>2.1 x/± 2.6</td>
<td>3.2 x/± 2.7</td>
<td>2.7 x/± 2.3</td>
<td>2.9 x/± 2.2</td>
<td>2.7 x/± 2.3</td>
<td>3.2 x/± 2.3</td>
<td>2.9 x/± 2.3</td>
<td>3.3 x/± 2.8</td>
<td>Likely ↓</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>14.7 x/± 2.7</td>
<td>18.7 x/± 3.0</td>
<td>15.0 x/± 3.3</td>
<td>15.5 x/± 2.7</td>
<td>32.7 x/± 4.8</td>
<td>23.1 x/± 6.5</td>
<td>16.0 x/± 6.0</td>
<td>25.5 x/± 3.9</td>
<td>Very Likely ↓</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>70.7 x/± 1.6</td>
<td>72.3 x/± 2.0</td>
<td>61.3 x/± 2.0</td>
<td>53.9 x/± 1.7</td>
<td>78.9 x/± 3.0</td>
<td>85.0 x/± 3.1</td>
<td>47.6 x/± 2.5</td>
<td>75.8 x/± 2.0</td>
<td>Likely ↓</td>
</tr>
<tr>
<td>TNF-α</td>
<td>17.2 x/± 2.9</td>
<td>21.6 x/± 2.7</td>
<td>14.6 x/± 3.5</td>
<td>14.6 x/± 2.7</td>
<td>23.2 x/± 2.4</td>
<td>15.4 x/± 4.2</td>
<td>19.0 x/± 2.5</td>
<td>23.8 x/± 2.3</td>
<td>Very likely ↓</td>
</tr>
</tbody>
</table>

SD: standard deviation expressed as a times/divide factor of the mean.
90%CL: 90% confidence limits expressed as a times/divide factor of the mean effect.
Table S4. Raw cytokine concentrations pre and post VO₂ at baseline and at the end of supplementation in males. Data are shown as a factor change, e.g. a mean difference in the change of means of 1.2 equates to a 20% difference. (Units: pg.ml).

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Probiotic</th>
<th>Placebo</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Inference*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre VO₂</td>
<td>Post VO₂</td>
<td>Pre VO₂</td>
<td>Post VO₂</td>
<td>Pre VO₂</td>
<td>Post VO₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td></td>
<td>x/SD</td>
<td>x/SD</td>
<td>x/SD</td>
<td>x/SD</td>
<td>x/SD</td>
<td>x/SD</td>
<td></td>
</tr>
<tr>
<td>Anti-inflammatory cytokines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1ra</td>
<td>114.4 x/ 1.8</td>
<td>106.9 x/ 2.1</td>
<td>111.5 x/ 2.2</td>
<td>100.6 x/ 2.1</td>
<td>119.2 x/ 1.8</td>
<td>107.8 x/ 2.2</td>
<td>934 x/ 2.1</td>
</tr>
<tr>
<td>IL-10</td>
<td>5.8 x/ 2.8</td>
<td>5.4 x/ 3.2</td>
<td>5.9 x/ 3.9</td>
<td>5.7 x/ 3.4</td>
<td>5.7 x/ 2.4</td>
<td>5.5 x/ 2.7</td>
<td>5.0 x/ 2.6</td>
</tr>
<tr>
<td>Immuno-regulatory cytokine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>5.0 x/ 1.75</td>
<td>4.8 x/ 2.0</td>
<td>4.2 x/ 2.5</td>
<td>4.5 x/ 2.1</td>
<td>4.9 x/ 1.8</td>
<td>4.2 x/ 2.6</td>
<td>4.2 x/ 2.0</td>
</tr>
<tr>
<td>Pro-inflammatory cytokines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>2.6 x/ 2.5</td>
<td>3.2 x/ 2.3</td>
<td>3.2 x/ 1.9</td>
<td>2.8 x/ 2.6</td>
<td>2.9 x/ 2.1</td>
<td>2.8 x/ 3.2</td>
<td>2.9 x/ 1.8</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>29.6 x/ 2.9</td>
<td>28.3 x/ 2.9</td>
<td>27.2 x/ 4.1</td>
<td>26.4 x/ 3.8</td>
<td>41.8 x/ 3.2</td>
<td>34.7 x/ 2.9</td>
<td>27.9 x/ 3.8</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>86.0 x/ 1.6</td>
<td>76.9 x/ 1.9</td>
<td>64.0 x/ 2.3</td>
<td>71.2 x/ 2.1</td>
<td>86.8 x/ 2.0</td>
<td>75.6 x/ 2.4</td>
<td>55.4 x/ 2.5</td>
</tr>
<tr>
<td>TNF-α</td>
<td>23.9 x/ 1.8</td>
<td>20.1 x/ 2.4</td>
<td>17.8 x/ 2.5</td>
<td>18.5 x/ 2.2</td>
<td>23.0 x/ 2.3</td>
<td>19.2 x/ 2.9</td>
<td>13.2 x/ 2.4</td>
</tr>
</tbody>
</table>

SD: standard deviation expressed as a times/divide factor of the mean.
90%CL: 90% confidence limits expressed as a times/divide factor of the mean effect
*Refer to Table 5 (page 92) in manuscript
**Mucosal immunity**

The mean changes in the concentrations of salivary proteins are detailed in Table S5 and Table S6. There was a small decrease in the concentration of lactoferrin and small increases in the concentration of SIgA with probiotic treatment in males. There were no substantial differences between treatment groups in the concentration of lysozyme. The salivary proteins were characterised by large variability in protein concentration.
Table S5. Concentration of salivary proteins pre and post supplementation, with the effect of the treatment expressed as the difference in the change of the means.

