Effects of milk fat globule membrane on gastrointestinal and immune responses to exercise

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

November 2025

Declaration

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

Acknowledgements

Firstly, I would like to acknowledge my supervisor, Professor Glen Davison. I started working with Glen in 2nd year of my undergraduate, and he has since supervised my undergraduate dissertation, MRes and now, PhD. Glen's attention to detail and remarkable expertise of nutrition and exercise immunology has been a privilege to work with. I am immensely grateful for all the support and guidance over the years.

I would like to thank Volac Whey Nutrition Ltd (now Arla Food Ingredients) for funding this research. I am especially grateful to Dr LooWee Chia and Dr Elisa Glover for their active engagement throughout my PhD. Collaborating with them has provided me with unique opportunities to connect academic research with industry and I am hugely grateful for the experiences and insights I've gained.

A huge gratitude goes to my fellow SERS PGR students. The motivation, support and comic relief provided over the years made the gruelling lab days far easier. I would also like to thank all the SERS staff that have contributed to my academic journey. In particular, I'd like to thank Professor John Dickinson, who I first met as my undergraduate academic advisor, but has since provided me with invaluable experiences within applied sports science.

I am profoundly thankful to my family. Completing a PhD is a marathon, and their unwavering support and encouragement have been a constant throughout the process.

Finally, I would like to thank all of the participants for donating their time and effort, without which this research would not have been possible.

General abstract

It is well established that exercise, particularly of a strenuous and/or prolonged nature, can cause perturbations to gastrointestinal (GI) integrity and immune functions. Many nutritional interventions have been researched in the context of mitigating these disturbances, of which dairy-based supplements have shown promising findings. Milk fat globule membrane (MFGM) has received considerable attention for its role in infant health, where it has demonstrated positive effects on the GI and immune systems. However, whether these results are also evident in an adult population is currently unclear, highlighting a clear gap in the research. The aims of this thesis were to investigate the short-term and long-term effects of MFGM on GI and immune functions, which was assessed across 3 randomised controlled trials. **Chapter 3** (study 1) assessed the effects of short-term (2 weeks) MFGM supplementation on gut damage and permeability following a strenuous exercise bout. The results found that MFGM prevented an increase in gut epithelial cell damage and permeability following exercise. **Chapter 4** (study 2) provided a mechanistic insight into the immunomodulatory effects of MFGM, investigating cellular and mucosal immune functions following prolonged cycling. It was found that MFGM enhanced resting neutrophil function and attenuated the exercise-induced depression of neutrophil function. Building on the promising findings of study 2, **Chapter 5** (study 3) investigated the effects of long-term (12 weeks) MFGM supplementation on upper respiratory tract infection (URTI) incidence/burden and mucosal immune functions. Despite the promising findings from the previous two chapters, there was no effects observed on URTIs or mucosal immunity. The findings of this thesis demonstrate that MFGM may help maintain GI and immune functions in response to exercise stressors. However, this did not translate to protection against URTIs. This thesis highlights the GI and immune benefits of MFGM and the need for further research into this potential nutraceutical.

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Presentations

To date, work from this thesis has been presented at two scientific meetings/industry summits:

Searle, W. J and Davison, G. (2024) Volac Whey BING Summit: Overview of gut and immune clinical research. Volac Whey Nutrition Limited, Herfordshire, UK, November 2024.

Searle, W. J. and Davison, G. (2023) Volac Whey BING Summit: Introduction to gut and immune clinical research. Volac Whey Nutrition Limited, Herfordshire, UK, November 2023.

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List of Abbreviations and Symbols

1 h Post-Ex	1 hour post-exercise
^{51}Cr-EDTA	^{51}CR-labeled ethylenediaminetetraacetic acid
ALA	α-linolenic acid
ALR	AIM2-like receptor
AMP	antimicrobial peptide
ANOVA	analysis of variance
APC	antigen-presenting cell
APP	acute phase protein
ASV	amplicon sequencing variant
BM	body mass
BMI	body mass index
C1q	complement component 1q
C1r	complement component 1r
C1s	complement component 1s
CD	cluster for differentiation
CHIPS	Cohen-Hoberman Inventory of Physical Symptoms
CKK	cholecystokinin
CL	chemiluminescence
CLA	conjugated linoleic acids
CLR	C-type lectin receptors
CO_2	carbon dioxide
CV	coefficient of variation

DAMP	damage-associated molecular pattern
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EEC	enteroendocrine cell
ELISA	enzyme-linked immunosorbent assay
FasL	fas ligand
FcεRI	high affinity IgE receptor
fMLP	n-formyl-methionyl-leucyl-phenylalanine
g	gram
GI	gastrointestinal
GIP	gastric inhibitory peptide
GLP-1	glucagon-like peptide 1
GSRS	gastrointestinal symptom rating scale
h	hour
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HMP	hexose monophosphate shunt
HOBr	hypobromous acid
HOCl	hypochlorous acid
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
HR	heart rate
HSC	hematopoietic stem cell
HSP70	heat shock protein 70
ICMJE	International Committee of Medical Journal Editors

I-FABP	intestinal-fatty acid binding protein
IFN	interferon
Ig	immunoglobulin
IgA	immunoglobulin a
IgE	immunoglobulin e
IgG	immunoglobulin g
IgM	immunoglobulin m
IL	interleukin
ISC	intestinal stem cell
kDa	kilodalton
km	kilometre
KOH	potassium hydroxide
L/R	lactulose/rhamnose
LAL	limulus amoebocyte lysate
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
M	molar
MASP	MBL-associated serine protease
MCT	medium chain triglyceride
MFG-E8	milk fat globule epidermal growth factor 8
MFGM	milk fat globule membrane
MHC	major histocompatibility complex
min	minute
mL	millilitre
MOF	multiple organ failure

MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NK	natural killer
NLR	nod-like receptor
NOD	nucleotide-binding oligomerization domain
NSAID	non-steroidal anti-inflammatory drug
O_2-	superoxide anion
OTC	over the counter
PAMP	pathogen-associated molecular pattern
PAR-Q	physical activity readiness-questionnaire
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCoA	principle coordinates analysis
PE	phosphatidylethanolamine
PEG 400	polyethylene glycol 400
PERMANOVA	permutational multivariate analysis of variance
PI	phosphatidylinositol
PLA	placebo
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear
PRR	pattern recognition receptor
PS	phosphatidylserine
PSS	perceived stress scale

PYY	peptide YY
QC	quality check
qPCR	quantitative polymerase chain reaction
REAG	research ethics advisory group
RIG	retinoic acid-inducible gene-
RLR	rig-like receptor
RLU	relative light unit
RM	repeated measures
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPE	rating of perceived exertion
Rq	relative quantification
rRNA	ribosomal ribonucleic acid
s	second
SCFA	short-chain fatty acid
SIgA	secretory immunoglobulin a
SIRS	systemic inflammatory response syndrome
SM	sphingomyelin
SOD	superoxide dismutase
sRPE	sessional rating of perceived exertion
STAI-S	state aspect of the state-trait anxiety inventory
Th2	t helper 2
TLR	toll-like receptor
TNF	tumour necrosis factor

TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
µL	microlitre
URI	upper respiratory illness
URS	upper respiratory symptom
URT	upper respiratory tract
URTI	upper respiratory tract infection
$\dot{V}O_2$	oxygen uptake
$\dot{V}O_{2max}$	maximal oxygen uptake
W	watt
WHO	World Health Organisation
°C	degrees celsius
α	alpha
β	beta
γ	gamma
δ	delta
Δ	delta (difference)
χ	chi

Chapter 1. Introduction and Literature Review

Over time, the human body has evolved complex systems dedicated to maintaining homeostasis and host defence. Among these, the GI and immune systems are two crucial components of the body that work to support health, as well as psychological and physiological function (Delves et al., 2011; Ogobuiro et al., 2024). Despite being independent systems, some functions overlap and provide synergistic effects, and when functioning properly, the GI and immune systems provide a robust foundation for host defence. Disruption to these systems, either individually or in combination, can compromise host defence, potentially giving rise to a range of clinical consequences. For example, impaired gut integrity is associated with conditions such as irritable bowel syndrome (Hanning et al., 2021), inflammatory bowel disease (Dunleavy et al., 2023), or in some severe cases, can contribute to endotoxemia and sepsis (Michelena et al., 2015; Potruch et al., 2022). Alternatively, impaired immune function is linked with an increased risk of illness, as well as being implicated in conditions such as cancer, and autoimmune or inflammatory diseases (Goyani et al., 2024).

While these examples highlight the clinical importance of maintaining gut and immune homeostasis, disturbance to these systems can also occur in response to physiological stressors such as exercise. Acute and chronic exercise, particularly when prolonged or performed at higher intensities, has been shown to transiently influence both gut integrity and immune function (Nieman and Wentz, 2019). Although these responses are typically temporary and not indicative of disease, they may still have practical relevance in athletic populations.

In athletes, exercise-induced perturbations in gut integrity are frequently observed during prolonged or strenuous exercise and are often accompanied by GI symptoms. These perturbations may also influence factors such as nutrient absorption and hydration status, which could negatively influence recovery (Costa et al., 2017). Similarly, exercise-related immune perturbations may also increase susceptibility to illnesses, particularly with URTIs, which could potentially interrupt training or competition (Gleeson and Pyne, 2016; Keaney et al., 2019). Therefore, maintaining gut integrity and immune function is considered important for supporting both athlete health and performance.

Milk-derived products have demonstrated strong bioactive potential, with supplements such as bovine colostrum having shown to attenuate exercise-induced GI and immune perturbations (Davison, 2021). Colostrum is produced only during the short period following calving, and collection is restricted to the excess remaining after the calf's requirements have been met, limiting its availability and increasing costs. In contrast, MFGM is a bioactive component found in both milk and colostrum that can be produced at lower costs from all milk and is also generated as a byproduct during whey protein production (see Figure 1.1). Therefore, this thesis intends to explore the GI and immunomodulatory potential of MFGM to determine whether it can exhibit effects comparable to those of bovine colostrum, while offering a more accessible and cost-effective alternative.

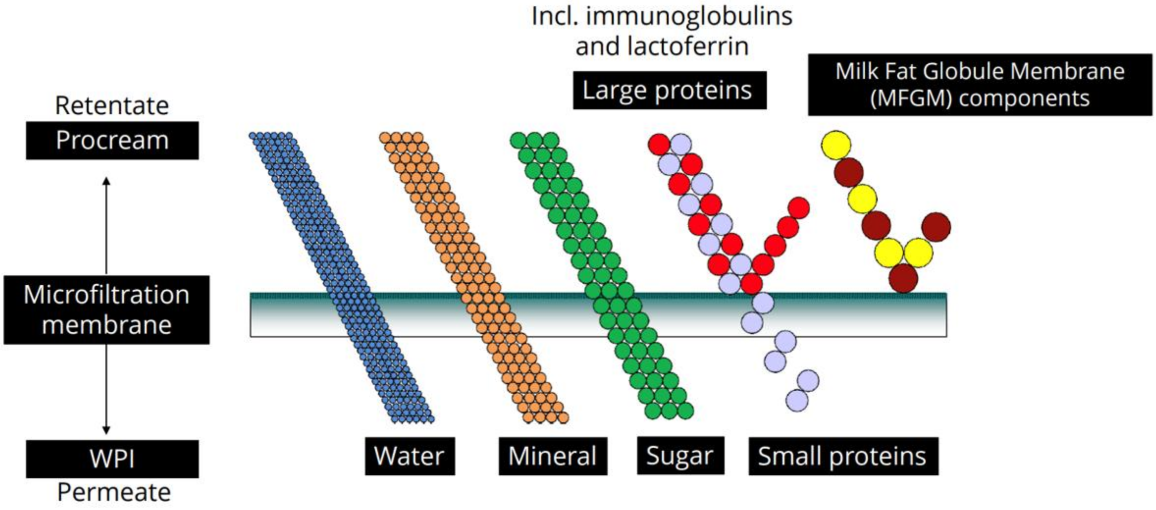

Figure 1.1. Diagram of MFGM production during whey protein manufacturing.

1.1 The gastrointestinal system

The GI tract is a complex system that consists of the oral cavity, pharynx, oesophagus, stomach, small intestine, large intestine and anal cavity. It serves as the entry point for orally consumed substances and is responsible for the ingestion and digestion of food, absorption of nutrients, secretion of water and enzymes, and excretion of waste products (Ogobuiro et al., 2024). The GI tract also serves as a barrier to potentially harmful pathogens and toxins inside the gut (lumen), preventing them from passing the intestinal barrier and entering systemic circulation (Gieryńska et al., 2022).

The gut microbiota refers to the large community of microorganisms (e.g. viruses, bacteria, fungi, parasites and archaea) that reside in the gut, which have influential effects on all GI functions and can act as one of the first lines of defence against harmful agents in the gut. Numerous factors such as age, sex, nutrition, genetics and stress (including exercise-induced) can affect and shape the composition of the microbiota, creating a usually stable, yet constantly changing environment (Vineis et al., 2020). There are also some factors, such as the use of antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs), that disturb and damage the gut microbiota, creating a window of vulnerability and allowing toxins a better chance of survival in the gut. However, the gut microbiota typically recovers fully within 1-2 months following treatment (Ng et al., 2019; Patangia et al., 2022).

The gut microbiota provides defence against intestinal toxins through numerous mechanisms, most notably through the production of short-chain fatty acids (SCFAs) such as butyrate, propionate, and acetate via fermentation of soluble dietary fibres (Kau et al., 2011). SCFAs serve as the primary energy source for colonocytes, whilst also supporting gut immunity through their ability to stimulate antimicrobial peptide (AMP) and mucus production from epithelial cells (Nogal et al., 2021; Tan et al., 2014). SCFAs also provide effects on the integrity of the gut barrier itself, having shown to upregulate the expression of mucin glycoproteins and contribute to the maintenance of tight junction proteins like claudin, occluding, and ZO-1 (Hiippala et al., 2018; Pérez-Reytor et al., 2021). Beyond SCFA-mediated effects, the gut microbiota also provides protection through colonisation resistance, where commensal bacteria occupy niches and consume available resources in the gut which would otherwise be utilised by pathogens (Lawley and Walker, 2013).

Regular moderate exercise is largely beneficial to the gut microbiota, with research showing greater cardiorespiratory fitness may correlate with higher microbial diversity and elevated butyrate levels due to increases in SCFA-producing organisms (Estaki et al., 2016). Similarly, Clarke et al. (2014) found that elite rugby players exhibited greater microbial diversity, influenced by both exercise and diet. More recently, it was found that gut microbial stability may underpin athletic performance, whereby highly trained individuals undergoing dietary periodisation (acute high-protein diet) performed better when the microbiota remained stable, but the same acute high-protein diet compromised performance in those with less stable microbiota (Furber et al., 2022). The authors also found that short-term high-carbohydrate diets improved performance, with subtle alterations to the gut microbiota. Though, again, stable microbial communities were associated with better performance.

In contrast, intense and/or prolonged exercise has been linked to shifts in the gut microbiota to a state of dysbiosis, including elevated succinate levels (Morishima et al., 2021) and shifts towards greater amounts of opportunistic pathogens (Karl et al., 2017). It has also been demonstrated that faecal metabolome of lesser trained individuals may exhibit a more pro-inflammatory profile following prolonged exercise (Zhao et al., 2018). Ultimately, moderate exercise appears to support desirable microbial environments (Conlon and Bird, 2014; Yang et al., 2017), whereas prolonged or exhaustive exercise may be detrimental (Ticinesi et al., 2019), with outcomes influenced by host conditioning, diet and microbial stability.

Whilst the gut microbiota plays a crucial role in modulating host health and performance, the focus of this thesis is the gut barrier itself. This emphasis is important, as many of the effects attributed to the microbiota ultimately depend on how these organisms influence gut barrier integrity and function. Understanding the gut barrier provides a more direct insight into how these microbial changes translate into physiological outcomes.

1.1.1 The gut barrier

The intestinal barrier is a multi-layered defence system tasked with not only enabling nutrient absorption but also protecting the host from potentially harmful luminal contents, such as pathogens, toxins and antigens (Peterson and Artis, 2014). The foundations of the intestinal barrier consist of a monolayer of intestinal epithelial cells connected by multiprotein complexes known as tight junctions (see Figure 1.2. Structure of the small intestine barrier. Intestinal epithelial cells include enterocytes, goblet cells, enteroendocrine cells and Paneth cells – each with specialised roles in digestion, barrier maintenance, immune signalling and microbial defence (Steinert et al., 2011).

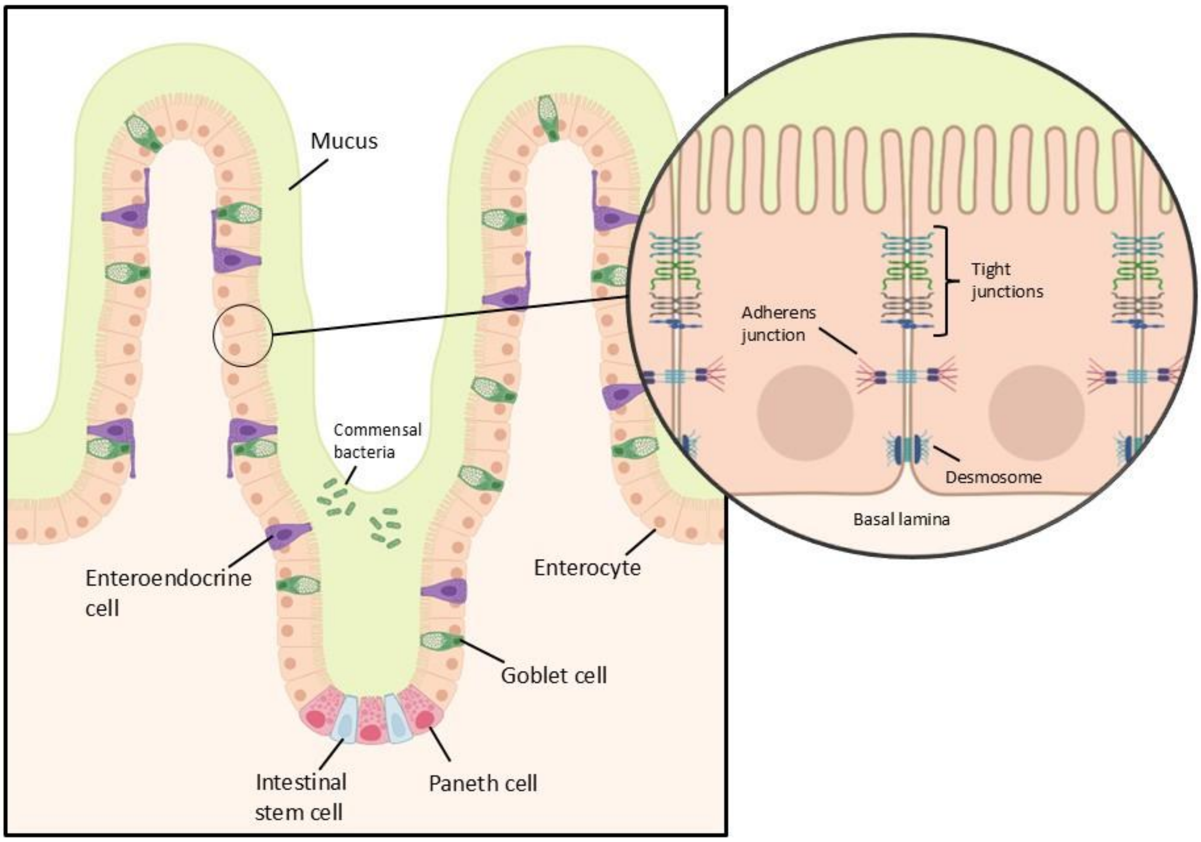

Figure 1.2. Structure of the small intestine barrier. Figure created using *Biorender*.

The most abundant cells bordering the intestinal lumen are absorptive enterocytes, constituting from 80% (ileum) to 95% (duodenum) of intestinal epithelial cells, and are largely responsible for basic intestinal functions; digestion of water, nutrients and vitamins, and the absorption of unconjugated bile salts (Miron and Cristea, 2012). Enterocytes are column-shaped cells that possess an apical cell membrane or brush border, consisting of microvilli (estimated at 3000-7000 per cell in the small intestine, although fewer in the large intestine), allowing for a greater surface area for absorption (DeSesso and Jacobson, 2001). A wide range of digestive enzymes are expressed by enterocytes, as well as transporters for nutrients such as amino acids, monosaccharides and fatty acids, among other molecules. These enzymes and transporters, located primarily at the apical membrane, facilitate the transcellular transport of nutrients from the intestinal lumen into the cells. Once inside, nutrients are then transported across the basolateral membrane into the bloodstream, completing the absorptive process (Hooton et al., 2015).

Following enterocytes, the next most abundant intestinal epithelial cell are goblet cells, distributed across both the small and large intestine. The primary function of these cells is to synthesise and secrete the mucus that lines the intestinal epithelium. Goblet cells also contain an apical surface, characterised by microvilli that allow for greater surface area for mucus secretion. They also contain an endoplasmic reticulum and Golgi apparatus crucial for the synthesis, packaging and transportation of mucins, the key protein component of mucus (Raya Tonetti et al., 2024). There are over 20 documented mucins (characterised MUC1 to MUC21), each with slightly different structures and functions, and it is the role of goblet cells to constantly secrete and renew the mucus layer in the intestinal epithelium (Konstantinidi et al., 2022). Goblet cells are also implicated in microbial defence due to their synthesis and secretion of AMPs such as resistin-like molecule β, regenerating islet-derived 3 proteins and trefoil factor, which combat commensal bacteria and pathogens that surpass the mucus barrier (Ferrand et al., 2019). Due to their antimicrobial properties, there is a notable abundance of goblet cells in the colon, where the exposure to harmful and potentially infectious luminal agents is highest (Bergstrom et al., 2010).

Enteroendocrine cells (EECs) make up a small amount (< 1%) of total intestinal epithelial cells; however, they comprise the largest endocrine organ in the body (Furness et al., 2013). They are thought to be the dominant chemosensory cell in the intestinal epithelium, with at least 12 subtypes identified, and are associated with the release of various hormones related to the regulation of satiety, GI motility and energy metabolism (Lee and Owyang, 2019). The cells were originally characterised based on the peptide secreted, on a "one cell type-one hormone" basis, however, this has since been disproved, with many EECs having shown to express messenger ribonucleic acid (mRNA) for multiple hormones (Engelstoft et al., 2013; Haber et al., 2017). With the exception of enterochromaffin cells (5-HT – serotonin), EECs are not evenly distributed across the GI tract. I cells (cholecystokinin [CKK]) and K cells (gastric inhibitory peptide [GIP]) are primarily located in the small intestine, whereas L cells (glucagon-like peptide 1 [GLP-1], peptide YY [PYY]) are mainly located in the ileum and colon. Alternative EECs such as D cells (ghrelin, somatostatin), G cells (gastrin), and ECL cells (histamine) are predominantly located in the stomach (Steinert et al., 2011).