<table>
<thead>
<tr>
<th>Males</th>
<th>Probiotic</th>
<th>Placebo</th>
<th>Effect of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Mean  x⁄± SD</td>
<td>Post Mean  x⁄± SD</td>
<td>Pre Mean  x⁄± SD</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2200 x⁄± 3.0</td>
<td>2500 x⁄± 3.0</td>
<td>3000 x⁄± 4.4</td>
</tr>
<tr>
<td>Lysozyme as a ratio to albumin</td>
<td>53 x⁄± 4.0</td>
<td>54 x⁄± 4.0</td>
<td>87 x⁄± 2.9</td>
</tr>
<tr>
<td>Lysozyme as a ratio to osmolality</td>
<td>27 x⁄± 2.8</td>
<td>31 x⁄± 3.4</td>
<td>37 x⁄± 2.9</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>2900 x⁄± 3.4</td>
<td>2700 x⁄± 3.3</td>
<td>3800 x⁄± 2.2</td>
</tr>
<tr>
<td>Lactoferrin as a ratio to albumin</td>
<td>75 x⁄± 2.9</td>
<td>57 x⁄± 2.7</td>
<td>110 x⁄± 2.3</td>
</tr>
<tr>
<td>Lactoferrin as a ratio to osmolality</td>
<td>37 x⁄± 2.5</td>
<td>32 x⁄± 4.0</td>
<td>43 x⁄± 2.0</td>
</tr>
<tr>
<td>SIgA</td>
<td>150 x⁄± 2.1</td>
<td>170 x⁄± 2.3</td>
<td>210 x⁄± 1.8</td>
</tr>
<tr>
<td>SIgA as a ratio to albumin</td>
<td>3.6 x⁄± 2.5</td>
<td>3.8 x⁄± 2.3</td>
<td>6.1 x⁄± 1.8</td>
</tr>
<tr>
<td>SIgA as a ratio to osmolality</td>
<td>1.8 x⁄± 2.0</td>
<td>2.0 x⁄± 2.9</td>
<td>2.5 x⁄± 1.7</td>
</tr>
</tbody>
</table>

SD: standard deviation expressed as a times/divide factor of the mean. 90%CL: 90% confidence limits expressed as a times/divide factor of the mean effect
There were moderate-large decreases in the concentration of lactoferrin with probiotic treatment in females (Table 4, page 91). Similar to the males, the concentrations of salivary proteins were characterised by large variability.

Table S6. Concentration of salivary proteins pre and post supplementation.

<table>
<thead>
<tr>
<th>Females</th>
<th>Probiotic</th>
<th>Placebo</th>
<th>Difference in change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Mean</td>
<td>Post Mean</td>
<td>Pre Mean</td>
</tr>
<tr>
<td></td>
<td>×/ SD</td>
<td>×/ SD</td>
<td>×/ SD</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2700 ×/ 6.6</td>
<td>3500 ×/ 4.6</td>
<td>1700 ×/ 2.7</td>
</tr>
<tr>
<td>Lysozyme as a ratio to albumin</td>
<td>41 ×/ 3.8</td>
<td>100 ×/ 4.8</td>
<td>58 ×/ 2.8</td>
</tr>
<tr>
<td>Lysozyme as a ratio to osmolality</td>
<td>26 ×/ 5.2</td>
<td>36 ×/ 3.8</td>
<td>22 ×/ 2.5</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>4900 ×/ 3.4</td>
<td>4200 ×/ 2.1</td>
<td>3500 ×/ 2.2</td>
</tr>
<tr>
<td>Lactoferrin as a ratio to albumin</td>
<td>90 ×/ 1.9</td>
<td>130 ×/ 2.2</td>
<td>130 ×/ 3.2</td>
</tr>
<tr>
<td>Lactoferrin as a ratio to osmolality</td>
<td>50 ×/ 2.9</td>
<td>44 ×/ 2.3</td>
<td>40 ×/ 1.9</td>
</tr>
<tr>
<td>SLgA</td>
<td>190 ×/ 2.5</td>
<td>160 ×/ 2.6</td>
<td>170 ×/ 1.4</td>
</tr>
<tr>
<td>SLgA as a ratio to albumin</td>
<td>4.0 ×/ 3.5</td>
<td>4.8 ×/ 2.2</td>
<td>6.2 ×/ 2.4</td>
</tr>
<tr>
<td>SLgA as a ratio to osmolality</td>
<td>2.3 ×/ 2.4</td>
<td>2.1 ×/ 2.4</td>
<td>2.0 ×/ 1.7</td>
</tr>
</tbody>
</table>
The effect of exercise on innate mucosal immunity

West NP*, Pyne DB*, Kyd JMδ, Renshaw GM†, Fricker PA**, Cripps AW‡‡.

*Department of Physiology, Australian Institute of Sport, †School of Physiotherapy and Exercise Science, Griffith Health, Griffith University, δSchool of Medicine, Australian National University, ‡School of Health Services, Central Queensland University, **Executive, Australian Institute of Sport, Canberra, Australia, ‡‡ Griffith Health, Griffith University, Australia.

To compare the concentration of salivary lactoferrin and lysozyme in elite rowers and sedentary individuals (controls) over a training season to determine the chronic effects of exercise and to examine the time course of changes in salivary lactoferrin and lysozyme in response to a graded exercise session. Saliva samples were taken fortnightly over 5 months from a cohort of elite male and female rowers (n=17, mean age 24.3 ± 4.0 y) and at three time points, baseline, midpoint and the end of the study, from a sedentary control group (n=18, mean age = 27.2 ± 7.1 y) over the same period. In the dose response study salivary lysozyme and lactoferrin concentrations were measured at rest, at a submaximal workload and after exercise to exhaustion. Lactoferrin and lysozyme concentrations were determined by ELISA and corrected for changes in albumin to control for salivary flow rate and total protein concentration differences. Values were log-transformed prior to analysis. Magnitudes of differences and changes were interpreted as a standardized (Cohen’s) effect size (ES). The rowers had approximately 60% lower concentration of lactoferrin than control subjects at baseline and at the midpoint of the longitudinal study: baseline (7,913 ± 4948 ng.ml−1 mean ± SD, 19,444 ± 22,035 ng.ml−1, P=0.05, ES=0.68, ‘moderate’), midpoint (6,437 ± 5,651 ng.ml−1 mean ± SD, 21,585 ± 16,634 ng.ml−1, P=0.001, ES=0.89, ‘moderate’). There was no significant difference between rowers or control subjects in the concentration of salivary lysozyme over the study. Correcting for albumin had little impact on the outcomes of the study. In the dose response study there was a significant increase in the concentration of both lactoferrin (15,322 ± 10,272 ng.ml−1, 30,209 ± 16,914 ng.ml−1, P=0.04) and lysozyme (13,509 ± 12,629 ng.ml−1, 29,693 ± 13,569 ng.ml−1, P=0.01) from pre-exercise to maximal exertion only. Lower lactoferrin and lysozyme concentration may be indicative of a possible impairment of innate protection of the upper respiratory tract during a training season in elite rowers. The increase in the concentration of lactoferrin and lysozyme following exhaustive exercise may be a protective response to limit damage to the body from exercise.
Appendix G – Abstract for the Griffith Institute for Health and Medical Research Conference, Gold Coast, December 2007

The effect of exercise on innate mucosal immunity

West NP*, Pyne DB*, Kyd JM*, Renshaw GM†, Fricker PA**, Cripps AW‡‡.

*Department of Physiology, Australian Institute of Sport, †School of Physiotherapy and Exercise Science, Griffith Health, Griffith University, ‡School of Medicine, Australian National University, δSchool of Health Services, Central Queensland University, ††Executive, Australian Institute of Sport, Canberra, Australia, ‡‡ Griffith Health, Griffith University, Australia.

Elite athletes may be at greater risk of upper respiratory tract illness from exercise-induced perturbations in immunity. We conducted a prospective observational study comparing salivary lactoferrin and lysozyme concentration over five months (chronic changes) in elite rowers (n=17, mean age 24.3 ± 4.0 y) with sedentary individuals (controls) (n=18, mean age = 27.2 ± 7.1 y) and a graded exercise test to exhaustion (acute changes) with a cohort of elite rowers (n=11, mean age 24.7 ± 4.1). Magnitudes of differences and changes were interpreted as a standardized (Cohen's) effect size (ES). Lactoferrin concentration in the observational study was approximately 60% lower in rowers than control subjects at baseline (7,913 ± 1,237 ng.ml⁻¹ mean ± SEM, 19,444 ± 5,690 ng.ml⁻¹, P=0.05, ES=0.68, ‘moderate’) and at the midpoint of the season (6,437 ± 1,413 ng.ml⁻¹ mean ± SD, 21,585 ± 4,286 ng.ml⁻¹, P=0.001, ES=0.89, ‘moderate’). The concentration of lactoferrin at the end of the study was not statistically significant (P=0.1). There was no significant difference between rowers and control subjects in lysozyme concentration during the study. There was a 50% increase in the concentration of lactoferrin (P=0.04, ES=1.04, ‘moderate’) and 55% increase in lysozyme (P=0.01, ES=3.0, ‘large’) from pre-exercise to exhaustion in the graded exercise session. Lower concentrations of salivary lactoferrin may be indicative of an impairment of innate protection of the upper respiratory tract. Increased salivary lactoferrin and lysozyme concentration following exhaustive exercise may be due to a transient activation response that increases protection in the immediate post exercise period.
Appendix H – Abstract for the International Society of Exercise and Immunology, Tubingen, 2009

Probiotic supplementation, illness and immunity in athletes

West NP1,2, Pyne DB1,3, Hopkins WG4, Fricker PA1,3, Jairath A5, Eskesen DC6, Cripps AW 2.