Paneth cells were originally discovered in the late 19th century, although it wasn't until a few years later when Joseph Paneth, the namesake of the cells, described the cells in depth (Paneth, 1887). Paneth cells are pyramidal-shaped, columnar, secretory cells located at the base of intestinal crypts, also known as crypts of Lieberkühn, in the small intestine. Although, at times, Paneth cells have been observed in the stomach and colon, this has mostly been accredited to mucosal inflammation instead of homeostasis (Bevins and Salzman, 2011). Paneth cells play critical roles in gut immunity, acting as key effectors for intestinal innate immune functions through the secretion of numerous antimicrobial proteins and peptides, such as immunoglobulin A (IgA), lysozyme, lactoferrin and α-defensins (Clevers and Bevins, 2013; Ouellette, 2010). Many granular components are synthesised and packaged into dense core granules in the endoplasmic reticulum and Golgi apparatus. However, other components may be synthesised elsewhere prior to storing in the granules. One example is IgA, which is produced by plasma B cells prior to being stored in Paneth cell granules (Lueschow and McElroy, 2020; Satoh et al., 1986). Alongside their valuable functions within microbial regulation, Paneth cells also stand out as key epithelial niche cells, expressing various niche components that support and enhance intestinal stem cell (ISC) function (Sato et al., 2011). However, whilst important, Paneth cells are not essential for this function, as ISCs have been shown to tolerate the absence of Paneth cells (Kim et al., 2012).

Tight junctions are multiprotein complexes located at the apical region between intestinal epithelial cells, where they connect adjacent cells and form a selective, semi-permeable paracellular barrier. Tight junctions contribute to intestinal barrier function through their regulation of the paracellular movement of ions, solutes and water across the intestinal epithelium (Lee et al., 2018). The tight junction complex is composed of transmembrane proteins, primarily claudins and occludin, which interact with cytoplasmic proteins such as zonula occludens (ZO-1, ZO-2 and ZO-3). ZO-1 acts as a scaffolding protein that links the transmembrane components of the tight junction to the actin cytoskeleton (MacNaughton, 2006).

Paracellular transport occurs via two distinct pathways: the highly selective ‘pore’ pathway, primarily regulated by claudins, which controls the passage of ions and small molecules based on charge and size; and the less selective ‘leak’ pathway, which facilitates the movement of larger solutes in response to physiological or pathological stimuli (Shen et al., 2011). This paracellular transport complements the aforementioned transcellular pathway, together regulating the selective permeability and integrity of the intestinal barrier.

Various protein kinases have been shown to regulate tight junction function through phosphorylation of tight junction-associated proteins, particularly ZO-1. Additionally, myosin light chain kinase (MLCK) has been shown to phosphorylate myosin light chain (MLC), leading to actomyosin contraction and cytoskeletal remodelling, which in turn compromises tight junction integrity (Cunningham and Turner, 2012). In fact, a common pathway for pathogens to compromise intestinal barrier function is by altering tight junction structure. One example is enteropathogenic *E. coli*, which activates MLCK, leading to contraction of the actin filament and dephosphorylation of occludin, both of which result in a reduction in transepithelial resistance (Glotfelty and Hecht, 2012).

1.1.2 Exercise and gut integrity

The selective, semi-permeable function is a crucial characteristic of the gut, allowing for the permeation of nutrients, water and ions, whilst preventing the permeation of pathogens and other harmful substances. Though, if the integrity of the gut barrier becomes compromised, such as through disruption to tight junctions or direct epithelial cell damage, the function of the barrier can become impaired, allowing larger, and potentially harmful molecules to pass from the lumen into circulation – this is commonly referred to as "leaky gut" (Chelakkot et al., 2018; Dmytriv et al., 2024).

Intestinal-Fatty Acid Binding Protein (I-FABP) is a 15 kDa cytosolic protein influencing cellular uptake and metabolism of fatty acids (Ockner and Manning, 1974). It is expressed almost exclusively in mature enterocytes of the small intestine, making it a highly specific biomarker for intestinal epithelial cell damage. Under conditions of epithelial injury or stress, I-FABP is rapidly released into circulation, with elevated levels indicating epithelial cell damage or necrosis (Evennett et al., 2010; Lau et al., 2016). I-FABP has become a common marker of epithelial injury and has shown potential in the detection of intestinal ischaemia, both in clinical (Kanda et al., 2011) and exercise settings (van Wijck et al., 2011). Numerous exercise models have shown to cause significant elevations in I-FABP (Costa et al., 2017). Interestingly, the magnitude of epithelial damage seems to be intensity- and temperature-dependent, with vigorous running and cycling bouts typically eliciting greater I-FABP concentrations, with the highest often accompanying vigorous exercise in the heat (March et al., 2019; Morrison et al., 2014).

This is further supported by Walter et al. (2021), who observed significant increases in I-FABP concentrations following a standard marathon. Though, I-FABP was substantially higher in individuals who experienced exercise-associated collapse with hyperthermia, and still significantly higher than pre-race levels at 1 hour after collapse. Core temperature taken immediately post-collapse/marathon was also 2.5 °C higher in the collapsed cases (39.7 °C) compared to the controls (36.2 °C). Not only do these findings support the temperature and intensity dependent nature of I-FABP but further enforces the evidence for an association between intestinal damage and heat illness.

Due to the impaired intestinal barrier function accompanying intestinal epithelial cell damage, elevated I-FABP concentration has also been strongly associated with exercise-induced increases in gut permeability (Costa et al., 2017). Current methods for assessing intestinal permeability typically involve either quantifying the urinary excretion of orally ingested probes (Davison et al., 2016; Marchbank et al., 2011), or measuring systemic indicators of barrier dysfunction, such as the translocation of luminal contents into circulation (March et al., 2019; Ogden et al., 2020).

Earlier research utilised single probe tests, using substances such as lactulose, polyethylene glycol 400 (PEG 400) or ^{51}Cr-labeled Ethylenediaminetetraacetic Acid (^{51}Cr-EDTA), although the results were often inconsistent due to inter-individual variations in transit time, fluid balance and renal clearance (Travis and Menzies, 1992). Nowadays, dual-sugar tests, involving non-metabolisable disaccharides (e.g. lactulose) and monosaccharides (e.g. rhamnose or mannitol) are the gold standard for measurements of intestinal permeability. Lactulose is a relatively large disaccharide (342 kDa) that should not permeate a fully functioning intestinal barrier. Though, when the intestinal barrier is impaired, lactulose can cross the intestinal epithelium through paracellular pathways. In addition, this test will also involve a monosaccharide, such as rhamnose or mannitol, to act as a control. Rhamnose (164 kDa), which permeates via transcellular pathways, and mannitol (182 kDa), which can permeate via both transcellular and paracellular pathways, are smaller probes that can pass the intestinal barrier largely unaffected by changes in permeability. Therefore, the monosaccharides provide an internal reference for factors of individual variability, such as gastric emptying rate and urine volume (Travis and Menzies, 1992).

The lactulose/rhamnose (L/R) test has proven useful in measuring changes in intestinal permeability across several interventions, including exercise (Davison et al., 2016; March et al., 2017), NSAID ingestion (Playford et al., 2001), as well as use in clinical settings, such as in those with irritable bowel syndrome (Marshall et al., 2004), cystic fibrosis (Leclercq-Foucart et al., 1987), celiac disease (Cobden et al., 1980) and immunodeficiency (Keating et al., 1995). Exercise has consistently shown to increase the L/R ratio, likely reflecting the disruption of tight junction integrity and epithelial cell damage associated with exercise (Davison et al., 2016; Lambert et al., 2008; March et al., 2017; Marchbank et al., 2011; Pals et al., 1997; Zuhl et al., 2014a).

In addition to urinary recovery of carbohydrate probes, systemic biomarkers have been investigated for their potential as indirect markers of gut barrier dysfunction. Increased gut permeability allows for the translocation of luminal microbes and their components into circulation – a process that would normally be prevented by a functioning gut barrier (Ghosh et al., 2020). LPS, a key component of gram-negative bacteria membranes, and anti-LPS IgG have been examined as markers of exercise-induced endotoxaemia (Barberio et al., 2014; Jeukendrup et al., 2000). The translocation of LPS from the gut into circulation has clinical importance for its role in systemic inflammation and conditions such as systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF) (Michelena et al., 2015). However, issues related to sample collection and subsequent analysis/detection of LPS have led researchers to establish more reliable methods of assessing gut permeability. To overcome these limitations, researchers may use alternative markers of exercise-induced endotoxaemia, such as LPS-binding protein (LBP) or cluster of differentiation (CD) 14 (CD14). LBP is a 50-

60 kDa glycoprotein responsible for binding and transferring LPS in the blood stream to receptors like CD14 and TLR-4 (Rossol et al., 2011). Alternatively, CD14 is a protein expressed predominantly by macrophages and monocytes and acts as the receptor for LPS and other PAMPs (Sharygin et al., 2023). It exists in two forms: membrane-bound CD14 (mCD14), anchored to immune cell membranes, and soluble CD14 (sCD14), which is either shed from the membrane or secreted directly into the bloodstream. Elevated levels of sCD14 have been used as an indirect marker of microbial translocation and systemic immune activation (Turgunov et al., 2024).

Using a similar principle to LPS translocation, quantifying bacterial load in blood has gained attention in recent years as an indirect marker of gut barrier dysfunction. The common techniques employed for measuring bacterial DNA are real-time quantitative polymerase chain reaction (qPCR), 16S ribosomal RNA (rRNA) gene sequencing and shotgun metagenomic sequencing. Though, shotgun sequencing is generally impractical for measuring bacterial load in blood due to the low bacterial biomass, combined with the high amount of host DNA in blood, which accounts for over 99% of total blood DNA (Tan et al., 2023). For this reason, 16S amplicon sequencing and qPCR are the preferred methods of bacterial DNA quantification in blood.

The 16S rRNA gene encodes the small subunit rRNA molecules of ribosomes, which has an important role in signalling genetic information to functional cell components via the translation of mRNA to proteins (Byrne et al., 2018). It is expressed exclusively in bacteria and archaea, and not found in eukaryotic organisms, making it an excellent target for bacterial analysis in humans without interference from host DNA (Kim and Chun, 2014). Briefly, 16S rRNA gene sequencing involves amplifying the 16S rRNA gene from extracted DNA samples using PCR with primers that bind to conserved regions and sequencing the resulting amplicons.

The sequencing data is then compared to reference databases to characterise the bacteria present in the sample (Clarridge, 2004; Janda and Abbott, 2007).

In comparison, qPCR is a highly specific technique used to detect DNA in a sample by amplifying specific, targeted DNA sequences in real-time. Unlike 16S sequencing, which amplifies all 16S rRNA in a sample, qPCR focuses on individual taxa or genes using specific primers and fluorescent probes (Ishmael and Stellato, 2008). This allows for precise quantification of bacterial load, and due its speed and sensitivity, is a popular method for measuring bacterial abundance in samples (Smith and Osborn, 2009). Whilst qPCR is more sensitive than 16S sequencing and provides an absolute quantification of bacterial DNA, it requires prior knowledge of specific bacterial targets present in a sample. In contrast, 16S rRNA sequencing performs broad detection of bacterial taxa, making it more applicable for tests where the bacterial composition is unknown. However, it typically provides only relative abundance data and may be less sensitive for detecting low-biomass microbes (Jervis-Bardy et al., 2015).

Although the use of 16S amplicon sequencing and qPCR are common practices for analysing blood bacterial DNA in microbiology-related fields, they are still somewhat new concepts within exercise science. For this reason, there is currently limited research using these methods, although a few studies do exist. At present, one study has investigated the effects of exercise on bacterial load in blood using 16S rRNA gene sequencing (Henningsen et al., 2024). This study found that an exercise bout of running for 2 h at 60% $\dot{V}O_{2max}$ in 35°C elicited significant increases in microbial DNA from pre- to post-exercise. Interestingly, the most abundant bacterial groups detected were phyla-Proteobacteria (88%), family-Burkholderiaceae (59.1%), and genus- *Curvibacter* (58.6%).

Whilst the results were promising, the study would have benefited from a more comprehensive consideration of potential contaminants, as *Chloroplast* and *Herbaspirillum* DNA were both present in the sequencing results, which are very rare in humans (X. Li et al., 2022). It's not impossible that these were present in the lumen pre-translocation due to diet, but they are also known contaminants in 16S rRNA sequencing. Thus, the authors should have located and removed any off-target sequences. This could also be a result of database contamination rather than sample contamination – it's possible that contaminated or erroneous sequences in reference databases may lead to incorrect taxonomic classification, further highlighting the requirement for eliminating off-target sequences. The study also assessed 16S rRNA in plasma, which poses its own limitations. There is strong evidence to suggest that the majority of bacterial DNA in blood is in the buffy coat, specifically within the white blood cells due to phagocytosis (Païssé et al., 2016). This is particularly important when considering diagnostic challenges with 16S sequencing, where sequencing accuracy is largely affected by the abundance of bacteria in the sample. In low biomass samples like blood, this limitation can reduce both accuracy and sensitivity of the analysis, highlighting the importance of persevering as much bacteria in the sample as possible.

There are several mechanisms underpinning the phenomenon of exercise-induced increases in gut permeability, the first of which is elevations in core temperature. Heat stress (hyperthermia) impairs gut integrity through direct thermal injury to epithelial cells and denaturation of tight junction proteins, leading to structural alterations and reduced barrier function (Dokladny et al., 2016; Ghulam Mohyuddin et al., 2022; Walter et al., 2021b). Given that exercise has consistently shown to increase core temperature (Gleeson, 1998), and even more so in heated conditions (Périard et al., 2021), it is logical to suggest that exercise may cause increases in GI damage and permeability through core temperature-related mechanisms. Supporting this, it has been shown that heating colon cells from 37°C to 39°C significantly reduces transepithelial resistance, highlighting the potential for heat to impair gut barrier function (Marchbank et al., 2011). Further demonstrating the influence of temperature-related mechanisms on gut barrier function, it was shown that passively heating rats to 41.5°C drastically increased (34-fold relative to ambient temperature) the translocation of ^{51}Cr-EDTA from the gut into circulation (Prosser et al., 2004).

It has also been speculated that exercise-induced heat stress may induce epithelial cell apoptosis, creating weaker points in the barrier. This stems from animal research, which has showed that overexpression of heat shock protein 70 (HSP70) activated cell adhesion and cell apoptosis signalling pathways (Yong et al., 2022). Given that heat shock proteins are activated by acute exercise (Henstridge et al., 2016), it is plausible that prolonged or repeated exercise could intensify these signalling cascades.

During exercise, and especially as exercise duration/intensity increases, the sympathetic nervous system is activated and prioritises blood flow away from the gut to the heart, lungs and skeletal muscles. The splanchnic hypoperfusion results in intestinal ischaemia, which can damage components of the gut, such as epithelial cells and tight junctions via hypoxia (Ulluwishewa et al., 2024). Impaired clearance of metabolic byproducts under hypoxic conditions may further intensify epithelial injury (van Wijck et al., 2012). Following exercise, the gut must be reperfused, which introduces oxidative and inflammatory stress, inflicting further epithelial damage (Grootjans et al., 2010; G. Li et al., 2022).

Mechanical stress is another contributing factor, particularly in high-impact sports such as running. Repetitive mechanical forces used in these sports may cause direct microtrauma to the intestinal lining, thus weakening the barrier (Costa et al., 2017). Further, the gastric jostling and mechanical stress imposed by high-impact sports may facilitate epithelial injury, and possibly the disruption of tight junctions (Chantler et al., 2022). Disruption of the tight junction arrangements may create paracellular gaps in the epithelial wall, facilitating the translocation of luminal contents into the bloodstream (de Oliveira et al., 2014; Zuhl et al., 2014b).

It has been suggested that the translocation of harmful luminal contents into circulation during periods of exercise-induced increased gut permeability may be one of the reasons underpinning elevations in GI symptoms incidence following exercise (Chantler et al., 2022; de Oliveira et al., 2014). However, GI symptoms cannot be explained by permeability alone. Other factors including nutrition, hydration and psychological stress are also likely implicated in determining the incidence and severity of GI symptoms (Costa et al., 2022a). Thus, GI symptoms arise from a complex interplay involving various physiological, psychological and environmental factors, with gut damage and permeability having significant but not exclusive effects.

1.2 Upper respiratory tract infections

Upper respiratory tract infections (URTI), defined as an illness caused by an infection (typically viral or bacterial) of the middle ear, nose, sinuses, pharynx, larynx or large airways, are the most common infection in a 'healthy' population, with adults typically experiencing two to five episodes per year, and children experiencing seven to ten, most commonly during winter (Eccles, 2005). The most common symptoms associated with URTI episodes are coughing, sore throats, headaches and nasal obstructions, and whilst most URTIs are mild by nature and short-lived, they may still pose a burden to society (Jin et al., 2021; Wang et al., 2021). In some extreme cases, URTIs have been seen to trigger further complications such as pneumonia, myocarditis, otitis media, and glomerulonephritis, therefore increasing the burden posed to society, and athletes especially (Bertino, 2002; Fung et al., 2016; Satoskar et al., 2020).

When pathology testing has taken place to identify whether upper respiratory symptoms (URS) in athletes were the result of an infection, it has been identified that bacterial infections account for roughly 5% of all episodes (Cox et al., 2008; Gleeson et al., 1995; Reid et al., 2004; Spence et al., 2007). Therefore, the most common cause of URS from an identified infection are of viral origin, which has shown to account for over 70% of URS episodes in recent studies (Davison et al., 2025b; Hanstock et al., 2016; Valtonen et al., 2021). Though, it is also possible that URS may be from non-infectious origin, resulting from allergic or environmental causes, underscoring the importance of laboratory identification of pathogens within clinical research.

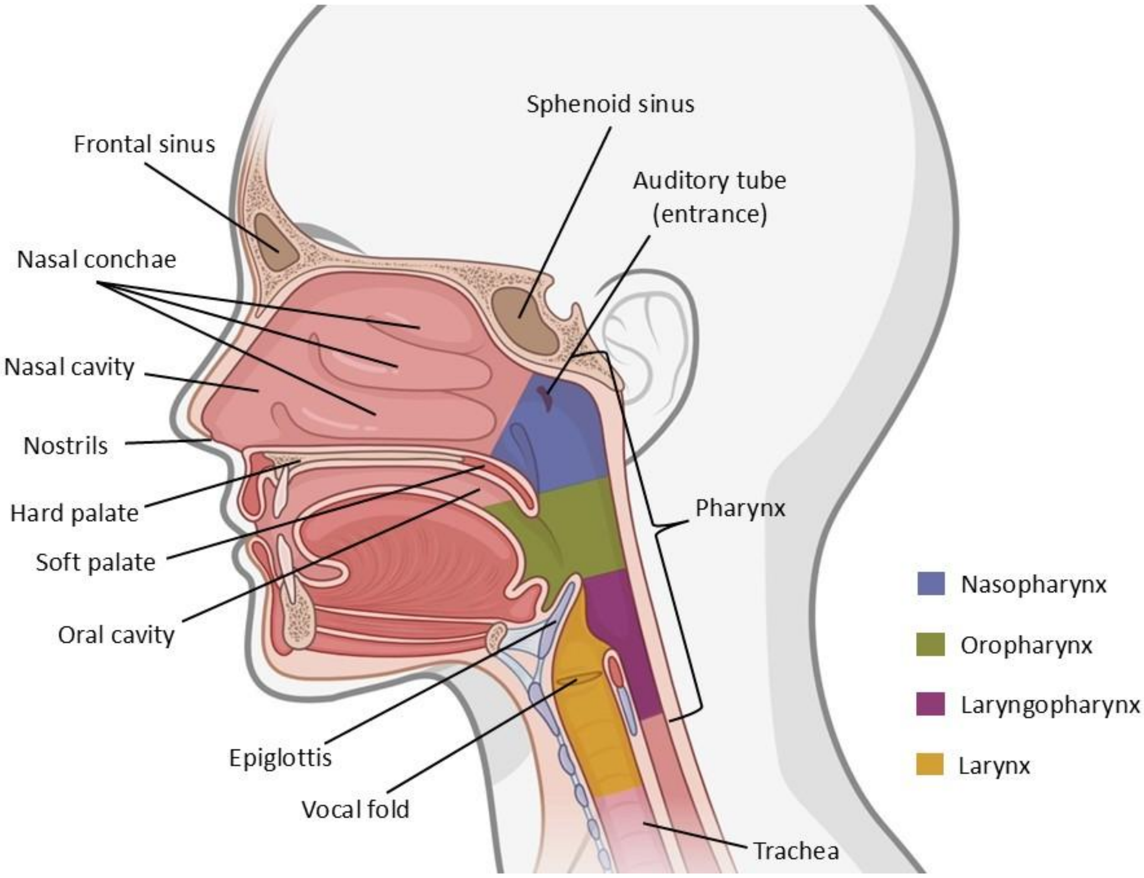

Figure 1.3. Anatomy of the upper respiratory tract. Figure created using *Biorender*.

There are more than 200 serologically different types of viruses responsible for URTIs, with rhinoviruses being the most common source, causing up to 80% of all respiratory infections during peak seasons (Eccles, 2005; Heikkinen and Järvinen, 2003). Other viral agents responsible for URTIs include adenovirus, coronavirus, enterovirus, parainfluenza virus, and respiratory syncytial virus (Thomas and Bomar, 2022). Infections of viral origin can infect the mucosa of the middle ear, paranasal sinuses, nose, pharynx, larynx, and the large airways, and are thought to be transmitted by any of the three major mechanisms: direct contact with infected secretions, inhalation of small-particle aerosols lingering in the air, or inhalation of large-particle aerosols received directly from an infected person (Chonmaitree et al., 2008; Heikkinen and Järvinen, 2003; Marom et al., 2014). However, although the aforementioned mechanisms are typically involved in the transmission of all respiratory viruses, the predominant modes of transmission seem to differ between viruses. The transmission of rhinoviruses via inhalation is well documented, though transmission via direct contact with infected secretions is thought to be the most effective method of contracting the viruses, whereas influenza viruses are primarily spread by inhalation of small particles lingering in the air (Dick et al., 1987; Gwaltney et al., 1978; Nicholson et al., 1998).

Viruses may not only have differing modes of transmission, but the pathogenesis of viral agents may vary too. For example, the replication of rhinoviruses is seen to start primarily in the nasopharynx, as opposed to influenza viruses which begin replication in the tracheobronchial epithelium (Nicholson et al., 1998; Winther et al., 1986). A common entry point for viruses may be either the anterior nasal mucosa or the eye, into which viral agents may be deposited via the aforementioned mechanisms of transmission. Once the viruses have passed through the lacrimal duct into the posterior nasopharynx, they will enter the epithelial cells and begin replicating quickly (Heikkinen and Järvinen, 2003). Viruses also differ in their impact on the epithelium – rhinoviruses have shown to cause direct damage to epithelial cells and reduce cell integrity using in vitro models such as high content screening, immunofluorescence analyses of pseudostratified epithelia and transepithelial electrical resistance (Davison et al., 2025b). Though, other viruses such as influenza and SARS-CoV-2 have shown considerably more damage to epithelial tissues in the same models (Posch et al., 2021; Zaderer et al., 2022). Variation in damage to the respiratory epithelium may also have implications in the development of URTI symptoms. Whilst some symptoms of URTIs arise from the damage to the epithelium and subsequent inflammation, others may be driven by the host's systemic inflammatory response to infection (Blaas and Fuchs, 2016; Warner et al., 2019). Following pathogen entry and replication in epithelial cells, signalling pathways are triggered, releasing an array of cytokines, chemokines, peptides and growth factors (Kennedy et al., 2012). This results in immune cell activation and translocation to the submucosa, leading to amplification of inflammatory processes and typical symptoms of URTIs (Blaas and Fuchs, 2016).