1Australian Institute of Sport, Australia, 2Griffith University, Australia, 3Australian National University, Australia, 4 AUT University, Auckland, New Zealand, 5Probiomics Ltd, Australia 6 Chr. Hansen A/S.

Probiotic supplements are proposed to enhance health and wellbeing. The aim of this study was to evaluate the effects of *Lactobacillus fermentum* VRI-003 (PCC) supplementation on symptoms of respiratory illness and medication use in well-trained athletes. Well-trained competitive cyclists (n=99, 64 males and 35 females, mean ± SD age 35 ± 9 and 36 ± 9 y, VO2max 56 ± 6 and 52 ± 6 ml.kg⁻¹.min⁻¹) were randomly allocated to either probiotic (minimum 1 × 10⁹ *L. fermentum* per day) or placebo treatment for 77 days. Athletes reported details of training and illness symptoms in a daily diary. Severity of illness was graded on a 3-point Likert scale based on the impact of illness on training (no impact, a reduction, complete cessation). A factor of 1.20 was chosen as the threshold for a substantial difference in the symptom load (severity × duration) and number of medication episodes between the treatment groups. For severity, the threshold was a difference of 0.20 of the between-subject standard deviation. Males in the probiotic group had a substantially lower intensity of self-reported symptoms of chest infection (0.43 of a scale step, 99% confidence limits –0.08 to 0.92) and a substantial reduction in symptom load for chest infections (by a factor of 0.31, 0.07 to 0.96) compared with males in the placebo group. There was also less use of cold and flu medications (by a factor of 0.29, 0.04 to 0.89) in males taking the probiotic vs placebo. Differences in clinical outcomes between females in the two groups were unclear. Supplementation with *L. fermentum* VRI-003 (PCC) may reduce the severity of chest infection, illness load and use of medications associated with respiratory infections in well-trained male cyclists.
Immunomodulating potential of nutrition supplements: a randomised trial comparing a prebiotic and symbiotic preparation

West NP1,2, Pyne DB1,3, Fricker PA4, Topping DL5, Conlon MA5, Clarke JA5 and Cripps AW6.

1Department of Physiology, Australian Institute of Sport, Australia, 2School of Physiotherapy and Exercise Science, Griffith Health, Griffith University, Australia, 3School of Medicine, Australian National University, Australia, 4Executive, Australian Institute of Sport, Canberra, Australia, 5CSIRO Human Nutrition, Adelaide, Australia, 6Griffith Health, Griffith University, Australia.

There is growing interest in the use of synbiotics and prebiotics to alter enteric microflora and enhance biomarkers of immune function in healthy people. Prolonged intense exercise causes acute and chronic perturbations in immunity that is proposed to heighten the risk of infectious illness. This makes exercise an ideal model to examine the efficacy of immune-modulating supplements in healthy people. The aim of this study was to compare a symbiotic supplement containing probiotics (L. paracasei, B. lactis, L. acidophilus, L. rhamnosus), prebiotics (Raftiline GR, Raftilose P95), lactoferrin and immunoglobulins with a known prebiotic supplement (acacia gum) on gut microflora and markers of mucosal and systemic immunity utilising exercise as a model of stress. Forty four competitive male cyclists (mean age = 33.9 ± 6.5y, VO2max = 57.3 ± 5.2 ml.kg⁻¹.min⁻¹) were randomised in a control trial to receive one of four supplements: a prebiotic, a symbiotic, a combination low dose prebiotic/high dose synbiotic (Low-P) or a combination high dose prebiotic/low dose synbiotic (High-P) for 21 days. Saliva samples, blood samples, urine samples and faecal samples were taken pre-, mid and post-intervention for measurement of immunological variables: Salivary lactoferrin concentration was determined by ELISA and corrected for changes in albumin to control for salivary flow rate and total protein concentration differences. GI permeability was measured by the percentage recovery of urinary lactulose and mannitol. Serum cytokines (TNF-α, IFN-γ, IL-16 and IL-18) were quantitatively measured on a Bio-Plex Suspension Array System. Total aerobic and anaerobic micro-organisms, lactobacilli and bifidobacteria were quantified by phylogenetic oligonucleotide microarray. Values were log-transformed prior to analysis. Magnitudes of differences and changes were interpreted as a mean standardized (Cohen’s) effect size (ES) ± standard deviation (SD).

Supplementation had no effect on salivary lactoferrin or GI permeability. IL-16 concentration increased 7.3 fold (ES 3.88 ± 0.61, P<0.01) in the prebiotic group, 4.2 fold (ES 4.62 ± 0.78, P<0.01) in the High-P group, 4.5 fold (ES 2.18 ± 0.67, P<0.01) in the Low-P group and 3.5 fold (ES 1.92 ±0.8, P<0.01) in the symbiotic group. There was a moderate increase of 107% in total bacteria (eubacteria) in the prebiotic group only (ES 0.49 ± 0.68, P=0.2). There was a large increase...
in total lactobacillus count in the prebiotic group (432% ×/÷ 5.3%, ES 1.11 ± 0.64, P=<0.01) and High-P group (259.6% ×/÷ 3.6%, ES 0.64 ± 0.70, P=0.13) from pre- to post-intervention. There was a large increase in *L. paracasei* from pre- to post-intervention in the Low-P (434% ×/÷ 5.3%, ES 0.90 ± 0.69, P=0.04) and a moderate increase in the synbiotic dose group (194% ×/÷ 6.6%, ES 0.46 ± 0.44, P=0.09). The proportions of samples containing *Lactis* and *L. rhamnosus* from pre- to post-intervention increased in all groups except the prebiotic group.

This study indicates that there is a differential effect between prebiotic and synbiotic supplementation on enteric microflora. Supplementation with both prebiotics and synbiotics may have a beneficial effect on systemic immunity by enhancing IL-16, with the synbiotic having the largest effect. Neither prebiotics nor synbiotics had an effect on mucosal immunity.
Probiotics, immunity and illness: a randomised clinical trial in athletes.

West NP 1,2, Pyne DB 1,3, Cripps AW 2, Hopkins WG 4, Jairath A 5, Eskesen DC 6, Fricker PA 1,3

1 Australian Institute of Sport, Australia, 2 Griffith University, Australia, 3 Australian National University, Australia, 4 AUT University, Auckland, New Zealand, 5 Probiomics Ltd, Australia 6 Chr. Hansen A/S.

Probiotic supplements are proposed to modulate immunity and reduce susceptibility to illness in healthy people. This study examined Lactobacillus fermentum VRI-003 (PCC) supplementation on mucosal and systemic immunity and on illness using exercise as a model of stress. Competitive cyclists (n=99, 64 males and 35 females, mean ± SD; age 35 ± 9 and 36 ± 9 y, VO2max 56 ± 6 and 52 ± 6 ml.kg\(^{-1}\).min\(^{-1}\)) were randomly allocated to probiotic (minimum 1 \(\times\) 10\(^9\) L. fermentum per day) or placebo treatment for 77 days. Saliva and blood samples were taken pre- and post-supplementation and pre- and post-exercise challenge for measurement of mucosal and systemic immunity. Salivary lactoferrin, lysozyme and IgA concentration were determined by ELISA. Serum cytokines were measured on a Bio-Plex Suspension Array System. Athletes reported training and illness symptoms daily. Values were log-transformed prior to analysis. Magnitudes of differences and changes were interpreted as a mean standardized (Cohens) effect size (ES) ± standard deviation (SD). Males in the probiotic group had a substantially lower intensity of self-reported symptoms of chest infection, a substantial reduction in symptom load for chest infections (by a factor of 0.31, 0.07 to 0.96) and less use of cold and flu medications (by a factor of 0.29, 0.04 to 0.89) compared with males in the placebo group. Differences in clinical outcomes between females in the two groups were unclear. Supplementation with L. fermentum reduced acute post-exercise perturbations in IL-1RA by 40% (\(P=0.03\)), in IL-10 by 22% (\(P=0.09\)), in GM-CSF by 80% (\(P<0.01\)) and TNF-\(\alpha\) by 41% (\(P=0.05\)) in males and in IL-1RA by 65% (\(P=0.06\)) and IL-6 by 110% (\(P=0.03\)) in females at the mean exercise training load. The effects of supplementation on mucosal immunity were unclear. Supplementation with L. fermentum VRI-003 (PCC) may be a useful nutritional adjunct in those undertaking physical activity.
Probiotics for Illness Prevention and Improved Exercise Performance

Cecilia M Shing1, Nick West2,3

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Department of Physiology, Australian Institute of Sport, Canberra, AUSTRALIA, 3
Griffith Health, Griffith University, Southport, Queensland, AUSTRALIA

Introduction
Probiotics are non-pathogenic and non-toxinogenic bacteria that have been defined as living organisms in food and dietary supplements which upon ingestion improve the health of the host beyond their inherent basic nutrition. Supplementation with probiotics has a variety of purported health benefits including improving nutrient synthesis and bioavailability, improving blood lipid profile, reducing the incidence and severity of gastrointestinal disorders (e.g. diarrhoea, Crohn’s disease), alleviating chronic inflammatory conditions (e.g. allergies) and reducing symptoms of upper respiratory illness (1). The chemical functions of probiotics include the synthesis of organic acids which reduce the pH of the gastrointestinal tract, competition for essential nutrients and the production and secretion of proteins with antibacterial properties. The physical functions of probiotics include prevention of colonization by pathogens and reinforcement of the intestinal lining. These functions create an unfavourable environment for foreign bacteria and help to prevent the translocation of foreign bacteria into the blood stream.