Due to the low incidence rate (~5% of URS episodes), there has been notably less research looking at URTIs of bacterial origin when compared to those of viral origin. However, bacterial URTIs account for a large proportion of antibiotic prescriptions, and chronic/recurrent infections may develop resistance to treatments (Butt et al. 2025; Dolk et al. 2018; Bartlett, 1997). Therefore, understanding the aetiology and processes of bacterial infections is crucial to developing effective methods for URTI management.

Given the low incidence rate for bacteria to be the primary cause of URTI, is it more common for them to be co-infections. In many cases, bacterial infection follows an initial viral infection, with the compromised immune defence (e.g. damage to respiratory epithelium and impaired mucociliary clearance) creating a favourable environment for opportunistic bacterial infections (Bakaletz, 2017). The most common bacterial pathogens responsible for URTIs are *Streptococcus* species*, Haemophilus influenzae and Moraxella catarrhalis* (Block, 1997; Gwaltney et al., 1978). Bacterial infections localised to the upper respiratory tract (URT) can display distinct symptoms. For example, *Streptococcus Pyogenes* can cause acute streptococcal pharyngitis, commonly termed 'strep throat' – typically characterised by pharyngeal inflammation, pain swallowing and swelling of the lymph nodes (Hamilton and McCrea, 2024). Alternatively, bacterial URTIs can also lead to other illnesses, such as rhinosinusitis and otitis media (Danishyar and Ashurst, 2025; Matera et al., 2022). Beyond the localised immune responses to bacterial URTIs, the activation of immune cells may initiate the release of proinflammatory cytokines and induce a systemic immune response. Release of such mediators into circulation can induce fever, leading to general and systemic illness symptoms such as malaise, myalgia and aching (Blomqvist and Engblom, 2018).

1.2.1 Moderate exercise and upper respiratory tract infections

The effects of moderate exercise on URTI incidence are well researched, with numerous randomised controlled trials and epidemiologic studies having been conducted. It is the general consensus that regular moderate exercise training is associated with reduced rates of URTIs and/or laboratory confirmed URI/URTI episodes, regardless of confounders such as age, education level, marital status, gender, body mass index (BMI) and mental stress (Barrett et al., 2018, 2012; Chubak et al., 2006; Fondell et al., 2011; Matthews et al., 2002; D. Nieman et al., 1990; Nieman et al., 2011, 1998, 1993; Zhou et al., 2018). This has also been supported in animal models, as moderate exercise performed before or immediately after inoculation with pathogens has been shown to reduce infection-related mortality and morbidity compared to inactivity (Davis et al., 1997; Lowder et al., 2005).

It is likely that enhanced immune function following moderate exercise occurs due to numerous mechanisms all working in unison. Several immune cells and proteins including NK cells, neutrophils, B and T cells, anti-inflammatory cytokines and immunoglobulins all experience increased recirculation following moderate exercise (Adams et al., 2011; Bigley et al., 2014; LaVoy et al., 2015; Simpson et al., 2017, 2015). Furthermore, hormones such as cortisol and proinflammatory cytokines may not reach the concentrations required to suppress the immune system following shorter exercise bouts at moderate intensities (Nieman et al., 2005).

1.2.2 Prolonged/strenuous exercise and upper respiratory tract infections

Whilst regular physical activity generally supports immune function, bouts of strenuous and prolonged exercise can, in some cases, lead to a transient depression of immune functions. This temporary dip may increase susceptibility to infections, particularly URTIs (Walsh et al., 2011). The population most likely to suffer from heavy exercise-induced immune depression is elite and high-performance athletes, due to their highly demanding training regimens and competition schedules (Schwellnus et al., 2022). For most people, URTIs such as the ‘common cold’ are usually mild and short-lived, however, these illness episodes can prove catastrophic for competitive athletes; affecting performance directly if suffered during/around competition, or indirectly if training is disrupted. Due to the importance of this topic, research into the increased risk of illness amongst athletes has been of high interest since the 1980s (Peters and Bateman, 1983).

This seminal study questioned 141 randomly selected runners from the 1982 Two Oceans Marathon in Cape Town. Subjects’ URTI symptoms were recorded on the day pre-race and 2-weeks post-race. Compared to 124 individually matched controls who didn’t run, 33.3% of runners experienced URTI symptoms, as opposed to only 15.3% of the control group. Interestingly, URTI symptom frequency was inversely related to marathon completion time, with prevalence reaching 47% among runners finishing in under 4 hours. These findings prompted the idea that there could be a relationship between exercise workload and risk of URTIs. Though, this interpretation assumes that faster finishing times reflected higher exercise workload during the race, and that faster runners would have also had higher training loads in the build-up to the race. As these variables were not assessed, the relationship between exercise workload and URTI risk was inferred rather than directly measured.

Over a decade later, Nieman (1994) hypothesised that a J-shaped relationship exists between exercise workload and risk of URTIs, which is still the most widely accepted theory in this field. This theory suggests that when compared to a sedentary lifestyle, moderate exercise reduces illness susceptibility, but that acute bouts of strenuous/prolonged exercise, or chronic heavy training loads may increase the risk of infection to a level above average. Over the years, revisions to this model have been proposed, including a revised J-shape model, suggesting that moderate exercise may only maintain immune function, as opposed to improving it from sedentary levels (Jeukendrup and Gleeson, 2018).

It has also been suggested that an S-shaped relationship may exist, implying that having an immune system able to tolerate and withstand the increased risk of infections associated with high level sport is a prerequisite for athletes to reach elite level (Malm, 2006). However, the S-shaped model is not as widely accepted as the J-shaped model, as elite athletes still suffer from far greater infection rates than sedentary and moderately active individuals (Engebretsen et al., 2013, 2010). In addition to this, those competing in elite level sport will usually have access to more support systems (e.g. medical, financial and nutritional) than most athletes, allowing them to implement preventative and treatment strategies for managing and avoiding illness. Therefore, it is more likely that reductions in illness incidence (and risk thereof) between elite athletes and non-supported counterparts are due (at least in part) to the support systems in place to mediate these risks, as opposed to a genetically stronger immune system (Jones and Davison, 2018). See for Figure 1.4 for stylised representations of these models.

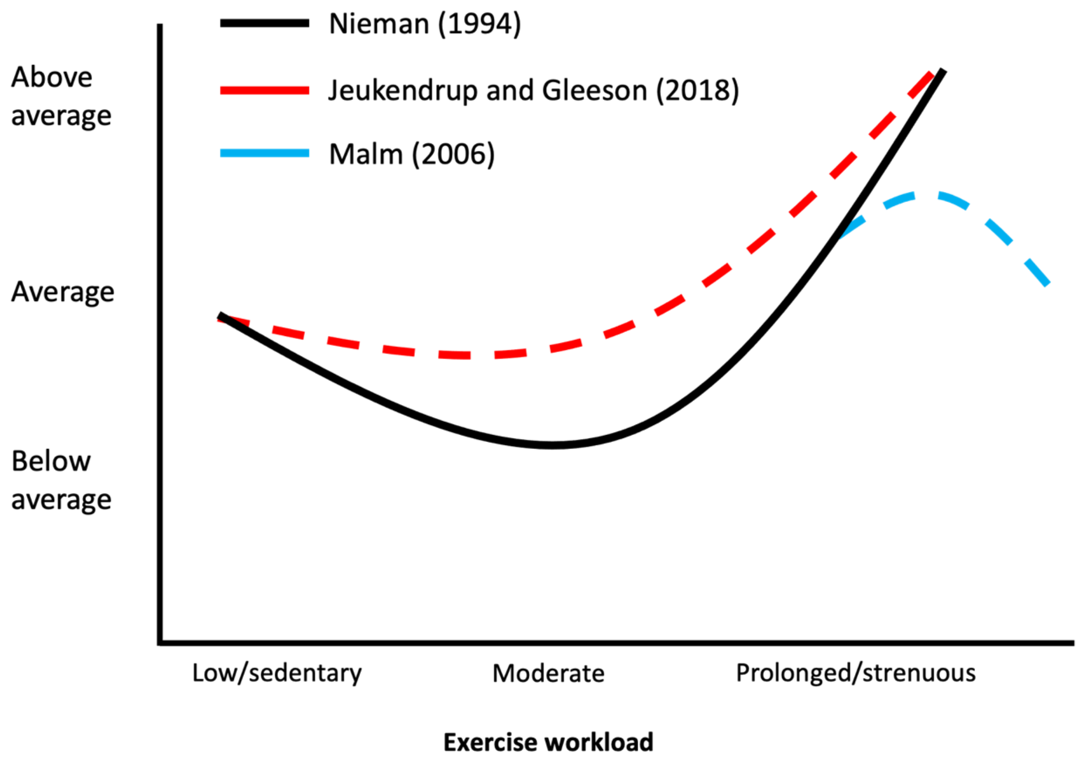

Figure 1.4. Proposed J-shaped curve models. Workload can refer to acute volume or intensity, or chronic exercise load.

Alongside early epidemiologic research (Peters and Bateman 1983; Heath et al. 1991; Nieman et al. 1990), there is a compelling body of evidence to support the J-shaped curve model, highlighting the association between prolonged, intensive exercise/heavy training workloads and increased illness susceptibility (Jones and Davison, 2018; Nieman and Wentz, 2019). However, the relationship between exercise and risk of URTIs is likely underpinned by numerous mechanisms, including various host immune functions, but also importantly, the extent of pathogen exposure. The contributions of such factors to the risk of URTIs has been an area of ongoing discussion, including the question of whether elevated URTI risk is influenced more by exercise-induced immune perturbations or greater exposures to pathogens (Campbell and Turner, 2018; Simpson et al., 2020). The likelihood is that exercise is a significant risk factor for susceptibility to URTIs, though, pathogen exposure will always be required to ‘catch’ the illness. Pathogen exposure remains the most difficult factor to standardise in exercise immunology, possibly explaining the lack of relationship seen in some cases, and the overwhelming relationship seen in others.

Another important consideration in this field is the confirmation of whether URS are of infectious or non-infectious origin. This is a key limitation for many studies in this area, as previously mentioned in this review, a large portion of URS come from non-infectious origins such as allergies and inflammation. This is further supported by Cox et al. (2008), who found that upon presentations of URS at a sports medicine clinic, physicians characterised 89% of cases as viral or bacterial URTIs, whilst after laboratory testing for pathogen identification or other parameters indicating infections (e.g. elevated total leukocyte and/or neutrophil count, lower vitamin D concentration), only 57% of cases tested positive for infections. Interestingly, recent studies have observed better success rates in identifying pathogens. For example, Davison et al. (2025) had a 100% detection rate in a total of 82 samples, alongside other recent studies which have found positive pathogen detection rates of 82% (Hanstock et al., 2016) and 75% (Valtonen et al., 2021). The greater success rates in recent studies could be a result of improved detection methods, or other confounders such as time of year, location and training level. Nonetheless, these studies highlight the importance of using laboratory screening methods to distinguish between URI/URS (unconfirmed) and URTIs (confirmed), increasing the reliability and validity of reported URTI episodes.

1.3 Exercise and the immune system

1.3.1 The immune system

Through contact, ingestion and inhalation, the human body undergoes daily exposure to pathogens (e.g. viruses, bacteria, parasites) with the potential to infect the host. The immune system (derived from the Latin *immunis* – meaning free from), which comprises a network of organs, tissues, cells, proteins and molecules, has evolved to protect against these potentially harmful microbes (Delves et al., 2011). White blood cells (leukocytes) are arguably the foundation of the immune system. Originating and maturing in bone marrow, with the exception of T lymphocytes, which mature in the thymus, these cells are tasked with defending the host from infections and other harmful agents, and are comprised of granulocytes (neutrophils, eosinophils and basophils; 60-70%), monocytes (5-15%), dendritic cells (<1%) and lymphocytes (15-25%), the latter of which can be separated into innate (Natural Killer [NK] cells) and adaptive (T and B cells) cells (Jones and Davison, 2018).

A notable strength of the immune system is that it possesses the ability to establish a memory of pathogens after the first exposure, meaning that upon subsequent encounters, a more specific and effective immune response will take place to eliminate the pathogen quickly, this is known as adaptive immunity. However, the adaptive immune system is slow to respond to new pathogens and relies on the immediate responses of the innate immune system (Delves et al., 2011). Although the innate immune system does not target specific pathogens, it plays a crucial role in the first line of defence against infectious agents. Upon encountering a new pathogen, it recognises potentially harmful agents through pathogen recognition pathways, initiating an immediate response and activating adaptive immune responses during subsequent exposures (Alberts et al., 2002). Although both innate and adaptive immune systems are highly complex and provide the host with valuable defence against infectious agents, each has limitations. It is the synergistic interplay between these systems that creates such an effective resistance.

1.3.2 Innate immunity

The innate immune system is the host's first line of resistance against potentially harmful and infectious pathogens. This arm of the immune system provides immediate but nonspecific defence against infectious agents, meaning that although the response is fast, it responds to all infections in a similar manner (McDonald and Levy, 2013). The innate immune system consists of an array of defences, including physical and anatomical barriers, as well as direct cellular and inflammatory responses (Marshall et al., 2018).

1.3.2.1 Physical and anatomical barriers

Before gaining entry to the host, any infectious agents must first pass the physical and anatomical barriers. The most obvious physical barrier is the skin, covering the outer surfaces of the body, but other physical barriers include the inner surfaces of the respiratory, gastrointestinal (GI) and reproductive tracts, which are lined with mucous secretions (Delves et al., 2011). The skin usually provides a strong barrier against pathogens, as most cannot endure the low pH (acidic) conditions generated by lactic acid and other fatty acids in sweat and sebaceous secretions. However, in events when the integrity of this barrier is compromised, such as lacerations or burns, the risk of infection is far greater (Delves et al., 2011). The epithelial cells that form the linings of the respiratory, gastrointestinal (GI) and reproductive tracts also provide a barrier to infections, due to their capacity to produce mucus, AMPs, metabolites and cytokines (Belkaid and Artis, 2013). Another mechanism to remove infectious agents from the body is used when these microbes are trapped or suspended in the adhesive mucosal secretions or bodily fluids such as tears, saliva, or urine. Once trapped within adhesive mucus, these particles can be removed via mechanisms such as mucociliary clearance, which uses ciliary movement to transport the mucus and pathogens away. Alternatively, the washing action of tears, saliva and urine can also transport suspended pathogens away from the body (Delves et al., 2011). However, in the event that an infectious agent is able to penetrate the physical and anatomical barriers, it will enter systemic circulation and trigger a cascade of immune responses.

1.3.2.2 Cellular defences

Once physical and chemical barriers have been breached, cellular defence mechanisms such as phagocytosis are activated. However, the first step in combating an infectious agent in systemic circulation is to distinguish between ‘non-infectious self’ and ‘infectious nonself’ components, or in other words, substances normally present in the body and infectious foreign agents (Janeway, 1992). The discrimination of these substances is performed by pattern recognition receptors (PRRs), which are primarily found on the plasma membrane of macrophages but also exist in other subcellular compartments such as endosomes and other immune cells such as granulocytes and dendritic cells (Jang et al., 2015). PRRs can be divided into five separate ‘families’: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain- (NOD-) like receptors (NLRs), retinoic acid-inducible gene- (RIG-) like receptors (RLRs) and AIM2-like receptors (ALRs) (Jang et al., 2015). Ultimately, PRRs are a network of proteins and ligands that are able to detect and respond to components of pathogens that are foreign to the host and needed for pathogen survival, known as pathogen-associated molecular patterns (PAMPs) (McDonald and Levy, 2013). If a PAMP is detected by PRRs, a cascade of proinflammatory and antimicrobial responses will ensue. This includes the activation of macrophages (enhanced phagocytosis) and the secretion of cytokines and chemokines, which can increase endothelial cell permeability in blood capillaries. This allows plasma proteins (some of which with antimicrobial properties) to enter the point of infection, whereas they would usually be limited to the blood. The increased permeability of blood capillaries will also allow neutrophils to enter the infected site, which similarly to macrophages, will begin to destroy any microbes they encounter via mechanisms such as phagocytosis and subsequent elimination mechanisms (Delves et al., 2011; Niedergang, 2016).

If a PRR engages with a PAMP, phagocytic receptors will begin a signalling cascade to defend against the pathogen. Phagocytosis, a key part of the innate immune response and refers to the process of when certain immune cells (phagocytes) engulf and subsequently destroy infectious agents (Rosales and Uribe-Querol, 2017). Through the remodelling of lipids in the cell membrane and manipulation of the actin cytoskeleton, the cell membrane will extend and connect around the particle. The result is that the microbe is now imprisoned within the macrophage/neutrophil (Delves et al., 2011). However, there are several mechanisms used for destroying the microbe once it is confined (Freeman and Grinstein, 2014; Rosales and Uribe-Querol, 2017).

One such method is known as oxidative burst (or respiratory burst), which refers to the release of reactive oxygen species (ROS) from leukocytes, in an attempt to damage and neutralise the detected pathogen. Whilst there are other pathways of ROS production in the body, these alternative sources of ROS fall beyond the scope of this immunological literature review, instead, this review will focus on the production of ROS by leukocytes such as neutrophils and monocytes (or macrophages), as a key function of the innate host defence system.

Upon the initiation of phagocytosis, hexose monophosphate shunt (HMS) activity is significantly increased, meaning that nicotinamide adenine dinucleotide phosphate (NADPH), a product of the HMP, also experiences an increase in production. Then, primarily catalysed by the NADPH oxidase enzyme, electrons are passed from NADPH to molecular oxygen, resulting in the creation of a superoxide anion (O_2^-), the first ROS in this process. Following this, superoxide anions are then converted into hydrogen peroxide (H_2O_2) via dismutation catalysed by superoxide dismutase (SOD), an enzyme present in all living cells (Delves et al., 2011; Fisher and Zhang, 2006; Sakamoto and Imai, 2017). An excess of H_2O_2 has shown to degrade methaemoglobin, releasing iron ions that then react with the H_2O_2 to produce hydroxyl radicals ($\cdot OH$), as seen in the Fenton reaction ($Fe^{2+} + H_2O_2$) (Fenton, 1894; Puppo and Halliwell, 1988). Lastly, within neutrophils, myeloperoxidase (MPO) can react with H_2O_2 and halide/pseudohalide ions (such as chloride, Cl^-) to produce hypohalous acids such as hypochlorous acid (HOCl) and hypobromous acid (HOBr) (Ulfig and Leichert, 2021). The products of the reactions above are known as ROS, which can be defined as highly reactive unstable forms of molecular oxygen, that easily react with other molecules (Hayat, 2015). It is the high reactivity that makes ROS such effective antimicrobial agents.

The primary mechanism used by ROS to damage their targets is through oxidation, which refers to the loss of electrons from a molecule. ROS are highly reactive molecules that act as oxidising agents, stripping electrons from other molecules indiscriminately. However, it is worth noting that not all ROS are free radicals, such as H_2O_2, which is still highly reactive and leads to the downstream production of free radicals (Delves et al., 2011). This oxidation can cause significant damage to DNA and proteins, by altering structures and functions, as well as damaging membrane lipids via peroxidation, thus disrupting membrane integrity (Juan et al., 2021; Milkovic et al., 2019). The molecular damage caused by ROS makes them formidable antimicrobial agents, disrupting or even eliminating pathogens. However, the indiscriminate nature of ROS also poses a danger to host DNA, proteins and lipids. It is the role of antioxidants within the body to combat ROS, preventing imbalances and limiting oxidative damage. Although, when there are not enough antioxidants, imbalances can occur, commonly known as oxidative stress, which is linked to numerous diseases and health conditions (Ashrafizadeh et al., 2020; Rotariu et al., 2022).

Alternatively, to the mechanism of ROS, phagocytosed microbes can also be combated by secreted granule proteins. Once a phagosome has formed surrounding the phagocytosed pathogen, neutrophil granules will fuse with the phagosome and release granule proteins into the vacuole to attack the pathogen, a process known as intracellular neutrophil degranulation (Gierlikowska et al., 2021; Othman et al., 2022). There are three types of granule proteins, each released at different time points. Initially, tertiary granules containing enzymes such as cathepsin and gelatinase are released. Next, secondary granules containing lactoferrin, lysozyme and collagenase are released. Lastly, primary granules with the greatest antimicrobial and proinflammatory activity are released, including the release of elastase, myeloperoxidase (MPO) and defensins (HNP-1, HNP-2 and HNP-3 peptides) (Faurschou and Borregaard, 2003; Othman et al., 2022).

The predominant leukocyte implicated in the production of ROS are neutrophils. Neutrophils are polymorphonuclear (PMN) leukocytes, meaning they are a type of leukocyte that possess multilobed nuclei, and in this case, three lobes (Ma et al. 2023). Derived from bone marrow, neutrophils are also known as granulocytes, which refers to PMN leukocytes that possess granules within their cytoplasm, containing a range of antimicrobial proteins, peptides, enzymes and other molecules that help to fight infection and mediate inflammation (Scher et al. 2013). Neutrophils express a wide range of PRRs, such as TLRs and NLRs, that are activated by PAMPs and damage-associated molecular patterns (DAMPs). This recognition step initiates several neutrophil functions, such as phagocytosis, degranulation and the release of neutrophil extracellular traps (NETs) (Jones et al. 2018).

NETs are a an antimicrobial mechanism of neutrophils, whereby following activation of PRRs, a cascade of intracellular responses ensue, resulting in a web-like structure containing numerous potent antimicrobial agents released into the extracellular space (Rada, 2019). NETs consist of extracellular strings of DNA that bind to pathogens, and contain various agents such as histones and granule proteins that damage entrapped microbes. The major type of NET formation is known as suicidal NETosis and is triggered by excess ROS from the NADPH oxidase cascade (Azzouz et al., 2021). Suicidal NETosis is characterised by a series of morphological changes: chromatin decondensation via enzymes such as neutrophil elastase and MPO entering the nucleus, breakdown of the nuclear envelope and rupture of the plasma membrane, releasing NETs into extracellular space (Rada, 2019). However, given the name and rupturing of cell components, suicidal NETosis results in the death of the neutrophil. Alternatively, vital NETosis is the other type of NET formation, by which NETs are released via vesicular transport rather than lysis, keeping the neutrophil alive and functional (Yousefi et al., 2009).

Phagocytosis is a fundamental action of many innate immune cells, such as neutrophils, macrophages and dendritic cells, and involves engulfing microbes, debris, and even dying cells, then subsequently eliminating them (Fond and Ravichandran, 2016). Initially, PRRs bind to PAMPs, DAMPs or opsonised particles on target microbes. Following this, pseudopods (fake arms) are wrapped around the microbe, engulfing it, before compartmentalising it in an intracellular vesicle known as a phagosome. The phagosome then fuses with a lysosome creating a phagolysosome, containing various agents such as hydrolytic enzymes, ROS and RNS, that kill and digest the microbe (Richards and Endres, 2014).