Athletes may have increased susceptibility to upper respiratory tract illness during heavy training periods and competition (2). Some athletes also report gastrointestinal dysfunction, including diarrhoea, digestive intolerance and bloating (3). Episodes of illness can impair training and there is evidence that illness during competition may compromise athletic performance (3, 4). Preventing illness is therefore a high priority for athletes and their coaches. In view of the potential benefits of probiotics to enhance mucosal immunity and reduce symptoms of gastrointestinal and upper respiratory tract illness, studies investigating the influence of probiotic supplementation on athletes are warranted.

Probiotics for Illness Prevention
There are only three studies published that have examined the effects of probiotics in athletes. A double-blind, placebo-controlled cross over trial investigated the use of L. fermentum VRI-003 (Progastrim, Probiomics Ltd) in 20 elite male runners over a four month winter training season. Athletes taking the probiotic supplement reported less than half the number of days of respiratory
symptoms during the supplementation period (30 days) compared with the placebo group (72 days). Illness severity was also lower for episodes occurring during the supplementation period (5). These findings are consistent with a recent study conducted at the AIS in a group of well-trained cyclists. In contrast, two studies, one in athletes and one in military cadets, report mixed findings. A randomised double-blind intervention study in which 141 runners took either a placebo or L. rhamnosus for three months leading into a marathon reported no significant difference in either respiratory tract illness, or GI symptom episodes, in the two weeks after the marathon (6). There was, however, a trend toward shorter duration of GI symptom episodes in the probiotic group. Probiotic supplementation with L. casei DN-114001 by 47 French commando cadets during a three week training course, followed by a five day combat course, had little effect on the incidence of respiratory tract illness (7).

Recent research at the Australian Institute of Sport, in collaboration with Griffith University, has extended the findings of these initial studies in athletes. A study of 99 well-trained male and female cyclists and triathletes found that males consuming probiotics had a substantially lower intensity of self-reported symptoms of chest infection, a substantial reduction in symptom load for chest infections (by a factor of 0.31, 99% confidence limits 0.07 to 0.96) and less use of cold and flu medications (by a factor of 0.29, 0.04 to 0.89) compared with males taking a placebo over a 13 week winter training period. Furthermore, males had a reduction in GI symptom intensity at higher training loads than their counterparts taking the placebo. There was no substantial clinical benefit in females taking a probiotic. Supplementation with probiotics also reduced acute post-exercise cytokine perturbations to exhaustive exercise by 20–40% in both males and females following the 13 week supplementation period, indicating that probiotic supplementation may reduce exercise-induced perturbations in immunity.

The mixed findings from studies examining probiotic supplementation on immunity in athletes suggest that probiotics may be a useful nutritional adjunct for athletes during training and competition.

**Inflammation and Exercise Performance**

Disruption of gut integrity may lead to the leakage of gram negative bacteria (lipopolysaccharide, LPS) from the gut into the circulation, a process known as endotoxaemia. LPS is an endotoxin that is harmless when contained within the gut, as the gut normally blocks movement of LPS into the circulation. However, strenuous exercise redirects blood flow away from the gut, increases oxidative stress and results in ischaemia/reperfusion of gastrointestinal tissues, which all act to increase gut permeability and the translocation of LPS across the intestinal wall. Several studies have reported an increase in the plasma concentration of LPS following exercise (8-10). Increases in circulating LPS stimulate systemic release of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α). Strenuous exercise also causes a decrease in circulating factors that neutralise LPS (anti-LPS IgG and IgM) (8, 11). Gastrointestinal permeability is exacerbated while exercising in
hot conditions and with fluid restriction (12); increasing circulating LPS that stimulate systemic release of pro-inflammatory cytokines that may in turn impair exercise performance (13) and heat tolerance (10).

Animal models and human trials suggest that probiotics reduce inflammatory exacerbated intestinal permeability (14, 15) by increasing the expression of tight junction proteins joining intestinal epithelial cells (16, 17) and modulating the secretion of cytokines of mucosal immune cells. Maintenance of the intestinal barrier may prove beneficial for athletes as a reduction in endotoxaemia may dampen systemic inflammation, improve heat tolerance and enhance recovery from strenuous exercise.

**Conclusion**

Probiotic strains that have proven efficacy in clinical studies should be further investigated in athletic populations. Supplementation with probiotics in athletes has the potential to contribute to improved immune competence and maintenance of gut integrity that may be compromised during periods of intense exercise, exercise in hot environments and fluid restriction during exercise. Before probiotic use by athletes is advocated, the efficacy of supplementation needs to be established, with specific reference to effective strains, dose and duration of supplementation, and mechanisms of action in this population.