Macrophages are specialised leukocytes that play a role in the detection and phagocytosis of pathogens and other targets, as well as other secondary mechanisms such as the coordination of inflammation and antigen presentation to T cells (Ross et al., 2021). Like all immune cells, macrophages originate from hematopoietic stem cells (HSCs) in the bone marrow. Though, where cells such as lymphocytes and NK cells develop via a lymphatic precursor cell, macrophages and other cells such as neutrophils, eosinophils and basophils all develop via a myeloid progenitor cell. In the case of macrophages, the myeloid progenitor cell develops into monoblasts, which later mature into monocytes. Monocytes are a leukocyte that that circulate in blood and act as precursors to macrophages – once monocytes leave the bloodstream and enter tissue, they differentiate into macrophages (Ross et al., 2021).

Depending on the microenvironment surrounding naïve, or 'resting' macrophages, upon activation, they will differentiate into either 'classically activated' M1 or 'alternatively activated' M2 macrophages through a process known as polarisation. Typically activated by proinflammatory stimuli such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and lipopolysaccharide (LPS), M1 macrophages are associated with increased phagocytic activity and antigen-presenting capacity (Yunna et al. 2020). Moreover, M1 macrophages are strong cytotoxic agents due to their increased synthesis of proinflammatory cytokines, adhesion molecules, chemokines and cyclooxygenase-2, as well as increased production of O_2^- through activation of the NADPH oxidase system (Ross et al., 2021). In contrast, M2 macrophages play a more supportive role in innate immunity. Activated by mediators such as interleukin-4 (IL-4), IL-10 and IL-13, M2 macrophages are more involved in anti-inflammatory and repair functions, such as the promotion of angiogenesis, increased tissue-repair molecules such as arginase-1, and stromal activation and remodelling (Rath et al., 2014; Ross et al., 2021).

Dendritic cells are important cells for connecting the innate and adaptive arms of the immune system. They are specialised leukocytes that act as antigen-presenting cells (APCs), by detecting pathogens, processing the antigens and presenting them to T cells (Liu, 2016). Similarly to macrophages, dendritic cells possess an array of PRRs and migrate to tissues where they patrol and sample the environment via phagocytosis. However, upon detection of a pathogen, where a macrophage would attack the microbe, dendritic cells flee to the nearest lymph node to begin antigen presentation. Briefly, APCs such as dendritic cells will internalise and break down protein antigens into smaller peptide fractions, before presenting the antigen to T cells in the lymph node through a membrane complex known as the major histocompatibility complex (MHC). T cells can only ‘see’ an antigen when presented within the cleft of an MHC molecule, so the MHC essentially serves as a serving platform (Delves et al., 2011). Whilst dendritic cells are the predominant and most efficient APC, other cells such as macrophages and B cells can also provide similar functions.

NK cells are a subset of lymphocytes and are a vital part of the innate immune response in combating infected and mutated cells. NK cells can be activated by two types of receptors: activating receptors, which activate NK cells upon identifying stress-induced molecules and ligands released from infected and mutated cells (Sivori et al., 2019). Alternatively, inhibitory receptors detect MHC class I molecules, which are usually released by healthy cells to prevent attacks. However, MHC class I expression is reduced when a cell becomes infected or mutated, which reduces the inhibitory signals that would usually prevent NK cells from attacking, thus triggering the NK cell response (Elliott and Yokoyama, 2011).

Following activation, NK cells exhibit various mechanisms used to combat infected and mutated cells. Firstly, NK cells release ligands such as Fas (FasL) or Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), which bind to their respective receptors on the surface of cells. Once bound, these ligands induce cell apoptosis via the activation of apoptosis signalling pathways (Caligiuri, 2008; Lanier, 2008). Fc receptors on NK cells bind to the Fc region on antibodies. If these antibodies are bound to the surface of infected cells, cytotoxic granules will be released (Aguilar et al., 2023; Wang, 2015). The cytotoxic granules contain perforin, a glycoprotein able to polymerise and form pores in cell membranes (Osińska et al., 2014). These pores then allow granzymes to enter the cell, which trigger cascades of proteolytic enzymes and induce cell apoptosis (Prager et al., 2019).

Mast cells and basophils are closely related innate leukocytes involved in immune responses to allergic inflammation and parasitic infections. Both are granulocytes that originate from

HSCs, containing histamine, proteases and inflammatory cytokines that are released following activation (Stone et al., 2010). Mast cells and basophils both express the high-affinity immunoglobulin E (IgE) receptor (FcεRI). Briefly, the allergic response happens in two main phases: the sensitisation phase and the activation phase. During sensitisation, allergen exposure leads to activation of T helper 2 (Th2) cells, which stimulate B cells to produce IgE. IgE then binds to FcεRI receptors on mast cells and basophils, which leaves them in a 'primed' or sensitised state (Stone et al., 2010). Upon re-exposure, the allergen binds to two or more IgE antibodies bound to FcεRI receptors on the mast cells and basophils, known as cross-linking, which triggers a rapid intracellular signalling cascade to begin the allergic response (Delves et al., 2011). Upon activation, the granules fuse with the plasma membrane and release histamine, serine proteases and other mediators that can cause a range of hypersensitivity reactions. These immediate reactions are determined by the pre-formed mediators and rapidly synthesised lipid mediators and can include erythema, oedema, itching, sneezing and rhinorrhoea in the upper respiratory tract and coughing to name a few, or in more serious cases, anaphylaxis and allergic asthma (Stone et al., 2010). Late phase reactions can also occur 6-24 hours after the immediate reaction. Typically mediated by cytokines and chemokines, late phase reactions are mostly characterised by oedema and influx of leukocytes but have also been suggested to affect chronic asthma (Galli et al., 2008).

Similarly to neutrophils, eosinophils are also polymorphonuclear leukocytes. They are major effector cells involved in the defence against parasitic infections, such as nematodes, as well as also being implicated as part of the inflammatory process of allergic disease (Delves et al., 2011). Large parasites are too large to be phagocytosed, so eosinophils have evolved over time to eliminate these infections by a different means. Eosinophils are granulocytes, containing eosinophil major basic protein, cationic protein and peroxidase within the granule matrix (Acharya and Ackerman, 2014). Eosinophils also contain histaminase, arylsulfatase B, and phospholipase D. Eosinophils express receptors for the C3b complement protein, and upon activation, are capable of producing a significant oxidative burst response alongside degranulation (Carlson et al., 2011; Lacy et al., 2003). Lastly, these cells also contain an array of granule proteins, such as perforin, allowing eosinophils to inflict pore formation on the membranes of target cells (Delves et al., 2011).

Gamma delta (γδ) T cells are a unique, unconventional subset of T lymphocytes that many believe to be ‘in-between’ the innate and adaptive immune systems, typically making up for 1-5% of total T lymphocytes in peripheral blood (Roden et al., 2008). Whereas most T cells express alpha beta (αβ) T cell receptors (TCRs), these cells express γδ TCR, which can be activated independently from antigen presentation on MHC complex I or II, allowing for a far quicker response than conventional T cells (Verkerk et al., 2024). Like αβ T cells, γδ T cells possess potent cytotoxic mechanisms such as the release of perforins and granzymes, as well as the release of cytokines such as TNF-α and IFN-γ. Since γδ T cells are fully matured upon leaving the thymus, and have a broad and efficient antigen recognition system, they are incredibly cytotoxic agents (Chan et al., 2022).

1.3.2.3 Soluble factors

Cytokines are small proteins secreted by various immune cells (macrophages, NK cells, dendritic cells etc.) in response to encountering infectious agents or following tissue damage. There are three families of cytokines: interleukins (ILs), interferons (IFNs) and tumour necrosis factor (TNF). ILs are a large group of cytokines, with an array of functions including the regulation of inflammation through pro- and anti-inflammatory cytokines. ILs can also boost immune defences by enhancing cell growth, replication and cytotoxicity (Al-Qahtani et al., 2024; Justiz Vaillant and Qurie, 2024). Type I IFNs (IFN-α and IFN-β) are released from infected cells to activate nearby immune cells and inhibit viral replication, whereas Type II IFN (IFN-γ) is typically secreted from NK and Th1 cells, enhancing antigen presentation, cytotoxicity of T lymphocytes and activating cells such as macrophages (Khanna and Gerriets, 2024). Lastly, TNF cytokines are crucial for numerous cellular events such as cell survival, proliferation and differentiation, as well as regulating inflammation (Wang and Lin, 2008).

Chemokines are a group of secreted proteins used in chemotaxis, the directed migration of immune cells to target areas. These proteins are characterised into CC, CXC, C, CX3C chemokines, depending on the pattern of conserved cysteine residues (Zweemer et al., 2014). Chemokines work by attracting leukocytes to areas of infection or inflammation, with each subset possessing chemoattractants for different leukocytes, as well as CX3C chemokines also being involved in the cell-to-cell adhesion of leukocytes to endothelial cells (Hughes and Nibbs, 2018; Kaiser and Balic, 2015; Lopez et al., 2007; Raman et al., 2011; Schey et al., 2015).

The completement system consists of a collection of plasma proteins that react with one another to combat infectious agents. Once activated via proteolytic cleavage, a series of proteolytic cascades work to opsonise and eliminate pathogens, whilst also initiating chemotaxis (Dunkelberger and Song, 2010; Rus et al., 2005). The complement system is activated via three pathways: the classical, lectin and alternative pathways. The classical pathway is activated upon the binding of antibodies (typically IgG and IgM) to antigens, which occurs when complement components 1q (C1q), 1r (C1r) and 1s (C1s) bind to the Fc region of pathogenic surfaces (Dunkelberger and Song, 2010; Janeway et al., 2001). The lectin pathway is activated when germline-encoded PRRs such as mannose-binding lectin (MBL) detect lectin residues on nonself-infectious entities (Medzhitov and Janeway, 2000). The lectin pathway contains MBL-associated serine proteases (MASP)-1, -2 and -3, which are structurally similar to the C1s and C1r components of the classical pathway. Once activated, the classical and lectin pathways work similarly, leading to the cleavage of C2, C3 and C4 (Bally et al., 2009; Dobó et al., 2009;

Gál et al., 2007). Lastly, the alternative pathway is activated by the spontaneous low-level hydrolysis of C3 into C3(H_2O). Then, following binding to factor B and subsequently Factor D, this pathway also results in C3 cleavage. The complement components produced throughout these pathways have several effector functions, influencing opsonisation, inflammation and cell lysis, thus 'complementing' the immune response to infections (Bader et al., 2016; Ricklin et al., 2010).

Other soluble mediators within the innate immune system include AMPs, proteins, and acute-phase proteins (APPs). AMPs are small molecular structures consisting of a short chain of amino acids (typically 10-50 amino acids) that aid in the immune response to pathogens. Often produced by leukocytes and epithelial cells, examples of AMPs include defensins, histatins and cathelicidins (Huan et al., 2020; Q.-Y. Zhang et al., 2021). Due to their cationic and amphipathic characteristics, AMPs are able to interact with and disrupt cellular membranes, leading to cell lysis (Huan et al., 2020). Additionally, certain AMPs are also able to fight pathogens by interacting with intracellular components, such as DNA and RNA (He et al., 2017). APPs refer to proteins that experience a fluctuation in plasma concentrations in response to inflammation. During periods of acute or chronic inflammation, proinflammatory cytokines can trigger the liver to produce these proteins, which exhibit several functions (Ehlting et al., 2021). APPs are important for maintaining homeostasis and promoting tissue repair but also have a role in immune defence. Specific functions include the recruitment of immune cells to sites of infection and promotion of opsonisation, alongside the prevention of oxidative damage, promotion of blood clotting and wound healing (Berry and Gillen, 2014; Güleç et al., 2022; Naryzny and Legina, 2021).

Lysozyme is a crucial protein for the innate immune response, found in predominantly in mucosal secretions (e.g. saliva, tears), but also in milk and colostrum (Gomes et al., 2021). Lysozyme is secreted by various epithelial and immune cells across the body, and exerts its enzymatic activity through hydrolysis, or cleaving, of the β-1,4-glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in the polysaccharide section of peptidoglycans, a major component of Gram-positive bacterial cell walls (Ferraboschi et al., 2021). Due to the weakening, or even destruction of the bacterial cell wall, lysozyme can inflict osmotic lysis, killing the bacterium (Baron et al., 2016). Another antimicrobial protein similar to lysozyme is lactoferrin. Also found in mucosal secretions and neutrophil granules, lactoferrin possesses various antimicrobial functions. Lactoferrin has shown to bind to LPS on the surface of Gram-negative bacteria, disrupting bacterial cell membranes and increasing permeation of other antimicrobial agents. Due to the binding of LPS, lactoferrin has also shown to modulate the release of cytokines and limit excessive inflammation (Drago-Serrano et al., 2012). An alternative function of lactoferrin relates to its ability to bind to free iron. Since iron is a significant factor in the growth and virulence of bacteria, the sequestering of iron by lactoferrin may ‘starve’ the bacteria, limiting development (Posey and Gherardini, 2000).

Although IgA is typically considered part of the adaptive immune system, there is evidence to suggest that it also has innate mechanisms. IgA possesses a heavy chain and secretory component, both of which are heavy glycosylated. The oligosaccharide side chains found on secretory IgA (sIgA) closely resemble those found on the intestinal epithelial surface, it is thought that sIgA, whether free or bound, can act as inhibitors or ‘decoys’ for pathogens binding to host cells in the intestinal epithelium (Mantis et al., 2011). In fact, numerous studies have found that secretory IgA carries oligosaccharide receptors capable of binding to various intestinal pathogens, such as *Escherichia coli*, *Clostridium difficile*, and *Streptococcus pneumoniae* (Dallas and Rolfe, 1998; Lu et al., 2003; Wold et al., 1990).

1.3.3 Adaptive immunity

Unlike the innate immune system, the adaptive immune system does not provide an immediate defence to unrecognised, or ‘new’ pathogens. Instead, the adaptive immune system develops a memory of pathogen antigens encountered and initiates a significantly faster and effective response upon subsequent encounters (Chaplin, 2010). The main components of the adaptive immune system are B and T lymphocytes, which are produced in bone marrow before migrating to other areas in the body (Gartner and Hiatt, 2011). As previously mentioned, there is an innate-adaptive immune system interplay. After capturing antigens from pathogens (usually via phagocytosis or those bound to surface receptors), APCs such as dendritic cells and macrophages will then present these antigens to naïve (cell prior to antigen exposure) lymphocytes to activate them (Gaudino and Kumar, 2019; Moini et al., 2020).

A good example of adaptive immunity is vaccination, which delivers specific antigens in either live-attenuated or inactivated (non-live) forms safely into the body to stimulate an immune response. Following antigen uptake and presentation to T and B lymphocytes by APCs, an array of lymphocyte responses ensues, including affinity maturation, a process by which antibodies gain an increased affinity for binding to the specific antigen, as well as the production of memory cells, in the form of long/short-lived plasma cells and memory B cells (Pollard and Bijker, 2021). This results in rapid and robust recall responses upon subsequent exposures to the antigen. So quick in fact, the potential pathogen is usually eliminated before any illness symptoms even develop.

1.3.3.1 B lymphocytes

B cells develop from HSCs within bone marrow before migrating to peripheral lymphoid tissues. They are responsible for providing humoral immunity to the host, which is the process of creating antibodies (Althwaiqeb and Bordoni, 2024). B cell receptors (BCRs) consist of a membrane-anchored immunoglobulin (Ig), which activates the cell upon binding to an antigen (Mauri et al., 2014). Once activated, naïve B cells are differentiated via clonal proliferation into plasma cells capable of creating Igs. Alternatively, naïve B cells can be differentiated into memory B cells, which possess a memory of the antigen that activated the cell and allow the body to quickly identify and combat the antigen upon any subsequent exposures (Akkaya et al., 2020). It is also possible for B cells to act as APCs to T cells. This occurs when the BCR internalises the antigen, processes it and presents it to T cells (LeBien and Tedder, 2008).

Igs, also known as antibodies, are a large family of glycoproteins that can be divided into five classes: IgM, IgG, IgA, IgE and IgD, each of which differ in molecular size, structure and function (Justiz Vaillant et al., 2024). IgM is the largest Ig and is the initial antibody produced by B cells following recognition of antigens. It typically functions as a complement system activator and as an agglutinin, promoting the coagulation and aggregation of particles. Whilst IgM is usually in pentameric (five monomers) form, a monomeric IgM can also act as a BCR for cells prior to activation (Vasilev et al., 2016).

IgG is the most abundant Ig in circulation and can be divided into four subclasses: IgG1, IgG2, IgG3 and IgG4, each of which elicit important functions in the immune response. IgG1 and IgG3 typically protect against protein antigens (such as viruses) and activate the complement system (Vidarsson et al., 2014). IgG2 provides strong defence against encapsulated bacteria, with IgG2 deficiency having been linked to a greater susceptibility to bacterial infections (Justiz Vaillant et al., 2024; Kuijpers et al., 1992). Alternatively, IgG4 predominantly functions as regulator of immune responses, eliciting anti-inflammatory and tolerance-inducing properties (Maslinska et al., 2022).

IgA can occur in two molecular forms, serum, which is usually found in the bloodstream, and secretory, which is typically found in mucosal secretions (e.g. respiratory tract, urogenital tract, GI tract), and provides potent defence against infectious agents via multiple mechanisms (Justiz Vaillant et al., 2024). Although briefly touched on earlier in this review, IgA is found in various structures. Serum IgA exists in monomeric form, composed of two heavy chains and two light chains, forming a Y-shaped structure. Additionally, monomeric IgA contains a single Fc region and two antigen-binding sites (de Sousa-Pereira and Woof, 2019). Dimeric IgA, on the other hand, is secreted by plasma cells and found primarily in mucosal surfaces. They are dimers comprising of two IgA monomers joined by an additional 15 kDa polypeptide, known as a J chain (Johansen et al., 2000). The final form of IgA is sIgA, found in external secretions and produced by Ig-producing cells in mucosal-associated lymphoid tissues (MALT). SIgA typically exists in dimeric form, consisting of two IgA monomers joined by a J chain, along with a secretory component. The secretory component is a polypeptide fragment of the polymeric immunoglobulin receptor (pIgR), which is proteolytically cleaved from epithelial cells during the process of transcytosis, and remains bound to the dimer (Sitia, 1998). IgA is known to elicit ‘immune exclusion’, the regulation of the local microbiota via binding of IgA to pathogens, blocking their access to epithelial cells and mucosal surfaces, thus preventing infections (Mantis et al., 2011). Other suggested mechanisms include IgA binding to antigens that have already passed the mucosal barrier, to transport them back into the lumen, or the intracellular killing of pathogens bound to pIgR within epithelial cells (Lamm, 1988).

The two least abundant Igs in circulation are IgE and IgD. IgE is recognised for its role in Type I hypersensitivity reactions. Upon exposure to an antigen, IgE binds to high-affinity receptors on mast cells and basophils, triggering the release of inflammatory mediators such as histamine, leukotriene and prostaglandins, more commonly known as an allergic reaction (Albuhairi and Rachid, 2021). IgE is also the most important antibody for protection against parasites, with concentrations spiking dramatically in response to parasitic infections (Mukai et al., 2016). For IgD, little is known about its functionality in microbial defence. A rodent study prompted the idea that IgD may influence antigen-stimulated lymphocyte differentiation, however, no further evidence supports this (Goding, 1978). IgD can also function as a BCR, although their antigen detection may be less efficient than that of IgM BCRs (Noviski et al., 2018).

1.3.3.2 T lymphocytes

Naïve T cells also originate from the bone marrow; however, they migrate to the thymus before differentiating into either helper T cells (CD4$^+$) or cytotoxic T cells (CD8$^+$) (Luckheeram et al., 2012; Philip and Schietinger, 2022). CD4$^+$ cells aid the immune response in several ways, including the activation of B cells, CD8$^+$ cells and macrophages, which is typically signalled through cell-to-cell contact or the release of cytokines. CD4$^+$ cells can be further categorised into subsets depending on the cytokines they release, such as T helper 1 (Th1) cells which secrete IL-2 and IFN-γ, or Th2 cells which release other cytokines such as IL-4, IL-5 and IL-13 (Yu et al., 2018; Zhu and Paul, 2008). The several actions of CD4$^+$ cells mean they are indispensable to adaptive immunity, influencing cell-mediated immune responses, humoral immunity and allergic responses (Kruse et al., 2023; Murayama et al., 2022; Sokol et al., 2009).

The other major subset of lymphocytes, CD8$^+$ T cells, are also important in the adaptive immune response as they are predominantly used to eliminate infected or mutated cells. The killing mechanisms of CD8$^+$ T cells are similar to those of NK cells, inducing apoptosis in target cells via two methods: the perforin and Fas pathways (Koh et al., 2023). Additionally, CD8$^+$ T cells can release cytokines, and similarly to CD4$^+$ T cells, can be categorised into subsets primarily depending on the cytokines released and their functions. Cytotoxic T cell 1 (Tc1) promotes the release of cytokines such as TNF-α and IFN-γ and exhibits the greatest cytotoxicity. Alternatively, cytotoxic T cell 2 (Tc2) secretes cytokines such as IL-4 and IL-5 and is more involved in inflammatory responses and humoral immunity (Haghshenas et al., 2016; Koh et al., 2023; Planas et al., 2015). Similarly to memory B cells, naïve T cells may also differentiate into memory T cells upon activation of their specific antigen, with the function to provide long-term protection against its antigen (Lin et al., 2015).

The diverse functions of B and T lymphocytes make them crucial components to the immune system and can be summarised into four phases. The **recognition phase**, which refers to the identification of specific antigens through receptors. Following the recognition of antigens, lymphocytes will differentiate into either effector or memory cells, known as the **activation phase** (Crotty, 2011; Zhu and Paul, 2010)**.** Next, during what is known as the **effector phase**, effector cells will perform their respective tasks: the production of antibodies by plasma B cells (Althwaiqeb and Bordoni, 2024); the secretion of cytokines from CD4$^+$ cells (Zhu and Paul, 2008); the killing of target cells by CD8$^+$ cells (Koh et al., 2023). Lastly, memory B and T cells are produced to enable a more rapid and robust response upon re-exposure to the pathogen, known as the **memory phase** (Akkaya et al., 2020; Lin et al., 2015).

1.3.4 The effects of exercise on the immune system

It is well-documented that a sedentary lifestyle may increase the susceptibility to contracting various infections and diseases, and it is commonly accepted that when compared to inactivity, moderate exercise is beneficial to immunity (Nieman and Sakaguchi, 2022). Moderate physical activity has been shown to reduce chronic low-grade inflammation, improve responses to vaccinations, and support various health conditions, including cardiovascular disease, diabetes, and cancer (Gleeson et al., 2011; Hojman et al., 2018; Suzuki, 2019). However, strenuous and prolonged exercise, typically performed by, but not limited to, elite athletes has been shown to transiently depress the immune system and increase the risk of infection to a greater level than that seen in the sedentary population – this is best represented by Nieman's J-shaped curve model (Nieman, 2000; Simpson et al., 2020; Spence et al., 2007). However, this depression is transient, and does not mean exercise is inherently detrimental for immune function. Whilst regular strenuous/prolonged exercise may appear to cause chronic immune depression in some, this effect is actually the results of repeated transient dips in immune function In fact, the athletes experiencing temporary exercise-induced immune depression are likely 'healthier' than the general population. When not under heavy training loads, or even across their lifespan, these athletes will likely be at a lower risk of illness and non-communicable diseases, and may even experience greater longevity (Batista and Soares, 2014).

1.3.4.1 Exercise and cellular immune functions

Research into the effects of exercise on leukocyte responses has been an area of interest among researchers for over a century (Isaacs and Gordon, 1924; Larrabee, 1902). It is widely known that exercise induces leukocytosis, an increase in circulatory leukocyte counts (primarily neutrophils and lymphocytes) (Neves et al., 2015). These changes can be significant as increases of up to 400% have been observed during and post-exercise. Moreover, this relationship is intensity- and duration-dependent, with endurance exercise bouts longer than 1.5 hours typically exhibiting greater (3- to 4-fold) rates of leukocytosis than shorter (20-40 min) high-intensity exercise (Gleeson, 2007; Robson et al., 1999). It has been suggested that the increased cardiac output in exercise causes a mechanical effect, such as shear stress within blood vessels, which may result in the demargination of leukocytes from marginated pools (Foster et al., 1986). However, due to the vast numbers of leukocytes in marginal pools within vital organs such as the liver, lungs and spleen (e.g. pools in the lung contain lymphocytes at a 10-fold greater amount than in circulation), and lymphoid organs such as bone marrow, it is likely that exercise-induced effects on these organs also contribute to the increased leukocytosis observed with exercise (Hogg and Doerschuk, 1995).

Exercise has been shown to increase activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis, causing elevations in catecholamines and glucocorticoids, such as cortisol, which both contribute to leukocytosis (Caplin et al., 2021; Davis et al., 1991; Karsai et al., 2023). Exercise-induced leukocytosis typically also results in neutrophilia. This is thought to occur due to catecholamine-induced demargination of neutrophils from marginal pools, which occurs immediately, as well as delayed cortisol-induced release of neutrophils from bone marrow, known as delayed neutrophilia (Allsop et al., 1992; Ince et al., 2019; McCarthy et al., 1991). The magnitude of exercise-induced neutrophilia is influenced by both exercise intensity and duration, which act through partially distinct mechanistic pathways. Exercise intensity influences neutrophil demargination via sheer stress, which is largely determined by cardiac output and blood flow. In addition, hormonal responses to exercise may modulate the magnitude of neutrphilia. For example, exercise-induced catecholamine release, which is primarily intensity-dependant, or glucocorticoid release, which is both intensity- and duration-dependant may affect the rate of neutrophil release from bone marrow. Whilst neutrophils that demarginate from vascular walls exhibit similar maturity to those already in circulation, neutrophils released from the bone marrow in response to glucocorticoid stimulation typically contain larger numbers of immature neutrophils, such as band cells (Hetherington and Quie, 1985; McCarthy et al., 1991).

Whilst exercise-induced changes in leukocyte and neutrophil trafficking are well documented, they are only a part of the immune response to exercise. Ultimately, it is the functional capacity of these cells that determines their contribution to host defence. The neutrophil response to exercise has been an area of interest among researchers, but early work displayed large variation in study design and caused conflicting results (Peake, 2002). In an attempt to provide clarity on this, Albers et al. (2005) evaluated various methods of testing the effects of exercise on immune functions. When accounting for biological relevance, sensitivity and practical feasibility, it was suggested that measuring markers such as neutrophil oxidative burst and degranulation were highly suitable for representing the immunomodulatory effects of exercise due to their pronounced responsiveness and inducibility in relation to exercise.

The general consensus is that neutrophil oxidative burst and/or degranulation responses to in vitro stimulation (e.g. N-formyl-methionyl-leucyl-phenylalanine [fMLP], LPS or Phorbol 12-myristate 13-acetate [PMA]) experience significant decreases following prolonged exercise (> 1.5 h) (Chinda et al., 2003; Davison et al., 2007; Davison and Diment, 2010; Davison and Gleeson, 2006, 2005; Jones et al., 2015; Laing et al., 2008; Suzuki et al., 2003). Although, the degree to which neutrophil functions are suppressed is likely underpinned by the balance of immunosuppressive and immunostimulating factors, which itself is indirectly influenced by the magnitude of neutrophilia, due to the greater proportion of functionally immature cells entering circulation (Peake, 2002). It has also been suggested that neutrophils may enter a "refractory period" following unstimulated activation from alternative stimuli, such as tissue or cell damage. Therefore, it is possible that unexpected activation and the following refractory state may limit the response to pathogenic challenges subsequently encountered (Chmielecki et al., 2022; Peake, 2002).

As previously stated, prolonged exercise is typically accompanied by a dramatic spike in leukocyte counts, which results in neutrophilia (Neves et al., 2015; Peake et al., 2017). The rapid influx of cells into circulation contains many immature neutrophils, which are known to exhibit lesser granular content and NADPH oxidase activity, which could be one mechanism for the reduced killing activity measured (Berkow and Dodson, 1986; Hetherington and Quie, 1985; Peake, 2002). It has also been theorised that some agents released into circulation during exercise may have priming (e.g. induction of NADPH complex assembly) or desensitising (e.g.

receptor internalisation) effects on neutrophils, thus influencing their functionality and capacity to respond to stimuli (Amulic et al., 2012; Peake, 2002; Pyne, 1994). Alternatively, some have suggested that neutrophil functions may be temperature dependent. The initial research into this idea found that higher temperatures (38-39°C) exhibited greater bactericidal activity in neutrophils (Nahas et al., 1971; Roberts and Steigbigel, 1977). This was also tested decades later, finding that exercise-induced neutrophilia was not parallel with increases in plasma myeloperoxidase, suggesting the possibility of heat stress influencing neutrophil activation (Niess et al., 2003). However, this is far from conclusive, with multiple studies finding little to no effect of heat stress on neutrophil functions (Laing et al., 2008; Pedersen et al., 1994; Roberts and Sandberg, 1979).

Some have debated whether activation of the hypothalamic-pituitary-adrenal (HPA) axis from exercise impairs neutrophil function (Laing et al., 2008). Elevated cortisol levels were shown to decrease in vitro neutrophil chemotaxis, as well as being implicated in a reduction in LPS-stimulated neutrophil degranulation following prolonged cycling (Robson et al., 1999; Salak et al., 1993). Supporting this, it has been demonstrated that carbohydrate supplementation prior to an exercise stressor truncated the reduction in LPS-stimulated neutrophil elastase release, simultaneously with reducing the spike in post-exercise cortisol levels, highlighting the possibility that the absence of cortisol may have affected neutrophil function (Bishop et al., 2003). Since cortisol is associated with the suppression of neutrophil activation and reduced numbers of primed neutrophils, it is possible that activation of the HPA axis is at least partly responsible for decreases in neutrophil functions following prolonged exercise (Bishop et al., 2003; Blalock, 1989).

The elevated cortisol release during exercise can promote the release of functionally immature neutrophils from bone marrow into circulation, highlighting an indirect effect of cortisol on neutrophil functions. Though, it is also worth noting that although cortisol has also been hypothesised to impair neutrophil function through cell receptor pathways, this would have little effect on synthetic stimulants such as PMA, which do not require surface receptor activation (Jones and Davison, 2018). For this reason, various other mechanisms have also been proposed to affect neutrophil functions. Exercise induces an inflammatory response which may cause greater levels of catecholamines, cyclic adenosine monophosphate (cAMP), growth hormones, complement proteins and direct cellular damage. Such responses could impair calcium signalling, which mediates neutrophil activation and function, or affect other intermediates of intracellular pathways (Immler et al., 2018; Laing et al., 2008; Lee et al., 2005; Robson et al., 2003; Suzuki et al., 1999; Tintinger et al., 2001).

In summary, the current evidence suggests that exercise-induced changes in neutrophil oxidative burst are mediated by a complex interplay of endocrine, metabolic and other signalling mechanisms, which are yet to be fully elucidated. The understanding of these mechanisms may help to explain both the transient depression and enhancement of immune functions seen under different exercise conditions. As the function of neutrophils and other immune cells represent a key component of host defence, it is important to consider how these responses integrate with other defence systems, such as mucosal immune factors, which also provide one of the primary barriers to pathogenic challenges.

1.3.4.2 Exercise and mucosal immunity

The mucosal immune system is the largest lymphoid organ in the body, covering a surface area of 400 m^2 (Brandtzaeg et al., 1999). The system consists of organised immune tissues associated with the mucosal secretions that line the upper and lower respiratory tracts, GI tract and urogenital tract (Clarke, 2013). However, these tissues can be categorised into effector and inductive sites based on their anatomical and functional properties. Key inductive sites include

Peyer's patches and isolated lymphoid follicles within the gut-associated lymphoid tissues (GALT), and the tonsils/adenoids and cervical lymph nodes within the nasopharyngeal-associated lymphoid tissues (NALT). These sites are associated with the activation and 'priming' of immune responses to antigen exposures (McGhee and Fujihashi, 2012).

Effector sites such as the lamina propria of the GI and respiratory tracts, and secretory glandular tissues (e.g. mucous and sebaceous glands) are responsible for secreting the substances that execute immune responses, such as neutralising pathogens and creating immune memory (Mowat and Agace, 2014). Effector sites contain antigen-specific effector cells, such as Ig-producing plasma cells and memory B and T lymphocytes (Brandtzaeg, 2007). In fact, over 80% of all plasma cells in the body are located within mucosal effector tissues, which is why antibodies provide such effective protection in these sites. The most abundant antibody within mucosal secretions is SIgA, which acts as the main effector function of the mucosal immune system (Bishop and Gleeson, 2009).

Within exercise immunology, unstimulated whole saliva samples (passive drool method) are frequently used to measure mucosal immunity, a method favoured for its reliability and simple reproducibility (Jones and Davison, 2018). Due to its importance in mucosal immunity and ease of collection, salivary SIgA is a common biomarker used in this field of research. Although not completely unanimous, it is generally accepted that that the absolute concentration of SIgA in saliva decreases following prolonged exercise (MacKinnon and Jenkins, 1993; Nieman et al., 2003, 2002; Palmer et al., 2003; Steerenberg et al., 1997; Tomasi et al., 1982), with a combination of high intensities and long durations eliciting the greatest suppression on salivary SIgA concentrations (Nieman et al., 2002). However, as stated, some research has found contradicting results, suggesting that salivary SIgA concentrations are not affected by prolonged exercise (Mackinnon and Hooper, 1994; Sari-Sarraf et al., 2007, 2006).

Walsh et al. (2011) noted that such discrepancies in results could likely be attributed to numerous factors, including variations in saliva collection methods and hydration status. However, discrepancies within the literature relating to how SIgA is reported are also likely to be influential. Following exercise, whilst SIgA concentrations typically decrease, other saliva proteins such as α-amylase may experience increases in secretion rates and activity (Walsh et al., 1999). For example, Ligtenberg et al. (2015), demonstrated that following running exercise at moderate (HR ~130 beats per minute) and high (to exhaustion) intensities, salivary amylase increased following the moderate bout. Exercise can influence saliva flow rate through a number of mechanisms including activation of the sympathetic and parasympathetic nervous systems, and hydrations factors (Ntovas et al., 2022). For this reason, reporting SIgA as absolute concentration may misrepresent the true host defence capacity. To account for changes in hydration status and saliva flow rate, it is advantageous to normalise SIgA relative to secretion rate or saliva osmolality (Blannin et al., 1998).

When expressed as secretion rate, it is generally reported that SIgA decreases following bouts of prolonged or intense exercise (Allgrove et al., 2008; Matsubara et al., 2010; Murase et al., 2016; Uchino et al., 2024). Interestingly, some have suggested that expressing SIgA as absolute concentration may be superior to secretion rate, as the latter may only justify changes in salivary flow rate (Bishop and Gleeson, 2009). However, numerous studies have found that SIgA, when expressed as secretion rate, has a significant correlation with incidence of URTIs or URS, highlighting its clinical importance as a predictor of URI (Fahlman and Engels, 2005; Ihalainen et al., 2016; Nieman et al., 2003; Tiernan et al., 2020). Though, alongside secretion rate, absolute SIgA concentration has also been shown to have a consistent relationship with risk of URTIs (Walsh et al., 2011). However, whilst SIgA concentration may be reliable when resting samples are taken over longer periods, the exercise-induced changes in saliva composition and SIgA concentration mean that when assessing acute responses to exercise, SIgA should be normalised relative to osmolality or secretion rate. Other Igs have also been investigated, albeit less, for their role in exercise-induced changes in mucosal immunity. This preliminary evidence suggests that exercise has no effect on IgG concentrations, whilst IgM decreases linearly with the reductions in sIgA (Bishop and Gleeson, 2009).

AMPs are also valuable components of the mucosal immune system. In particular, lactoferrin and lysozyme are the predominant AMPs found in the mucosal secretions of the URT (Giansanti et al., 2016; Ragland and Criss, 2017). Limited research exists exploring the effects of acute and chronic exercise on salivary lysozyme (sLys) and lactoferrin (sLac) concentrations. However, from the data available, high intensity exercise of both short and prolonged durations has often been shown to increase sLys (Allgrove et al., 2008; West et al., 2010, 2006) and sLac (West et al., 2010). Additionally, following a 2.5 h cycling bout at ~60% VO_2 max, significant increases in concentrations, concentration:osmolality ratios and secretion rates were observed for human neutrophil peptides 1-3 and LL-37 (Davison et al., 2009). In contrast, other work has shown that sLys concentration, secretion rate and relative to osmolality decreases following prolonged exercise (2 h cycling at ~65% VO_2 max), with levels returning to baseline after 1 h recovery (Davison and Diment, 2010). There is some good evidence to suggest that AMP absolute concentrations may increase following prolonged exercise, but normalised reporting methods such as secretion rate and osmolality experience decreases. Though, the variation among findings at present make it difficult to draw definitive conclusions. The likely intensity- and duration-dependent nature of AMPs underscores the importance of normalised reporting methods and standardised protocols in accurately interpreting exercise-induced changes in AMPs.

The mechanisms underpinning the exercise-induced changes in mucosal parameters have been debated but are yet to be fully understood. Exercise-induced alterations in IgA production by plasma cells and polymeric immunoglobulin receptor (pIgR) availability are thought to be key components of the sIgA response to exercise (Bosch et al., 2002). Rodent studies have shown that stimulation of β-adrenoreceptors above a certain threshold elicited increased saliva sIgA concentrations via increased sIgA transcytosis, which was associated with greater pIgR availability (Carpenter et al., 2004; Proctor, 2003). Although such mechanistic studies are yet to be proven in humans, this mechanism of increased sympathetic stimulation could account for the increases in sIgA concentrations observed following short, intense exercise (Davison, 2011). Whilst this would not explain the decreases in sIgA concentrations seen with prolonged exercise, it has been theorised that repeated pIgR mobilisation could reduce the amount of available IgA, or there could be an additional stimulation threshold in which pIgR availability becomes impaired (Walsh et al., 2011). Though it should be noted that these theories are speculative at present.

Given that AMP and protein concentrations are more frequently observed to increase with exercise, it is also plausible to suggest that these may occur due to increased sympathetic stimulation (Usui et al., 2011). However, increased HPA activity could explain reductions in AMPs following prolonged exercise, as increased salivary cortisol has been linked with reduced concentrations of sLys (Perera et al., 1997). In contrast to endocrine influences on AMPs, exercise-induced activation of granulocytes in the oral cavity may directly initiate the release of granule proteins such as lysozyme and lactoferrin into saliva Furthermore, systemic activation of granulocytes may result in increased circulatory AMP levels, which may translocate into saliva more readily. This highlights that changes in salivary AMPs may not only reflect local (oral) responses, but systemic granulocyte activation and degranulation (Gillum et al., 2017; Morozov et al., 2003).

To summarise, strenuous/prolonged exercise has been shown to inflict perturbations to inflict GI damage and depress several immune functions. Such perturbations carry significant consequences for the health and performance of athletes and may also reflect clinical conditions related to the GI and immune systems. These reasons have led researchers to explore various nutritional countermeasures in attempt to mitigate these effects, with milk-based supplements having shown some promising results, possibly due to the bioactive components within.

1.4 Milk Fat Globule Membrane

Milk is a cornerstone in the nutrition of mammalian species, serving as the primary source of nutrients in early life, helping to provide the foundations of an infant's immune system, intestinal bacteria and various other components crucial to survival (Melnik, 2015; Stock and Wells, 2023). Once a mammal is able to forage and support themselves independently, milk consumption is typically weaned off, resulting in a reduction in lactase (the enzyme responsible for breaking down lactose) activity. However, in some human populations, lactase activity continues throughout life, allowing for milk (typically bovine) to remain a part of the diet (Barreiro, 2024).

Dairy nutrition is a field that has been extensively studied due to its importance in early life nutrition and ability to support healthy ageing, largely attributed to its richness in bioactive components that contribute to human metabolism and health (X. Zhang et al., 2021). Bovine milk contains 18 of 22 essential nutrients (Michaelsson et al., 2014), including a wide range of bioactive proteins/peptides and fatty acids, including whey and casein proteins, milk polar lipids, α-linolenic acid (ALA), conjugated linoleic acids (CLA), palmitic acid 16:0, and various other components (e.g. vitamins and minerals) (Bouglé and Bouhallab, 2017; Fulgoni et al., 2011). Among the numerous components of milk, the milk fat globule membrane (MFGM) has been predominantly studied for its role in early life development, but has recently been receiving attention for its potential to promote healthy ageing.

Milk fat globules are presented as a core of neutral glycerides (primarily triglycerides), stabilised by a trilayer (inner lipid monolayer and an outer layer bi-layer) membrane containing various bioactive components, such as glycoproteins and polar lipids (Huppertz and Chia, 2021). Whilst the main function of MFGM is to protect milk fat globules from digestion, if administered at an efficacious dose, its bioactive components may offer numerous nutraceutical benefits.

Of particular note, polar lipids within milk, including phospholipids and sphingolipids are recognised far beyond their initial role of providing structure to and maintaining the integrity of biological membranes, but also for a wide range of physiological and health benefits (Venkat et al., 2024). Milk PL consist of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and their lysophospholipid and plasmalogen forms. Alternatively, SL consist of sphingomyelin (SM), gangliosides, cerebrosides, sulfatides, ceramides and sphingosines (Vesper et al., 1999).

MFGM proteins have been studied extensively in proteomic research, making up ~1% of the total milk fat globule mass, and 1-4% of the total protein fraction in milk (Liao et al., 2011). Major proteins in MFGM include butyrophilin, lactadherin, mucins, xanthine oxidase, CD proteins and adipophilin. These proteins are found in all mammalian species, suggesting they are important for various biological functions. Interestingly, however, the concentrations seem to vary significantly between species (Yang et al., 2015). Lactadherin, also known as milk fat globule epidermal growth factor 8 (MFG-E8), is particularly interesting due to its higher abundance in bovine milk compared to humans and other mammals. It is an anti-inflammatory protein implicated in the clearance of apoptotic cells. This is important for immune regulation, as lactadherin deficiency or dysfunction has been linked to various autoimmune diseases (Yi, 2016). Additionally, lactadherin has shown to promote intestinal epithelial repair, maintaining barrier integrity (Shen et al., 2019).

1.4.1 Health effects of MFGM: Gut and immune

To date, there have only been two studies investigating the effects of MFGM on gut/immune responses in healthy adults. The first of which, from Ten Bruggencate et al. (2016), assessed the effects of MFGM on an oral *E. coli* challenge. Following two weeks of supplementation using a milk protein concentrate rich in MFGM (phospholipids supplemented at 3.2g/day) or a placebo consisting of sodium caseinate, participants were orally challenged with live, attenuated diarrhoeagenic *E. coli*. The *E. coli* challenge resulted in increased stool frequency, however, the MFGM group experienced significantly lower stool frequency than the placebo group, largely being attributed to the bioactive phospho- and sphingolipids found in the MFGM supplement. Interestingly, however, these results were not replicated in a recent, but similar study (Ulfman et al., 2022). Ulfman et al. (2022) also examined the effects of a whey protein concentrate rich in MFGM (phospholipids supplemented at 2.42g and 3.92g/day), supplemented for two weeks, on an oral diarrhoeagenic *E. coli* challenge. However, this study observed no differences in *E. coli*-induced stool frequency and Gastrointestinal Symptom Rating Scale (GSRS) scores between MFGM and placebo groups.

Both MFGM supplements from this study and that used by Ten Bruggencate et al. (2016) were similar, especially in phospholipid contents, suggesting that the results are unlikely to have varied due to inconsistencies in trial supplements. However, most notably, the placebo supplement used by Ulfman et al. (2022) was a whey protein hydrolysate, which could be a significant confounding factor as whey (and many other) protein hydrolysates have shown to exhibit numerous immunomodulatory effects in rodent models, interacting directly with immune cells (Meulenbroek et al., 2013; Pan et al., 2013; Rodríguez-Carrio et al., 2014; van Esch et al., 2011) and enhancing gut barrier integrity (Visser et al., 2012, 2010). Therefore, it is possible that the contrasting results between Ten Bruggencate et al. (2016) and Ulfman et al. (2022)'s studies were influenced by the different placebos used. However, as most of the research supporting the possible discrepancy comes from rodent models, which has its own limitations and may not accurately imitate human responses, further research is required to fully understand the relationship between MFGM and immune/gut health in healthy adults.

As previously highlighted, MFGM has been researched for its role in early life nutrition, often supplemented in infant formulas, possibly in attempt to replicate the bioactive properties found in human breast milk (Ambrożej et al., 2021). MFGM-supplemented infant formulas have demonstrated numerous benefits to the immunity of infants, reducing incidence of acute otitis media and subsequent antipyretic use (Timby et al., 2015), number of respiratory-associated adverse events (Li et al., 2019), and total febrile episodes when comparing to various unsupplemented control formulas (Veereman-Wauters et al., 2012). However, there is some contrasting literature on this, with some findings suggesting that MFGM-rich formulas do not affect the incidence or severity of adverse events (Billeaud et al., 2014; Breij et al., 2019).

To further elucidate the mechanisms behind the health benefits of MFGM, numerous in vitro studies have been conducted (Martínez-Sánchez et al., 2024). It has been reported that MFGM and its components within (such as PL, SL and gangliosides), possess antiviral properties, having shown to reduce rotavirus infectivity in cell lines such as MA-104 and Caco-2 (Fuller et al., 2013; Kvistgaard et al., 2004; Monaco et al., 2021). Of particular note, Fuller et al. (2013) further demonstrated that extracted lipid fractions from the MFGM displayed strong inhibition of rotavirus infectivity, suggesting that the lipid component of MFGM (largely PL and SL) may be a large contributor to the antiviral activity seen.

Also supporting the antimicrobial potential of MFGM and the lipids within, Sprong, Hulstein and Van der Meer (2001) showed that sphingosine – a metabolite of sphingomyelin, displayed strong bactericidal activity in vitro, decreasing the viabilities of *E. coli, Salmonella enteritidis, Campylobacter jenuni, Listeria monocytogenes, and Clostridium perfringens* following incubation with sphingomyelin. It is possible that another mechanism underpinning the antipathogenic properties of MFGM may be related to the inhibition of pathogens virulence factors binding to their target receptors in the gut. Herrera-Insua et al. (2001) found that a lipid fraction derived from human milk was able to bind to the Shiga toxin produced by Shiga toxigenic *E. coli*. Specifically, glycolipid globotriaosylceramide (GB3) has been shown to bind to both Stx1 and Stx2 Shiga toxins, which would prevent them from binding to their target sites in the gut (Gallegos et al., 2012). Therefore, toxin-binding lipids, present within MFGM, may also contribute to a reduction in Shiga toxigenic illnesses, such as Shiga toxigenic *E. coli*-induced diarrhoea.