**References**

Appendix L – Abstract for the European Congress of Sports Science, Turkey, 2010

Modulation of immunity in athletes with butyrylated starch supplementation.

West NP1,3, Christophersen CT2, Kang S2, McSweeney CS2, Aguirre de Carcer D2, Morrison M2, Clarke J2, Conlon MA2, Pyne DB1, Topping DL2, Cripps AW3.

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Introduction
Illness during athletic competition may negatively affect performance (Pyne et al., 2005). Butyrylated starch supplementation promotes the growth and activity of the microbial flora in the gastrointestinal tract (Topping et al., 2003). This flora is proposed to modulate the immune system (West et al., 2009). The aim of this study was to examine the effect of a butyrylated starch supplement in ameliorating exercise-induced perturbations in immunity.

Methods
The study was a double-blind placebo-controlled parallel trial. Male and female athletes were randomly divided into two groups and ingested 60 g of Protein Plus Protein powder (Powerbar Oceania, Rhodes, Australia) with either 20 g high amylase maize starch with butyrate HAMSB (n=22; age 37.9 ± 7.8 y; mean ± SD) or low amylase maize starch (LAMS) (n=18; age 36.9 ± 9.5 y) twice daily for 28 d. Serum, saliva and faecal samples were collected at Days 0, 14 and 28 for assessment of immunity. A daily training and illness diary was completed. Values were log-transformed prior to analysis. Magnitudes of differences and changes with supplementation were interpreted as a mean standardised (Cohen's) effect size with 90% confidence intervals (Hopkins et al., 2009).

Results
Athletes in the HAMSB group had a substantial 59% (24% to 104%; mean, 90% confidence interval) smaller reduction in interleukin-10 over the supplementation period. There was a small 28% (~28% to 60%) decrease in the resting concentration of tumour necrosis factor alpha in the LAMS group and a small 53% increase (5% to 124%) in the HAMSB group over the study, which is a moderate 60% (8.3% to 318%) difference between the groups. There were trivial but unclear changes in mucosal immunity.

Discussion
Prolonged intense exercise results in a shift in the immune system toward a “Th2” profile (reductions in IL-6 and TNF-α and increases in IL-1RA and IL-10) and the down-regulation of
cell-mediated immunity, which may increase susceptibility to infectious illness. HAMSB appears to have maintained a cell-mediated immune profile. The results of our project justify examining the effects of Butyrylated starch on illness patterns in athletes.

References

Clinically beneficial effects of supplementation with *Bifidobacterium lactis* Bl-04™ in healthy physically active individuals.

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Healthy physically active individuals represent a key target group for health claims and sales of probiotics. The effects of probiotics on upper respiratory and gastrointestinal tracts illness, immune function and faecal microbiology in healthy physically active individuals are unclear. The aim of this study was to determine the clinical, immunological and microbiological effects of *Bifidobacterium lactis* Bl-04™ supplementation in healthy active adults. A total of 117 males and 109 females (age 36 ± 10 y; mean ± SD) ingested either *B. lactis* Bl-04 (dosage 2 × 10^9 live cells per day) or placebo in powder form dissolved in a beverage daily in a double-blind placebo-controlled design over 150 d during autumn, winter and spring. Subjects recorded self-reported symptoms of illness (symptom type, duration and severity), medication usage and daily physical activity patterns on a Web-based questionnaire. Blood and faecal samples were collected at baseline and post-supplementation. *In vitro* analysis of whole blood phagocytosis (granulocytes and monocytes) and natural killer cell activity was undertaken by flow cytometry while faecal microbiology was determined by quantitative PCR. The effect of supplementation on illness episodes was modelled on symptom durations of one, three, five and ≥seven days to account for infectious and non-infectious aetiology. A reduction in illness symptoms >10% and an odds ratio of clinical benefit to harm greater than 66 (i.e. > 25% likelihood of benefit and <0.5% likelihood of harm) were determined a-priori as criteria for a substantial clinical improvement between treatments. There was a trend for increasing benefit of probiotics on number of episodes of upper respiratory tract illness of longer duration, with episodes seven days or longer being 46% lower (−39 to 79%; mean and 99% confidence interval; *P*=0.09) in the probiotic compared to placebo group. Episodes of chest infection lasting five days duration or longer were 45% (−27 to 76%; *P*=0.06) fewer in those taking Bl-04 than placebo. Subjects on *B. lactis* Bl-04 had 45% (1 to 70%; *P*<0.01) fewer total days of cold and flu medication usage those on placebo. No substantial effects of supplementation were evident in measures of immunity between the probiotic and placebo groups. Total *Bifidobacterium*
species declined by 60% (~80 to ~18%; \( P=0.03 \)) in the \textit{B. lactis} group. The probiotic \textit{B. lactis} BI-04 appears to have small but clinically beneficial effects on respiratory symptoms when delivered in powder form particularly for episodes of illness lasting 5 days or longer. Supplementation with \textit{B. lactis} Bl-04™ appears to be a useful nutritional strategy to limit the effects of upper and lower respiratory illness and medication usage in healthy active adults. The absence of substantial effect in blood and faecal markers indicates that probiotics delivered as a powder may mediate their clinical effects by modifying immune parameters at airway surfaces rather than through the gut.
Health promoting and immune enhancing effects of *Bifidobacterium lactis* BL-04: a randomised controlled trial