Although there is a lack of research on the relationship between MFGM and gut health and integrity in adult populations, several studies have been conducted using infant, rodent and in vitro models. As highlighted in an excellent review by Anto et al. (2020), milk polar lipids, which include PL/SLs and make up a major component of MFGM, have been shown to increase the abundance of some beneficial bacteria in the gut, such as Bifidobacteria and some Lactic Acid Bacteria (LAB). However, the mechanisms underpinning these phenomena are less understood. Kosmerl et al. (2021) suggested that the interactions between MFGM and Bifidobacteria/LAB occur due to bacterial surface adhesion effects. Elaborating on this, it has been suggested that phospholipids, such as those found in MFGM, bind directly to the surface of some bacteria during cell growth, instead of internalising them for further metabolism. This could subsequently affect several functions of these bacteria, including growth rate and adhesion properties. MFGM has also been shown to promote the colonisation and growth rate of SCFA-producing bacteria within the gut, resulting in elevated production of SCFAs, such as acetate, butyrate and propionate (Feng et al., 2025; Gong et al., 2024; Wu et al., 2021). SCFAs, particularly butyrate, are crucial to maintaining gut health and function, and low SCFA levels have been directly linked with several inflammatory and allergic conditions (Adolph and Zhang, 2022). Therefore, a connection could be made between the consumption of MFGM and possible effects on these functions, as a result of the upregulation of SCFA production within the gut.

At present, there is a gap in the literature for the relationship between MFGM and gut permeability measures, although there are a small number of studies showing promising results, using rodent and in vitro models. Jiang et al. (2022) orally supplemented sucking rat pups with MFGM from postnatal day 1-20, observing that intestinal differentiation and tight junction protein expression was increased in the colon and jejunum, compared a control supplement. It was then proposed that the intestinal development associated with MFGM may be due to increased activation of various signalling pathways, as it was also shown that PI3K/Akt/mTOR, mitogen-activated protein kinases, and myosin light chain kinase signalling pathways were all significantly activated compared to the control group. Additionally, the authors incubated Caco-2 cells with MFGM (400 µg/mL for 72 h), finding that MFGM increased transepithelial electrical resistance, and increased expression of tight junction proteins such as claudin-4 and ZO-2. Also using human enterocyte models, Milard et al. (2019) incubated milk polar lipids and milk SM with Caco-2 and TC7 cells, finding that milk SM increased the expression of tight junction proteins Occludin and ZO-1. It was also found that milk SM incubation increased the secretion and gene expression of IL-8, so the authors incubated the cells with IL-8, finding that this resulted in overexpression of tight junction proteins, and highlighting another possible mechanism underpinning the effects of MFGM and its components on intestinal functions.

1.5 Summary and aims

To summarise, strenuous and/or prolonged exercise has been shown to cause transient depression of various cellular and mucosal immune functions. Whilst these effects are only temporary, they may increase the risk of contracting illnesses such as URTIs, which may have more lasting effects. Exercise of this nature has also shown to induce transient gut perturbations, leading to periods of elevated GI 'leakiness'. Microbial leakage across the gut barrier into circulation has various consequences, including increased incidence GI symptoms and inflammation. It has also been suggested that severe cases of elevated GI permeability may also be a contributing factor in exertional heat stroke and systemic inflammation. Such gut and immune perturbations pose a risk not only to athlete performance, but also the health of the individual. For this reason, ways of mitigating these effects are of interest to researchers and practitioners, and nutritional strategies have often been a popular method due to their accessibility, ease of implementation and potential to influence a diverse range of health effects.

Within sports nutrition, dairy supplements have received consistent attention due to the natural bioactive composition of milk. In fact, some dairy-based products have shown considerable benefits to both the immune system and gut, in some cases attenuating the exercise-induced gut and immune perturbations. MFGM is a component of milk that has shown potential in aiding various health outcomes, including those related to gut and immune functions. However, there is a clear lack of research on MFGM in healthy, adult populations, with most research investigating infants, or using in vitro or rodent models. Consequently, this thesis explored the effects of short-term and long-term MFGM supplementation on exercise-induced gut and immune perturbations in healthy adult populations. Therefore, the aims of the thesis were as follows:

1. To determine whether MFGM attenuates exercise-induced gut damage and subsequent permeability (Chapters 3 and 4).
2. To determine whether MFGM improves cellular immune functions, including attenuation of exercise-induced depression of immune cell function (Chapter 4).
3. To determine whether MFGM improves mucosal immune functions and mitigates exercise-induced depression of mucosal immunity (Chapters 4 and 5).
4. To establish whether MFGM reduces the risk of contracting URTIs (Chapter 5).

Considering these aims, the following null (H_0) and alternate (H_1) hypotheses were made:

Hypothesis 1

H_0 = MFGM will not truncate exercise-induced gut damage and reduce gut permeability.

H_1 = MFGM will truncate exercise-induced gut damage and reduce gut permeability.

Hypothesis 2

H_0 = MFGM will not reduce the magnitude of exercise-induced depression of immune functions.

H_1 = MFGM will reduce the magnitude of exercise-induced depression of immune functions.

Hypothesis 3

H_0 = MFGM will not reduce the incidence and severity of URTIs.

H_1 = MFGM will reduce the incidence and severity of URTIs.

Hypothesis 4

H_0 = MFGM will not improve chronic mucosal immune functions.

H_1 = MFGM will improve chronic mucosal immune functions.

Chapter 2. General Methods

2.1 Ethical approval

All studies in this thesis were approved by the University of Kent Sport, Exercise, and Rehabilitation Sciences Research Ethics Advisory Group (REAG), and all procedures demonstrated were in accordance with the Declaration of Helsinki (2013). Prior to participation, all participants were fully briefed on the procedures required in the study and provided both written and verbal informed consent. All studies were prospectively registered on a clinical trial database, with details provided in the corresponding chapter.

2.2 Inclusion/exclusion criteria

All participants were required to be between 18-65 years old. The inclusion criteria required participants to be non-smokers, have no allergies or hypersensitivities to the study supplements, not be on medications or dietary supplements, and be free of illness or illness symptoms for the four weeks prior to participation. To be eligible, participants were required to be deemed 'healthy' by a physical activity readiness questionnaire (PAR-Q) was completed with the initial enrolment documents (Appendix A).

2.3 Physiological and perceptual measurements

For analysis of oxygen uptake ($\dot{V}O_2$) and maximal oxygen uptake ($\dot{V}O_{2max}$), all measurements of breath-by-breath ventilation and gas exchange were recorded using an online gas analysis system (MetaLyzer 3B, Cortex GmbH, Leipzig, Germany). Prior to every measurement, the system was calibrated in accordance with the manufacturer's instructions. Briefly, this involved calibration of the gas sensor, starting with a measurement of fresh ambient air, followed by a measurement of a calibration gas. The flow sensor was calibrated using a 3 L calibration syringe (Hans Rudolph, KS, USA).

Heart rate (HR) was measured using a chest band and monitor (Polar T31, Polar Instruments, Kempele, Finland) and rating of perceived exertion (RPE) was measured using the 6-20 Borg scale (Borg, 1982). Height was measured to the nearest 0.01 m using a portable stadiometer (Seca, Birmingham, UK) and body mass (BM) was measured to the nearest 0.1 kg using a weight scale (Seca, Birmingham, UK). All anthropometric measurements were taken wearing minimal clothing (t shirt and shorts) and no shoes.

2.4 Sample preparation and analysis

2.4.1 Blood sampling

To ensure standardisation of procedures and that participants were at rest, and to minimise the effects of movement or orthostatic stress, participants undertook a period of 10 min seated rest, with the exception of immediately post-exercise samples, which were taken as soon as possible following completion of exercise (no later than 5-min post-exercise). All blood samples were taken via standard venepuncture, using a 21-gauge needle (Becton-Dickinson, Oxford, UK), from a vein within the antecubital fossa. Samples were drawn into vacutainers pre-treated with lithium heparin or K_2EDTA. In accordance with the Human Tissue Act (2004), all measures and uses of whole blood were performed on the same day of collection. Samples were processed as soon as possible and rendered acellular by centrifugation at 1500 × *g* for 10 min at 4°C (Centrifuge 5702 R; Eppendorf, Hamburg, Germany), and the plasma supernatant was then aliquoted into 1.5 mL microcentrifuge tubes and stored at -80°C for later analysis.

2.4.2 Leukocyte counts

Total and differential (neutrophil, lymphocyte, monocyte, eosinophil and basophil) leukocyte counts were performed using whole blood from a K_2EDTA-treated vacutainer. A volume of 10 µL whole blood was drawn into a microcuvette (WBC Diff Microcuvette; HemoCue, Angleholm, Sweden) and used in a portable photometric analyser for the quantitative determination of leukocytes (WBC Diff System; HemoCue, Angleholm, Sweden). Basophils occur at inherently low proportions relative to other leukocytes (Pellefigues and Karasuyama, 2023), and were rarely detected. As a result, basophil data were not reported or analysed. Using 10 repeated measurements of the same sample intra-assay coefficient of variations (CV) were the following: total leukocytes 2%; neutrophils 4.4%; lymphocytes 3.5%; monocytes 28.7%; eosinophils 15.1%. Higher CVs for monocytes and eosinophils are attributable to their low counts, which may render them more susceptible to proportional fluctuations.

2.4.3 Plasma I-FABP

I-FABP was measured at pre-exercise (Pre-Ex), post-exercise (Post-Ex) and 1 hour post-exercise (1 h Post-Ex) on main trial days in chapters 3 and 4. A commercially available solid-phase sandwich enzyme-linked immunosorbent assay (ELISA; DY3078, DuoSet, R&D systems, Minneapolis, USA) was used on plasma (1:4 plasma dilution) from K_2EDTA-treated vacutainers. The capture antibody was reconstituted and diluted to the working concentration using phosphate buffered saline (PBS). The standard reagent diluent provided was used to reconstitute the detection antibody and I-FABP standard and dilute the Streptavidin-HRP to the working concentration. The detection antibody was diluted to the working concentration in reagent diluent supplemented with 2% normal goat serum (DY005, Reagent Additive 1, R&D

systems, Minneapolis, USA). All samples and standards were diluted using the standard reagent diluent with 10% normal goat serum. Based on 130 duplicate samples, the intra-assay CV was 4.8%.

2.4.4 Bacterial load

Bacterial load was measured via qPCR in DNA isolated from whole blood in Chapters 3 and 4. Bacterial DNA was quantified using a commercially available bacterial DNA quantification kit (E2006, Zymo Research, Irvine, CA, USA). Reactions were prepared in a 96 well plate and analysed using a real-time qPCR system (LightCycler 96, Roche, Basel, Switzerland). Using 128 repeated samples, intra-assay CV was 11.7%.

To account for potential variation in the efficiency of DNA extractions, bacterial DNA quantification was normalised to total human DNA. Human DNA was quantified in the same DNA samples using the same real-time qPCR system and a commercially available human DNA quantification kit (E2005, Zymo Research, Irvine, CA, USA). Based on 129 duplicate samples, intra-assay CV was 6.3%. Due to the influence of exercise-induced leukocytosis on human DNA, human DNA concentration was normalised against leukocyte count prior to normalising bacterial DNA concentration.

The thermocycling parameters in all experiments were in accordance with the manufacturer's instructions and a melting curve analysis was performed to detect any off-target amplifications or primer dimers, reaching 95 °C at a rate of 0.2°C/s and acquiring fluorescence every second. Thermocycling parameters are detailed in Table 2.1.

Table 2.1. qPCR thermocycling parameters.

Assay	Step	Temperature °C	Time	Number of Cycles	Acquisition mode
Bacterial	Initial denaturation	95	10 min	1	None
	Denaturation	95	30 s	40	None
	Annealing	50	30 s		None
	Extension	72	1 min		Single
	Final extension	72	7 min	1	Single
Human	Initial denaturation	95	10 min	1	None
	Denaturation	95	30 s	40	None
	Annealing	59	30 s		None
	Extension	72	1 min		Single
	Final extension	72	7 min	1	Single

2.4.5 16S rRNA gene sequencing

16S ribosomal ribonucleic acid (rRNA) gene amplicon sequencing was performed on whole blood DNA samples in Chapters 3 and 4 to provide compositional insights following bacterial translocation. Following quality check (QC), high-throughput amplicon sequencing was performed on the V3-V4 region of the 16S SSU rRNA gene by Biomarker Technologies (BMKGENE). Amplification was performed using the Illumina NovaSeq platform with the 515F (GTGCCAGCMGCCGCGGTAA) and 907R (CCGTCAATTCCTTTGAGTTT) primer set. A paired-end 2x 250 bp sequencing process was used. The raw reads provided had been purified and demultiplexed, and adaptors had been removed from the sequences.

2.4.5.1 16S data preparation

The raw sequencing reads from BMKGENE were processed into taxa for analysis using the LotuS2 software. Raw reads were processed using a DADA2-based workflow to infer amplicon sequencing variants (ASVs). Prior to denoising, reads were dereplicated using a tiered abundance filter to remove low-frequency sequences. Minimap2 was used to remove chimeric and unsuitable reads (stunted or fusions), and to map ASVs back to reads with a 99% identity cutoff to ensure accurate sequence assignment. Taxonomic classification of ASVs was performed with Lamda against the SILVA reference database. This database was selected as it is a 16S rRNA database with extensive coverage of bacterial diversity and is checked for chimaeras and contaminated sequences.

2.4.5.2 16S data analysis and visualisation

All 16S data analysis and visualisation were performed using R (v.4.5.1, R Core Team 2025). The following R packages were used: *devtools, phyloseq, ggplot2, dplyr, ggpubr, BiocManager, microbiome, vegan, microViz, Polychrome, ape, corrplot, microbial and ggrepel*. ASV counts were first rarefied to 5,000 reads to avoid data errors related to inconsistent sequencing depths. Rarefying to 5,000 reads is generally a low rarefaction level, but due to the low bacterial biomass of blood, this was required to maintain a sufficient number of samples while still ensuring comparability (Villette et al., 2021). The data was then trimmed to remove any taxa with less than 100 reads, as they would likely be a result of contamination or PCR error. The following water- and soil-associated genera were identified as likely contaminants (Salter et al., 2014) and purged from the dataset: Pseudomonas; Delftia; Bradyrhizobium; Acetobacter; Acinetobacter; Brevundimonas. Following rarefaction and trimming, data was analysed for relative abundance. To assess overall differences in bacterial composition (beta-diversity) in blood, a Bray-Curtis dissimilarity matrix was used to plot a Principal Coordinates Analysis (PCoA). A Permutational Multivariate Analysis of Variance (PERMANOVA) was used for statistical analysis of the PCoA.

2.4.6 Phospholipid quantification

In Chapters 4 and 5, subgroup analyses were performed to quantify phospholipids in red blood cells. Lipids were extracted from 100 µL of heparinised packed red blood cells, acquired following the centrifugation process detailed in section 2.4.1. A commercially available lipid extraction kit (STA-612, Cell Biolabs, CA, USA) was used in accordance with the manufacturer's instructions. The lipid extract was placed in a 37 °C hot plate overnight to evaporate the kit reagents and the remaining pellet was frozen at -80 °C for later analysis.

Phospholipids were quantified using the Stewart Assay, which is based on the ability of phospholipids to form a complex with ammonium ferrothiocyanate. Ammonium ferrothiocyanate was prepared by dissolving 27.03 g of ferric chloride hexahydrate (F2887, Sigma-Aldrich, Massachusetts, USA) and 30.4 g ammonium thiocyanate (221988, Sigma-Aldrich, Massachusetts, USA) in Milli-Q water volumised up to 1 L. A phospholipid stock concentration of 0.1 mg/mL was prepared by dissolving soy phospholipids (11145, Sigma-Aldrich, Massachusetts, USA) in chloroform. A 6-point standard curve was generated at concentrations of 0.10, 0.08, 0.06, 0.04, 0.02 and 0.00 mg/mL. Extracted lipid samples were reconstituted in 1 mL chloroform and vortexed for 1 min.

For the assay, 1 mL of each standard or sample was combined with 1 mL of ammonium ferrothiocyanate and vortexed for 20s. Following phase separation, 100 µL of the lower chloroform layer was transferred to a 96 well plate and all standards and samples were analysed in duplicate. Absorbance was measured at A_{490} using a microplate reader (FLUOstar OPTIMA; BMG Labtech, Aylesbury, UK). Based on 34 duplicate samples, intra-assay CV was 14.5%.

2.4.7 Saliva sampling

To ensure standardisation of procedures and that subjects were fully at rest, participants performed a period of 10 min seated rest prior to giving the saliva sample, with the exception of Post-Ex samples, which were taken as soon as possible after completion of exercise (no later than 5-min post-exercise). Participants were instructed not to drink any water, or chew gum during the 10 min rest period prior to giving the sample. They were then instructed to swallow, thus emptying the mouth, before beginning the sample collection. The participant was told to remain seated, with the head and body tilted slightly forward and to passively dribble into a pre-weighed 7 mL bijou tube (ThermoFisher Scientific, Roskilde, Denmark) for 2 min. Following the 2 min, if there was an insufficient amount of saliva, the process was repeated for another 2 min, until a maximum of 8 min or once an acceptable sample (~1 mL) was obtained. If a sufficient sample could not be obtained then the collection was stopped after 8 min. For an estimation of saliva flow rate, the saliva density was assumed to be 1.0 $g \cdot mL^{-1}$, as used in previous research (Davison et al., 2009), and was calculated with the total duration of the sample.

Once the saliva samples were obtained, they were centrifuged at 17,000 × *g* for 5 min (AccuSpin Micro 17R; Fisher Scientific, Hampton, New Hampton, USA) to pellet any cellular debris, and the remaining acellular supernatant was then aliquoted into 1.5mL microcentrifuge tubes and stored at -80°C for later analysis.

Saliva osmolality was measured using a freezing point osmometer (Osmomat 030 Series M, Gonotec, Berlin) which had been calibrated in accordance with the manufacturer's instructions. Based on 10 repeated measurements of the same sample, intra-assay CV was 3.2%.

2.4.8 Salivary SIgA

The concentration of salivary SIgA was measured using a commercially available ELISA kit (Salimetrics, State College, PA, USA). Saliva samples and SIgA standards were added to wells in a 96 well microplate, and absorbance at A_{450} was measured, subtracting a secondary filter correction at A_{490}, using a microplate luminometer (FLUOstar OPTIMA; BMG Labtech, Aylesbury, UK). Based on 404 duplicate samples, intra-assay CV was 4.1%.

SIgA was reported in 3 ways: absolute concentration; SIgA:osmolality and SIgA secretion rate. SIgA:osmolality was calculated by dividing absolute SIgA concentration by saliva osmolality, expressed as $mg \cdot mOsmol^{-1}$. Secretion rate was calculated as the product of absolute SIgA concentration and saliva flow rate ($mL \cdot min^{-1}$), expressed as $\mu g \cdot min^{-1}$.

2.4.9 Salivary lysozyme activity

The activity of salivary lysozyme was measured using an enzymatic assay based on the work of Shugar (1952). The principle of the assay is that when a lysozyme-containing sample (saliva) is added to a suspension of *Micrococcus lysodeikticus* cells, it causes cell lysis, reducing the turbidity of the solution—as measured by a decrease in absorbance (A_{450}) using a microplate luminometer (FLUOstar OPTIMA; BMG Labtech, Aylesbury, UK).

A 50mM potassium phosphate buffer was prepared, consisting of 20.125 mL of 1 molar (M) potassium phosphate monobasic solution (Sigma-Aldrich, Massachusetts, USA), 7.375 mL of 1 M potassium phosphate dibasic solution (Sigma-Aldrich, Massachusetts, USA) and ultrapure water, to reach a final volume of 550 mL. The pH of the solution was adjusted at 25°C using either 1 M potassium hydroxide (KOH) or 1 M hydrochloric acid (HCl), to a final pH of 6.24. A substrate suspension (0.015% [w/v] *Micrococcus lysodeikticus* cell suspension) was then prepared using lyophilised *Micrococcus lysodeikticus* cells (Sigma-Aldrich, Massachusetts, USA).

The following reagents were then prepared in a low-bind 96 well microplate equilibrated to 25°C: 250 µL of substrate solution in all wells, 20 µL of potassium phosphate buffer in sample blank wells, and 20 µL of saliva in test wells. The wells were then immediately mixed and the decrease in A_{450} was measured for 5 min in the microplate luminometer. The first minute of the reaction was excluded, and the subsequent 4 min were used to calculate the lysozyme activity using the following equation:

$$Units\ per\ mL = \frac{\left(\left(\frac{\Delta A_{450}}{min\ Test}\right) - \left(\frac{\Delta A_{450}}{min\ Blank}\right)\right)}{(0.001)(0.02)}$$

Where 0.001 represents the change in A_{450} as per the unit definition, and 0.02 represents the volume (in mL) of saliva used.

Using 286 duplicate samples, intra-assay CV was 17%.

2.5 Monitoring of illness, training load and compliance

Throughout studies 2 and 3, participants received a daily email sent using an online automated survey platform (Qualtrics; Utah, USA) that included the Jackson common cold questionnaire (Jackson et al., 1958) and a self-report training log (see Appendix B). The survey also contained a question confirming whether the participant had consumed their protein shake that day. Participants were sent a personalised link via email (automatically sent by the software) each day at either 19:00 **(Chapter 5)** or 20:00 **(Chapter 4).** A reminder was then sent out 24 h later if the survey had not been completed. Training load was calculated as the product of session RPE (sRPE) and duration, and totalled weekly. It has been demonstrated that sRPE is a simple and reliable tool for monitoring training load (Foster et al., 2001), and has been shown to correlate with variables such as $\dot{V}O_2$ and HR (Herman et al., 2006).

2.6 Validation of upper respiratory illness

A URTI episode was defined according to the Jackson criteria: whereby scores for each of eight symptoms were added up per day to generate a total Jackson score. An episode was defined as an illness lasting ≥3 days, and with either (i) a total Jackson score of ≥6 and perception of having an illness, (ii) a total Jackson score of ≥6 with nasal discharge for at least 3 days, or (iii) a total Jackson score of < 6 but with the perception of having an illness and nasal discharge present for at least 3 days.

For validation of illness, participants were given a set of self-swab kits, which contained a sample swab and a tube with 1 mL stabilisation buffer within (DNA Genotek, Stittsville, Canada), along with an instruction sheet for swab collection. Participants were instructed to collect a throat and nasal swab sample on days 1 and 3 when they believed they were experiencing a URTI episode, with day 1 being the first day of symptoms, a method that has proved successful in other surveillance studies using self-swabs (Davison et al., 2025b; Goff et al., 2015).. Swab samples were returned to the lab within 30 d of collection, in accordance with manufacturer guidelines, and stored at -80°C until analysis.