Horn P¹, Brun M², Cripps AW³, Fricker P¹, Hopkins WG⁴, Pyne DB¹, Warren H² and West NP¹,³

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Healthy active individuals are consumers of probiotic supplements and probiotic-enriched foods to enhance immunity and reduce susceptibility to illness. There is a paucity of data on the efficacy of probiotics in this sub-group of the population. This study investigated whether probiotics reduce upper respiratory tract (URTI) and gastrointestinal (GI) illness symptoms in a double-blind placebo-controlled trial over 150 d. Physically-active healthy adults (117 males, 109 females, age 36 ± 10 y; mean ± SD) were randomly assigned to either probiotic (2x10⁹ *Bifidobacterium lactis* Bl-04™) or placebo. Probiotic and placebo were ingested as a powder dissolved in a drink taken daily. All subjects kept an illness and training diary for self-reporting of the frequency, type, duration, severity, and load (duration x severity) of illness symptoms on a daily basis, as well as daily patterns of exercise and athletic training. A cohort of 87 individuals (47 males, 40 females, age 35 ± 10 y) provided a blood sample for analysis of innate immune parameters. A reduction of 10% in illness symptoms was established as the threshold value for a substantial difference between treatments - ratio 1.0 ± 0.2 (or ratio interval 0.83–1.2). Males and females taking *B. lactis* Bl-04 had a substantial ~26% (~17 to 55%; 99% confidence interval) lower upper respiratory tract load and symptom duration than the placebo group. The clinically beneficial effect of *B. lactis* Bl-04 on URTI symptom load and duration was evident in females (45% lower; ~14 to 73%) but not males. The severity of chest illness symptoms for males and females was ~17% (~23 to 45%) lower in the *B.lactis* Bl-04 group than the placebo group. Males and females on *B.lactis* Bl-04 had 32% (~9% to 57%) fewer total days of medications compared with those on the placebo. *B. lactis* Bl-04 enhanced neutrophil phagocytic activity by 25% (~5 to 65%) and monocyte phagocytic activity by 27% (~6 to 73%). This study provides evidence of clinical benefit using probiotics to reduce respiratory illness in healthy active individuals. Maintenance of phagocytic activity may be one mechanism underpinning the beneficial clinical outcomes with *B. lactis* Bl-04™ supplementation.
Appendix O – Abstract for the International Society of Exercise and Immunology, Oxford, England, July 2011

Granulocyte and Monocyte Phagocytic Activity as Mechanisms Facilitating the Effects of Probiotic Supplementation in Active, Healthy Adults.

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Administration of a probiotic supplement combining two strains has shown efficacy in reducing clinical symptoms of cold symptoms in healthy (3–5 y age) children (154). Less is known regarding the efficacy of multi-strain probiotics in healthy active individuals. This study investigated whether probiotics reduce upper respiratory tract (URTI) and gastrointestinal (GI) illness symptoms in a double-blind placebo-controlled trial over 150 d. A secondary aim was to investigate the effects on innate immunity. Healthy physically-active adults (111 males aged 37 ± 11 y and 109 females aged 38 ± 11 y; mean ± SD) were randomly assigned to either probiotic (Lactobacillus acidophilus NCFM™ and Bifidobacterium lactis Bi-07™ at 1 × 10¹⁰ (5 billion each)), or placebo, taken daily as a powder dissolved in a drink over 150 d. A sub-group of 43 males (aged 37 ± 9 y) and 38 females (aged 36 ± 10 y) provided pre- and post-supplementation blood samples. Clinical benefit was established where the likelihood of 10% reduction in symptoms was >25% provided the likelihood of harm (a 10% increase in symptoms) was <5%. Precision of estimation for clinical benefit and harm was reported with a 99% confidence interval. Compared to the probiotic group, the placebo group had shorter duration of mild GI symptoms (–26%; –50 to 9%; 99% confidence intervals) and symptom load (–31%; –55 to 5%). In comparison to males in the probiotic group males in the placebo group had a 45% (–9 to 130%) higher number of total days of medication use. There was a relative 11% (–17 to 32%) greater increase in monocyte phagocytic activity in the combined probiotic group compared to placebo. No substantial effects of supplementation were evident in neutrophil phagocytic activity or NK cell activity. Decrements in clinical indices of URTI conflict with previous reports while increased mild GI symptoms may be associated with adaptive responses to the probiotics. Further work is required to clarify whether combined NCFM™ and Bi-07™ probiotic-induced enhancement of phagocytic activity elicits substantial clinical benefits in adult population.