2.7 Monitoring of physical wellbeing

During chapters 4 and 5, subjects completed the Cohen-Hoberman Inventory of Physical Symptoms (CHIPS; see Appendix C). In study 2, this was completed on the morning of the first experimental trial of each study arm, and in study 3, it was completed on every visit. The

CHIPS is a list of 33 common physical symptoms where subjects rate how each item has bothered or distressed them on a 5-point Likert scale ranging from “not bothered” to “extremely bothered”. A sum of the 33 items was used to create a total score, which was indicative of physical well-being (Cohen and Hoberman, 1983). This survey was administered in-person using an online survey platform (Qualtrics; Utah, USA).

2.8 Supplementation

All supplements used throughout the thesis were instructed to be consumed at a dose of 40 $g \cdot day^{-1}$, and to be taken on an empty stomach. Each study contained an MFGM-rich whey protein supplement and placebo, which were administered randomised and double-blinded. Supplements were only unblinded once all statistical analysis for the study had been completed. Nutritional analyses of study supplements, including phospholipid contents, are included in each experimental chapter.

Chapter 3. Study 1 – Effects of 2 weeks of milk fat globule membrane supplementation on exercise-induced increases in gut damage and permeability

3.1 Abstract

Exercise has been associated with damage to gut epithelial cells and tight junctions, leading to a transient state of increased gut permeability. Previous research indicates that MFGM may be beneficial for GI integrity, so the purpose of this study was to determine whether short-term MFGM supplementation affects gut damage and permeability following strenuous exercise. Twelve healthy active males (age 27.4 ± 9.0 years; height 178.1 ± 8.6 cm; BM 74.1 ± 11.9 kg; $\dot{V}O_{2max}$ 57.3 ± 6.4 $mL \cdot kg^{-1} \cdot min^{-1}$) participated in a double-blind, crossover, randomised controlled trial. Participants consumed MFGM or a placebo daily for 2 weeks before completing an exercise trial (20 min run at 80% of $\dot{V}O_{2max}$). Urinary L/R was measured at baseline (pre-supplementation) and Post-Ex, as a marker of gut permeability. Venous blood samples were taken at Pre-Ex, Post-Ex and 1 h Post-Ex for analysis of I-FABP, sCD14 and bacterial composition. GI symptom incidence was measured on days 7, 14 and 21 of each arm. Urinary L/R significantly increased from baseline in the PLA trial ($p = 0.048$), but not in the MFGM trial ($p = 0.556$), and was significantly lower in the MFGM trial compared to PLA ($p = 0.033$). The change in plasma I-FABP from Pre-Ex to Post-Ex ($p = 0.001$) and Pre-Ex to 1 h Post-Ex ($p = 0.036$) was significantly lower in the MFGM arm. No changes were observed for plasma sCD14, blood bacterial composition and GI symptoms ($p > 0.05$). These findings demonstrate that MFGM is able to protect the gut from increases in gut damage and permeability following strenuous exercise.

3.2 Introduction

During exercise, various physiological responses occur, such as an increase in core temperature and redirected splanchnic blood flow to working skeletal muscles and other tissues, such as the skin for thermoregulation. Whilst these are normal responses to exercise, they have shown to contribute to transient intestinal injury, causing a temporary state of elevated gut permeability (Marchbank et al., 2011). Although the semi-permeable function of the gut is crucial for controlling the passage of substances, including nutrients and water across the gut into circulation, a compromised gut barrier due to epithelial or tight junction damage may allow undesirable and potentially harmful luminal contents across the gut barrier (Chelakkot et al., 2018). Translocation of contents such as bacteria and endotoxins across the gut barrier is also often coupled with higher incidence of GI symptoms (Costa et al., 2022a). Furthermore, cases of elevated bacteraemia or endotoxaemia may contribute to systemic inflammation or in some severe cases, sepsis (Butler-Laporte et al., 2018). It has also been suggested that exercise-induced increases in gut permeability may be a potential factor contributing to exertional heatstroke (Lim and Mackinnon, 2006).

A range of nutritional supplements/experiments have been researched for their effects on GI integrity following exercise including acute macronutrient supplementation (e.g. carbohydrates, amino acids), dietary changes (e.g. high and low fermentable oligosaccharides, disaccharides, monosaccharides and polyols [FODMAP] diets), and specialised supplements (e.g. biotics, curcumin) (Chantler et al., 2022; Costa et al., 2025). Dairy-derived supplements have also received considerable attention, demonstrating protective effects on the gut, possibly due to their richness in bioactive components. At present, there is little research on the immune and gut effects of MFGM in healthy adults, and the findings that do exist are contrasting (Ten Bruggencate et al., 2016; Ulfman et al., 2022). However, another bioactive dairy supplement, bovine colostrum, has shown to mitigate increases in gut damage and permeability following strenuous exercise (Davison et al., 2016; March et al., 2019, 2017; Marchbank et al., 2011), and many of the nutraceutical effects of bovine colostrum may be attributed to the low molecular weight (< 50 kDa) components within (Davison, 2021). Since MFGM and bovine colostrum may possess some similar low molecular weight components, especially since MFGM is a significant component of bovine colostrum, it is possible that some similar results may be seen. MFGM also contains other bioactive components that may influence gut integrity, like lactadherin, a protein known for its anti-inflammatory properties and implication in the removal of apoptotic cells (Ait-Oufella et al., 2007), as well as being associated with maintenance of gut barrier integrity (Shen et al., 2019).

The present study aimed to assess whether 2-week MFGM supplementation attenuates the exercise-induced increase in gut permeability, as measured by urinary L/R. Secondary aims of the study were to determine whether MFGM affects exercise-induced changes in I-FABP, 16S bacterial translocation, sCD14, and whether these changes influence gut symptom incidence. It was hypothesised that exercise would increase these markers of gut damage and permeability, but MFGM would attenuate these perturbations.

3.3 Methods

3.3.1 Ethical approval

All procedures in the present study were approved by the University of Kent Sport, Exercise, and Rehabilitation Sciences REAG (Prop 75_2024) and were in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to enrolling on the study. The study was prospectively registered on the ISRCTN registry (ISRCTN10822766, https://doi.org/10.1186/ISRCTN10822766). ISRCTN is a primary clinical study registry recognised by the World Health Organisation (WHO) and the International Committee of Medical Journal Editors (ICMJE).

3.3.2 Power calculation

No previous research has used MFGM for a study of this kind, so a preliminary power calculation was performed (G*Power version 3.1.9.7, Kiel, Germany) using data from a previous study of bovine colostrum (Marchbank et al., 2011), which may have some similar mechanisms to MFGM. For the primary outcome of this study, urinary L/R ratio, there was a large effect size for the effect of bovine colostrum vs placebo (Cohen's $f = 2.77$). Using a conservative assumption that the effect size of MFGM was 20% of that of bovine colostrum, an estimated effect size of Cohen's $f = 0.55$ was used. Power calculations incorporating a correlation among repeated measures of 0.59 and an epsilon value of 0.514 indicated that a sample size of $n = 10$ would be required to detect a significant difference between supplements from baseline to post-exercise (ANOVA, repeated measures, within-subjects) at 80% power and alpha level 0.05. A target of $n = 12$ was set to account for a dropout rate of up to 20%, although no participants dropped out, so the final sample size was $n = 12$.

3.3.3 Participants

Males and females were eligible to participate: No females volunteered. Twelve healthy, recreationally active males participated in the study (age 27.4 ± 9.0 years; height 178.1 ± 8.6 cm; BM 74.1 ± 11.9 kg; $\dot{V}O_{2max}$ 57.3 ± 6.4 $mL \cdot kg^{-1} \cdot min^{-1}$; peak speed in incremental test 17.6 ± 1.5 $km \cdot h^{-1}$; treadmill speed at 80% $\dot{V}O_{2max}$ 11.9 ± 1.8 $km \cdot h^{-1}$). All participants were free from injury or known medical conditions and were screened prior to enrolling via a standardised health questionnaire (see Appendix) to determine study suitability.

3.3.4 Study design

A double-blind, placebo-controlled, randomised controlled design was employed. Participants attended the laboratory on 6 occasions: at baseline, a $\dot{V}O_{2max}$ trial, and a main trial, for 2 crossover arms. At baseline, subjects completed a permeability measurement via urinary L/R recovery and were randomly assigned to consume either 40 g·day^{-1} of MFGM-rich whey protein concentrate (MFGM) or a placebo protein (PLA) daily for 2 weeks. $\dot{V}O_{2max}$ was assessed between days 7-11. On day 15 of each arm, participants attended the laboratory for the main trial, consisting of a 20 min run at the speed equivalent to 80% of $\dot{V}O_{2max}$ followed by assessments of gut damage and permeability. Prior to the first main trial, participants completed a 24 h food diary and were instructed to replicate this diet for the subsequent main trial. Following the first main trial, a 2-week washout period was performed, after which participants began the second crossover arm with the alternative supplement (Figure 3.1).

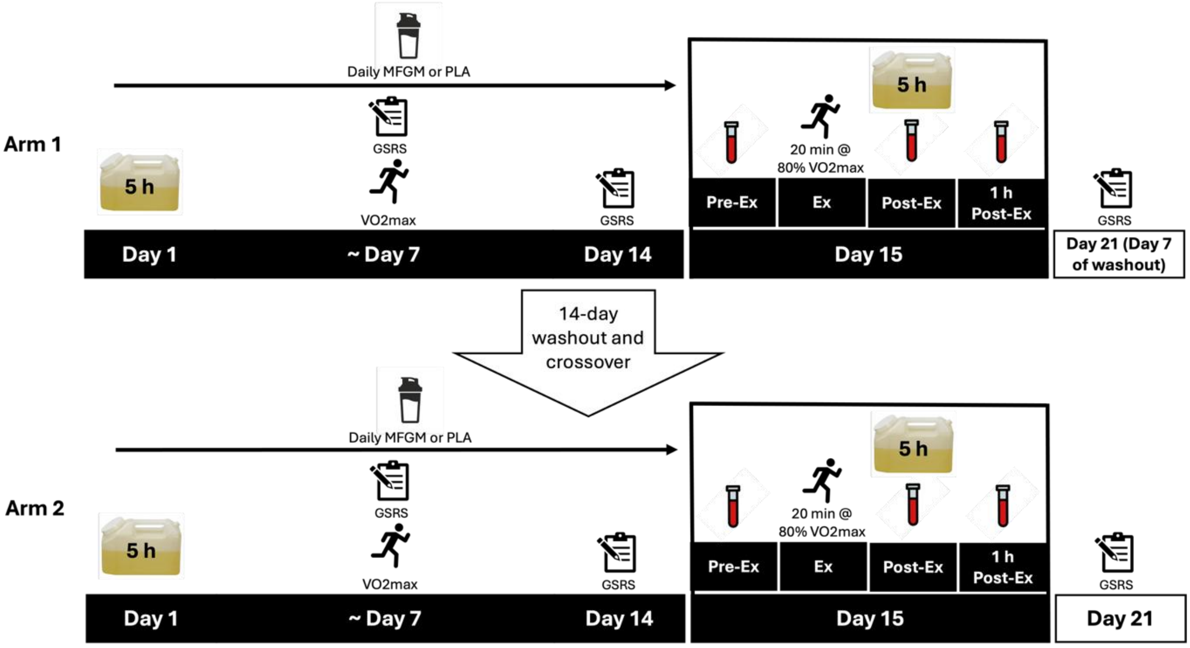

Figure 3.1. Schematic of Chapter 3 protocol. GSRS, gastrointestinal symptoms rating scale.

3.3.5 Supplementation

Participants consumed an MFGM-rich whey protein concentrate (MFGM) or a placebo (PLA) consisting of a whey protein isolate (Table 3.1). Supplements were consumed at a dose of 40 $g \cdot day^{-1}$ from day 0-14. Both supplements were flavoured so they were indistinguishable and were allocated double-blind and randomised. Randomisations were performed using an online randomisation tool (https://www.graphpad.com). Phospholipids are generally not present in whey protein supplements unless added during the instantisation process. The PLA designed did not match the fat profile of MFGM due to potential gut-modulatory effects associated with fat substitutes (Rial et al. 2016). Moreover, the difference in fat is likely to have very little effect on daily fat intake and thus no biological effect on the study outcomes. Supplements were provided in anonymous food-grade tubs and returned upon completion of each arm; the volume of powder returned was visually inspected to monitor supplement compliance.

Table 3.1. Nutritional analysis of Chapter 3 supplements.

	PLA	MFGM
Energy (Kcal/40 g)	146	157
Carbohydrates (%)	2	Not determined
Protein (%)	92	70.44
Fat (%)	0.4	11.11
Total phospholipids (mg·g^{-1})	3.1	53.1
Sphingomyelin (mg·g^{-1})	0.9	14.0
Phosphatidylcholine (mg·g^{-1})	0.8	14.7
Phosphatidylethanolamine (mg·g^{-1})	0.7	13.9
Phosphatidylinositol (mg·g^{-1})	0.2	3.2
Phosphatidylserine (mg·g^{-1})	0.3	4.8
Other (mg·g^{-1})	0.1	2.7

3.3.6 Urinary L/R test

Gut permeability was assessed using the previously established urinary L/R ratio technique (Davison et al., 2016; March et al., 2017; Marchbank et al., 2011; Playford et al., 2001). The urinary L/R method involves the oral ingestion of non-metabolisable sugars – lactulose, rhamnose and mannitol – followed by measurement of their urinary excretion over the following 5 hours. Rhamnose and mannitol are monosaccharides that cross the gut barrier via transcellular and paracellular pathways, reflecting normal gut barrier function. Lactulose is a larger disaccharide that does not usually permeate a 'healthy' gut barrier, but in periods of elevated gut permeability can permeate through paracellular pathways (Travis and Menzies, 1992). Thus, an elevated L/R (or L/M) is indicative of increased intestinal permeability, reflecting a compromised gut barrier.

All permeability assessments were performed following an overnight fast of at least 10 hours. Participants were instructed to empty their bladder before drinking a standardised beverage containing 5 g lactulose, 2 g mannitol and 1 g rhamnose in 450 mL of water. Urine was then collected over the next 5 h, with participants remaining fasted and resting (but allowed to continue their usual day) throughout the collection period. Water was consumed ad libitum, providing a minimum of 2 L was consumed, to ensure adequate urinary output. Aliquots were centrifuged at 3,000 × *g* for 10 min at 4 °C and ~14 mL of supernatant was frozen at -80 °C for batch analysis at a later date. As previously described (Marchbank et al., 2011), the sugars were separated using high performance liquid chromatography (HPLC) and quantified using pulsed amperometric detection in an ISO 15189-accredited laboratory (Clinical Chemistry, Royal Cornwall Hospitals NHS Trust).

3.3.7 Preliminary visits

On day 0, participants attended the laboratory to enrol on the study (complete all documentation and screening) and complete a baseline urinary L/R permeability test using the previously described urinary L/R test. Between days 7-11, $\dot{V}O_{2max}$ was measured using an incremental step test (1 $km \cdot h^{-1} \cdot min^{-1}$ step rate) on a motorised treadmill (Pulsar 3P; h/p/cosmos Sport and Medical, Nussdorf-Traunstein, Germany). All participants were required to wear a harness throughout the duration of the test and began with a self-paced warm up equivalent to 11 (light) on the 6-20 Borg RPE scale (Borg, 1982). The step test then began at 1 $km \cdot h^{-1}$ faster than the warmup and increased by 1 $km \cdot h^{-1}$ every minute until volitional exhaustion. Measurements of gas exchange were conducted as described in Chapter 2.3 (General Methods).

Exercise intensity for the main trial was calculated as a treadmill speed that would elicit 80% $\dot{V}O_{2max}$. Using the final 20 s of each step, the (submaximal) linear relationship between $\dot{V}O_2$ and treadmill speed was used to calculate the speed equivalent of 80% $\dot{V}O_{2max}$. The speed was then reduced by two thirds of the step rate (0.66 $km \cdot h^{-1}$) to account for the lag in $\dot{V}O_2$ response. This model has been proven safe and effective at inducing desired responses (gut injury) by previous studies in this area (Davison et al., 2016; March et al., 2017; Marchbank et al., 2011).

3.3.8 Main trials

On day 15 of each arm, participants attended the laboratory between 08:00 and 10:00. Participants were seated for 10 min with minimal movement prior to a resting blood sample (Pre-Ex) being drawn – 6 mL into a lithium heparin-treated vacutainer and 6 mL into a K_2EDTA-treated vacutainer. All exercise in this trial was performed on the same treadmill used in the $\dot{V}O_{2max}$ test, and at temperate conditions (temperature 21.6 ± 0.8 °C and relative humidity 20.5 ± 3.6%). A 5 min warmup was performed prior to the main exercise protocol, involving 1 min walking at 4 km·h^{-1}, 2 min running at (speed equivalent to) 45% $\dot{V}O_{2max}$ and 2 min running at 55% $\dot{V}O_{2max}$. Following this, a 20 min run at 80% $\dot{V}O_{2max}$ was performed. RPE and HR was measured at 5 min intervals. HR was measured using a HR telemetry band and monitor (Polar T31, Polar Instruments, Kempele, Finland). Expired gases were measured breath-by-breath (Cortex Metalyser 3b; Leipzig, Germany) throughout the test and mean $\dot{V}O_2$ was calculated for every 5 min of the test. Following the exercise test, a blood sample was taken immediately Post-Ex, after which participants began the gut permeability assessment previously described. A final blood sample was also taken after 1 h of seated rest (1 h Post-Ex).

3.3.9 Leukocyte counts

WBC differential counts were analysed at Pre-Ex, Post-Ex and 1 h Post-Ex on main trial days using the methods described in Chapter 2.4.2 (General Methods).

3.3.10 Plasma I-FABP

I-FABP was measured at Pre-Ex, Post-Ex and 1 h Post-Ex on main trial days as described in Chapter 2.4.3 (General Methods).

3.3.11 Plasma sCD14

Plasma sCD14 concentration was analysed in lithium-heparin treated plasma samples taken at Pre-Ex, Post-Ex and 1 h Post-Ex using a commercially available ELISA kit (Quantikine, DC140; R&D Systems, MN, USA). All tests were performed in accordance with the manufacturer's instructions, and all samples were performed in duplicate. Over 71 duplicate samples, intra-assay CV was 5.75%.

3.3.12 DNA extraction

Following the manufacturer’s guidelines, DNA was extracted from 200 µL lithium heparin-treated whole blood using a Quick-DNA Fungal/Bacterial Miniprep Kit (D6005. Zymo Research, Irvine, CA, USA). Individually packaged, sterile (DNase, RNase, endotoxin and pyrogen-free) pipette tips and microcentrifuge tubes were used throughout the extraction process to minimise contamination. Following the final elution, the isolated DNA was frozen immediately and stored at -80 °C for later analysis.

3.3.13 Bacterial translocation

Bacterial load was measured in whole blood at Pre-Ex, Post-Ex and 1 h Post-Ex using qPCR, and 16S rRNA gene sequencing was performed to provide compositional insights into bacterial translocation, as outlined in Chapter 2.4.4 and 2.4.5 (General Methods).

3.3.14 Gut symptoms

Weekly GI symptoms were measured using the GSRS, completed on days 7, 14 and 21 of each arm. The GSRS is a 15-item questionnaire assessing the severity of known GI symptoms over the previous week. The questionnaire rates the symptoms on a 7-point scale from "no discomfort" to "very severe discomfort". The GSRS was administered via an online automated survey platform (Qualtrics, Utah, USA). Survey links were sent by e-mail at 09:00, and a reminder want sent 24 h later if the survey had not been completed.

3.3.15 Statistical analysis

Statistical analyses were performed using SPSS v31.0 (IBM, Armonk, NY), with the exception of 16S amplicon analysis which was conducted as described in Chapter 2.4.4 (General Methods). Shapiro-Wilk tests and Z-scores calculated for skewness and kurtosis were used to assess data for normal distribution. Data with a non-normal distribution was normalised prior to analysis with parametric tests. Missing data were imputed using estimation-maximisation and Little's test was performed to verify that data were missing completely at random (Grove and Cipher, 2024).

The following transformations were used to normalise data for parametric tests:

Log: I-FABP (absolute and % of Pre-Ex). Square root: GSRS scores.

One-way repeated measures (time) ANOVA was used for the analysis of urinary L/R. Bonferroni-adjusted post-hoc *t*-tests were used to follow up a significant main effect of time, comparing values across baseline and main trials. Two-way (trial [within] × time [within]) repeated measures (RM) analysis of variances (ANOVAs) was used for analysis of the following variables: $\dot{V}O_2$ ($L \cdot min^{-1}$ and % of $\dot{V}O_{2max}$); HR; RPE; total and differential leukocyte counts; I-FABP (absolute and % of Pre-Ex); sCD14, bacterial load and GSRS scores. Post-hoc contrasts were performed to follow up any significant trial × time interactions, comparing time points to Pre-Ex. Post-hoc *t*-tests were used following significant main effects of time, to compare adjacent time points. The proportion of samples with bacterial DNA present in each trial was analysed using chi-squared analysis.

Analysis of 16S rRNA gene sequencing data was performed as described in Chapter 2.4.4.2 (General Methods). Analysis of 16S rRNA gene sequencing was performed using R (version 4.5.1; R Core Team 2025). The following packages were used: devtools, phyloseq, ggplot2, dplyr, ggpubr, BiocManager, microbiome, vegan, microViz, Polychrome, ape, corrplot, microbial and ggrepel.

3.4 Results

3.4.1 Exercise physiological and perceptual measures

Due to a technical issue with indirect calorimetry, $\dot{V}O_2$ could not be measured in one trial. Thus, all $\dot{V}O_2$ analyses were performed on $n = 11$. There was no main effect of trial, $F(1, 10) = 0.125$, $p = 0.731$ for $\dot{V}O_2$ ($L \cdot min^{-1}$). There was a significant main effect of time, $F(1.145, 11.446) = 47.451$, $p < 0.001$. There was no significant trial × time interaction, $F(3, 30) = 0.222$, $p = 0.880$ detected. Post-hoc Bonferroni-corrected *t*-tests, to follow-up the main effect of time, revealed a significant increase in $\dot{V}O_2$ (L·min^{-1}) from 5 min to 10 min ($p < 0.001$) and 10 min to 15 min ($p = 0.004$) (Figure 3.2). No main effect of trial, $F(1, 10) = 0.053$, $p = 0.823$ for $\dot{V}O_2$ (% of $\dot{V}O_{2max}$) was detected. There was a significant main effect of time, $F(1.099, 10.986) = 37.125$, $p < 0.001$. No significant trial × time interaction, $F(3, 30) = 0.324$, $p = 0.808$ was detected. Post-hoc Bonferroni-corrected *t*-tests revealed a significant increase in $\dot{V}O_2$ (% of $\dot{V}O_{2max}$) from 5 min to 10 min ($p < 0.001$) and 10 min to 15 min ($p = 0.002$) (Figure 3.2).

There was no main effect of trial, $F(1,11) = 0.248$, $p = 0.629$ for trial HR. A significant main effect of time was observed, $F(3, 33) = 18.234$, $p < 0.001$. There was no significant trial × time interaction observed, $F(3, 33) = 0.517$, $p = 0.673$. Post-hoc Bonferroni-corrected *t*-tests revealed significant increases in HR from 5 min to 10 min ($p = 0.003$), 10 min to 15 min ($p < 0.001$) and 15 min to 20 min ($p < 0.001$) (Figure 3.2).

No main effect of trial, $F(1, 11) = 0.249$, $p = 0.627$ was observed for trial RPE. A significant main effect of time was detected, $F(1.421, 15.628) = 45.353$, $p < 0.001$. There was no significant trial × time interaction observed, $F(3, 33) = 2.151$, $p = 0.113$. Post-hoc Bonferroni-adjusted *t*-tests revealed significant increases in RPE from 5 min to 10 min ($p < 0.001$), 10 min to 15 min ($p < 0.001$) and 15 min to 20 min ($p = 0.006$) (Figure 3.2).

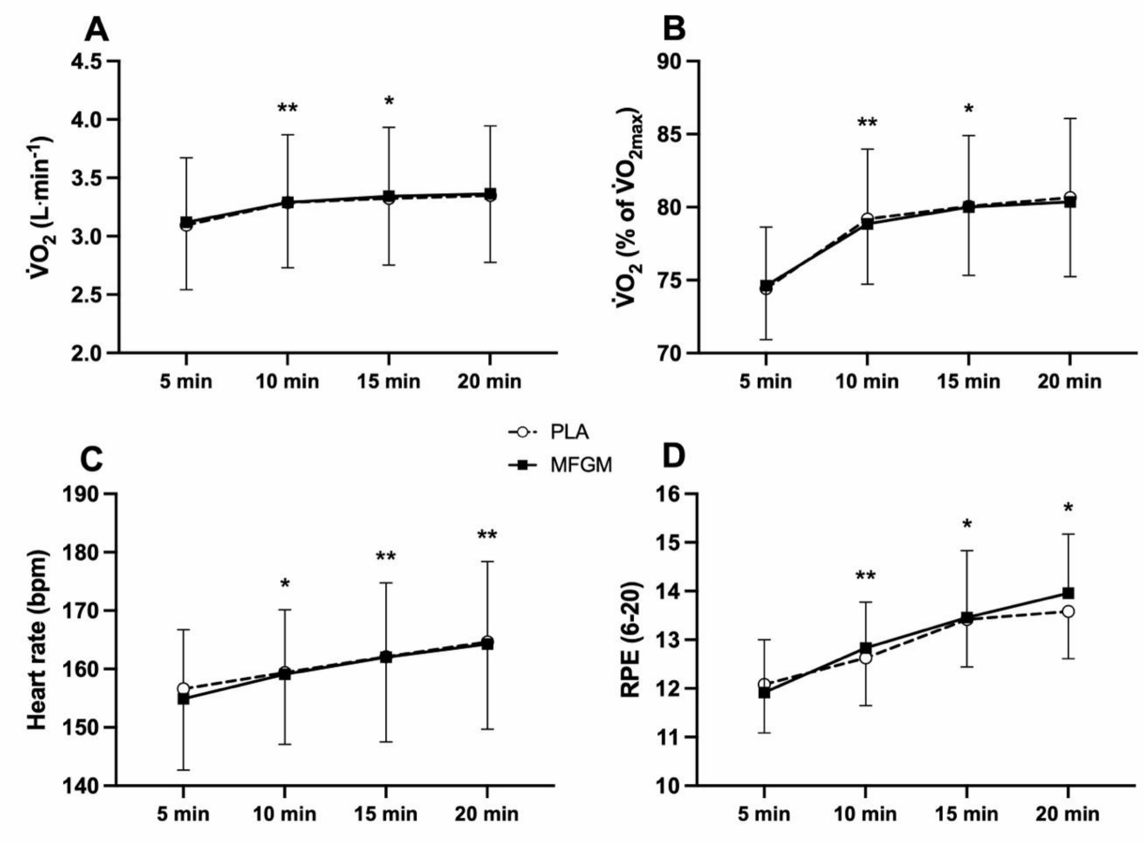

Figure 3.2. Exercise physiological and perceptual responses. A- $\dot{V}O_2$ (L·min^{-1}).. Two-way ANOVA main effects: $p = 0.731$ (trial); $p < 0.001$ (time); $p = 0.880$ (trial × time interaction). B- $\dot{V}O_2$ (% of $\dot{V}O_{2max}$). $p = 0.823$ (trial); $p < 0.001$ (time); $p = 0.808$ (trial × time interaction). C- Heart rate (bpm). Two-way ANOVA main effects: $p = 0.629$ (trial); $p < 0.001$ (time); $p = 0.673$ (trial × time interaction). D- RPE. Two-way ANOVA main effects: $p = 0.627$ (trial); $p < 0.001$ (time); $p = 0.113$ (trial × time interaction). * Mean values were significantly different from previous time point ($p < 0.05$; post-hoc analysis). ** Mean values were significantly different from previous time point ($p < 0.001$); post-hoc analysis).

3.4.2 Leukocyte counts

There was a significant main effect of time, $F(1.212, 13.331) = 36.547$, $p < 0.001$ for total leukocyte count. There was no significant main effect of trial, $F(1, 11) = 0.121$, $p = 0.735$ or trial × time interaction, $F(2, 22) = 0.974$, $p = 0.393$. Bonferroni-adjusted post-hoc tests showed a significant increase from Pre-Ex to Post-Ex ($p < 0.001$) (Table 3.2).

There was a significant main effect of time, $F(1.245, 13{,}699) = 29.650$, $p < 0.001$ for neutrophil count. No significant main effect of trial, $F(1, 11) = 0.014$, $p = 0.908$ or trial × time interaction, $F(2, 22) = 1.802$, $p = 0.188$ was detected. Post-hoc Bonferroni-corrected *t*-tests showed significant increases from Pre-Ex to Post-Ex ($p < 0.001$) and 1 h Post-Ex ($p = 0.004$). There was a significant main effect of time, $F(1.203, 13.235) = 34.449$, $p < 0.001$ for lymphocyte counts, but no significant main effect of trial, $F(1, 11) = 0.349$, $p = 0.566$ or trial × time interaction, $F(1.358, 14.937) = 2.823$, $p = 0.105$. Post-hoc Bonferroni-adjusted *t*-tests revealed a significant increase from Pre-Ex to Post-Ex ($p < 0.001$).

There was a significant main effect of time, $F(2, 22) = 31.274$, $p < 0.001$ and a significant trial × time interaction, $F(2, 22) = 4.914$, $p = 0.017$ for N/L ratio. There was no main effect of trial, $F(1, 11) = 0.836$, $p = 0.380$. Post-hoc contrasts revealed that the increase from Pre-Ex to 1 h Post-Ex was significantly higher in the PLA arm ($p = 0.044$). One-way ANOVAs performed on each arm showed a main effect of time, $F(1.229, 13.519) = 30.596$, $p < 0.001$ for the PLA arm, with a significant increase from Pre-Ex to 1 h Post-Ex ($p < 0.001$). A one-way ANOVA performed on the MFGM arm showed no main effect of time, $F(2, 22) = 3.091$, $p = 0.066$.

There was a significant main effect of time $F(2, 22) = 3.569$, $p = 0.045$ for monocyte counts, but there was no significant main effect of trial, $F(1, 11) = 0.008$, $p = 0.931$ or trial × time interaction, $F(2, 22) = 0.950$, $p = 0.402$. Post-hoc Bonferroni-adjusted *t*-tests showed a significant increase from Pre-Ex to Post-Ex ($p = 0.038$). There was a significant main effect of time, $F(2, 22) = 6.302$, $p = 0.007$ for eosinophil count. There was no main effect of trial, $F(1, 11) = 1.719$, $p = 0.217$ or trial × time interaction, $F(2, 22) = 1.47$, $p = 0.252$ observed. Post-hoc revealed a significant decrease from Pre-Ex to 1 h Post-Ex ($p = 0.004$).

Table 3.2. Leukocyte responses to strenuous exercise.

$10^9 \cdot L^{-1}$	Pre-Ex	Post-Ex	1 h Post-Ex	*p* trial time interaction
Total leukocytes				0.735
PLA	4.46 ± 0.79	6.83 ± 1.16	5.06 ± 1.20	< 0.001**
MFGM	4.63 ± 0.79	6.68 ± 1.09	5.20 ± 1.24	0.393
Neutrophils				0.908
PLA	2.09 ± 0.46	3.42 ± 0.64	3.10 ± 0.87	< 0.001**
MFGM	2.28 ± 0.67	3.33 ± 0.72	2.94 ± 0.89	0.188
Lymphocytes				0.566
PLA	2.02 ± 0.47	3.03 ± 0.59	1.69 ± 0.40	< 0.001**
MFGM	2.02 ± 0.35	2.90 ± 0.69	1.94 ± 0.46	0.105
N/L ratio				0.380
PLA	1.05 ± 0.23	1.18 ± 0.23	1.89 ± 0.45	< 0.001**
MFGM	1.15 ± 0.35	1.22 ± 0.44	1.45 ± 0.39	0.017*
Monocytes				0.931
PLA	0.20 ± 0.08	0.26 ± 0.09	0.22 ± 0.06	0.045*
MFGM	0.21 ± 0.09	0.28 ± 0.12	0.20 ± 0.04	0.402
Eosinophils				0.217
PLA	0.16 ± 0.08	0.15 ± 0.07	0.09 ± 0.05	0.007*
MFGM	0.13 ± 0.09	0.13 ± 0.08	0.10 ± 0.07	0.252

* Significant main effect ($p < 0.05$). ** Significant main effect ($p < 0.001$).

3.4.3 Urinary L/R ratio

Some baseline samples showed greater variability than expected, so the mean of both baselines was used for comparison with the main trials. A one-way ANOVA revealed a significant main effect of time, $F(2,22) = 7.781$, $p = 0.003$, for urinary L/R. Post-hoc *t*-tests with the Bonferroni correction applied showed a significant increase in urinary L/R from baseline to PLA trial ($p =$

0.029) but not the MFGM trial (*p* = 1.000). Urinary L/R was also significantly greater in the PLA trial compared to the MFGM trial (*p* = 0.042) (Figure 3.3).

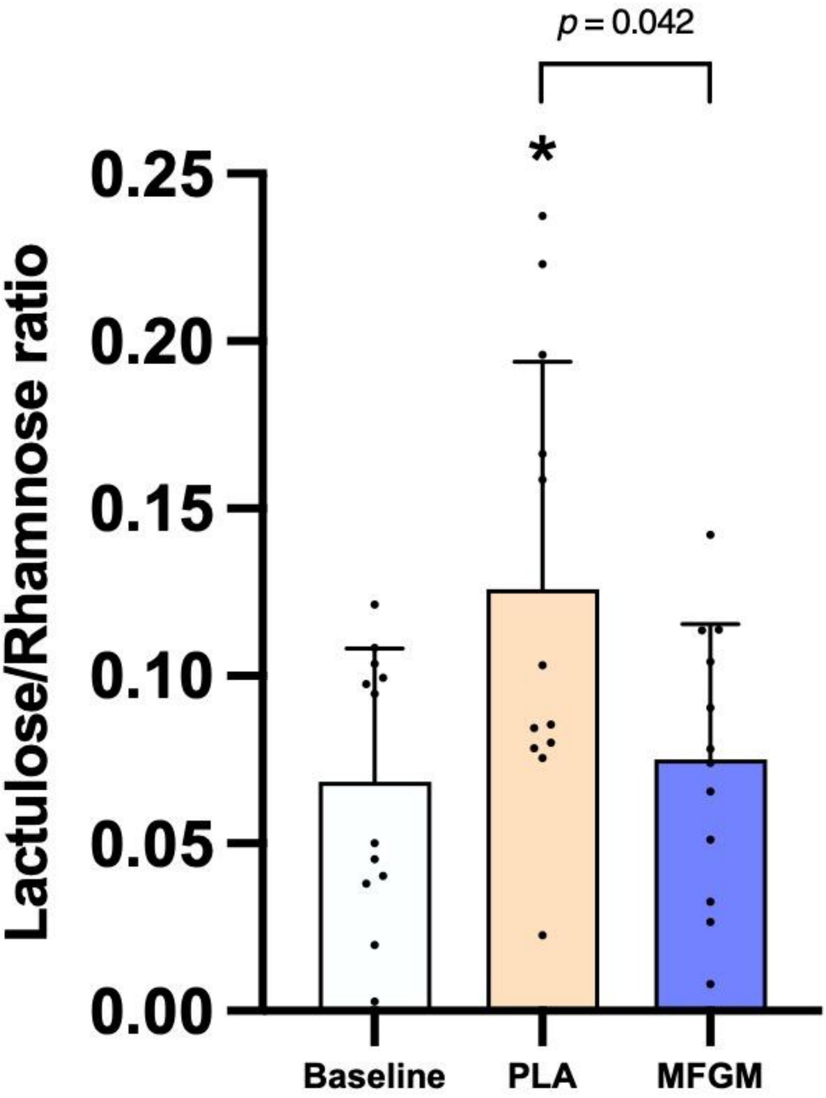

Figure 3.3. Urinary L/R ratio at baseline and Post-Ex on main trials. One-way ANOVA main effect: $p = 0.003$ (time). * Mean values were significantly higher than baseline ($p < 0.05$; post-hoc analysis). PLA trial was significantly higher than MFGM trial ($p = 0.042$; post-hoc analysis)

3.4.4 Plasma I-FABP

There was a significant main effect of time, $F(2, 22) = 6.541$, $p = 0.006$, but not trial, $F(1, 11) = 2.511$, $p = 0.141$ for absolute I-FABP concentrations. There was a significant trial × time interaction, $F(1.332, 14.654) = 7.693$, $p = 0.010$. Bonferroni-adjusted post-hoc tests revealed that the changes from Pre-Ex to Post-Ex (p = 0.001) and 1 h Post-Ex (p = 0.026) were significantly higher in the PLA arm. One-way ANOVAs performed on each arm showed a significant main effect of time, $F(1.262, 13.886) = 7.750$, $p = 0.011$ in the PLA arm, with Bonferroni-adjusted post-hoc tests showing a significant increase from Pre-Ex to Post-Ex ($p < 0.001$). A significant main effect of time, $F(2, 22) = 5.292$, $p = 0.013$ was detected in the MFGM arm, post-hoc tests did not show any time-specific significant comparisons (Figure 3.4).

There was a significant main effect of trial, $F(1, 11) = 13.589$, $p = 0.004$ for I-FABP% change from Pre-Ex, with MFGM significantly lower than PLA. A significant main effect of time, $F(2, 22) = 7.281$, $p = 0.004$ and trial × time interaction, $F(1.310, 14.413) = 7.199$, $p = 0.013$ were also detected. Bonferroni-adjusted post-hoc tests revealed that the changes from Pre-Ex to Post-Ex ($p = 0.001$) and 1 h Post-Ex ($p = 0.036$) were significantly higher in the PLA arm. One-way ANOVAs performed on each arm showed a main effect of time, $F(1.363, 14.991) = 8.431$, $p = 0.007$ in the PLA arm, with Bonferroni-adjusted comparisons revealing a significant increase from Pre-Ex to Post-Ex ($p < 0.001$). A significant main effect of time, $F(2, 22) = 5.292$, $p = 0.013$ was seen in the MFGM arm, but no significant Bonferroni-adjusted post-hoc comparisons were detected (Figure 3.4).

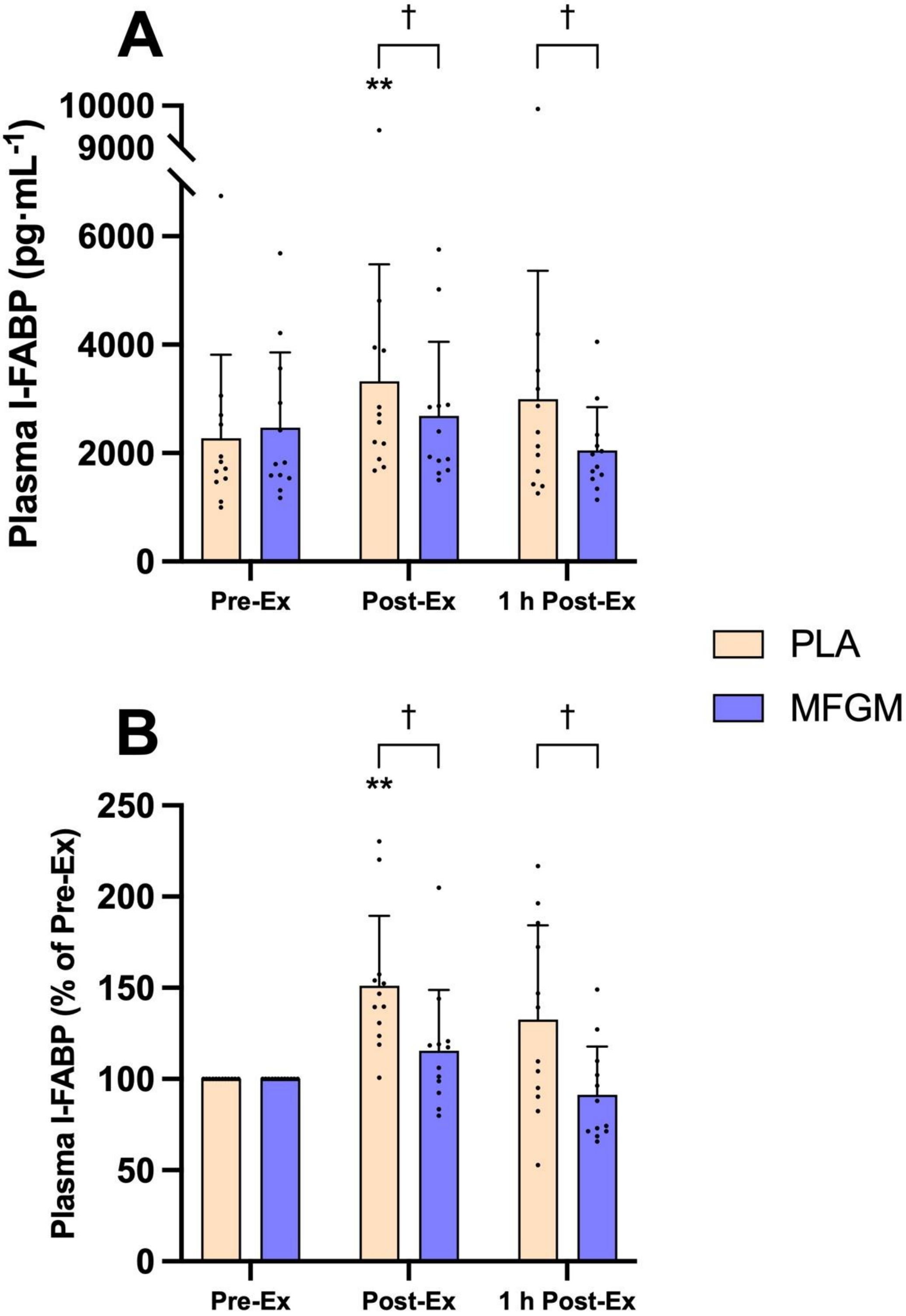

Figure 3.4. I-FABP responses following strenuous exercise. A- Plasma I-FABP concentration (pg·mL^{-1}). Two-way ANOVA main effects: $p = 0.141$ (trial); $p = 0.006$ (time); $p = 0.010$ (trial × time interaction). B- Plasma I-FABP% of mean values at Pre-Ex. Two-way ANOVA main effects: $p = 0.004$ (trial); $p = 0.004$ (time); $p = 0.013$ (trial × time interaction). ** Mean values were significantly different from Pre-Ex ($p < 0.001$; post-hoc analysis). † Change from Pre-Ex was significantly different between trials ($p < 0.05$; post-hoc analysis).

3.4.5 Plasma sCD14

A two-way ANOVA detected a significant main effect of time, $F(1.291, 14.197) = 6.328$, $p = 0.019$ for plasma sCD14 concentration. No main effect of trial, $F(1, 11) = 0.103$, $p = 0.755$ or trial × time interaction, $F(1.309, 14.394) = 1.273$, $p = 0.292$ was detected. Bonferroni-adjusted post-hoc *t*-tests revealed a significant increase from Pre-Ex to Post-Ex ($p = 0.003$) (Figure 3.5).

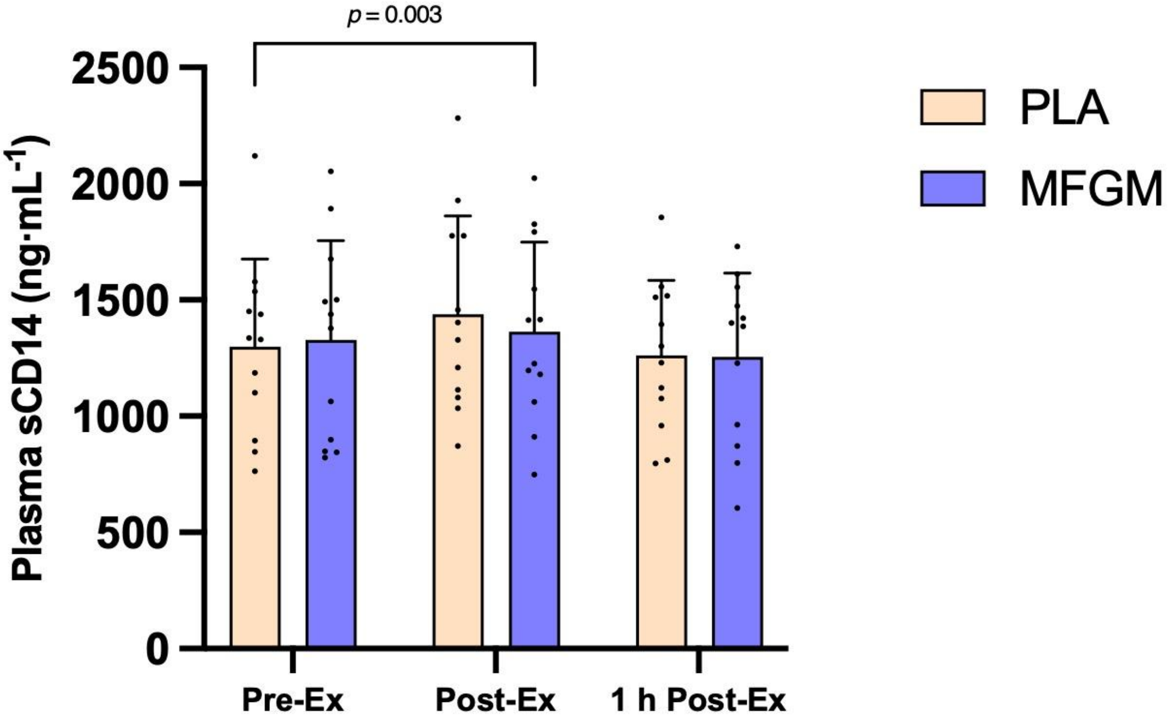

Figure 3.5. sCD14 responses to strenuous exercise. sCD14 concentration (ng·mL). Two-way ANOVA main effects: p = 0.755 (trial); p = 0.019 (time); p < 0.292 (trial × time interaction). Significant change from Pre-Ex to Post-Ex (p = 0.003; post-hoc analysis).

3.4.6 Bacterial translocation

There was a significant main effect of time, $F(2, 22) = 5.821$, $p = 0.009$ for blood bacterial load. There was no main effect of trial, $F(1, 11) = 0.785$, $p = 0.395$ or trial × time interaction, $F(2, 22) = 0.098$, $p = 0.907$. Bonferroni-adjusted post-hoc t-tests showed a significant increase from Pre-Ex to Post-Ex ($p = 0.009$) (Figure 3.6).

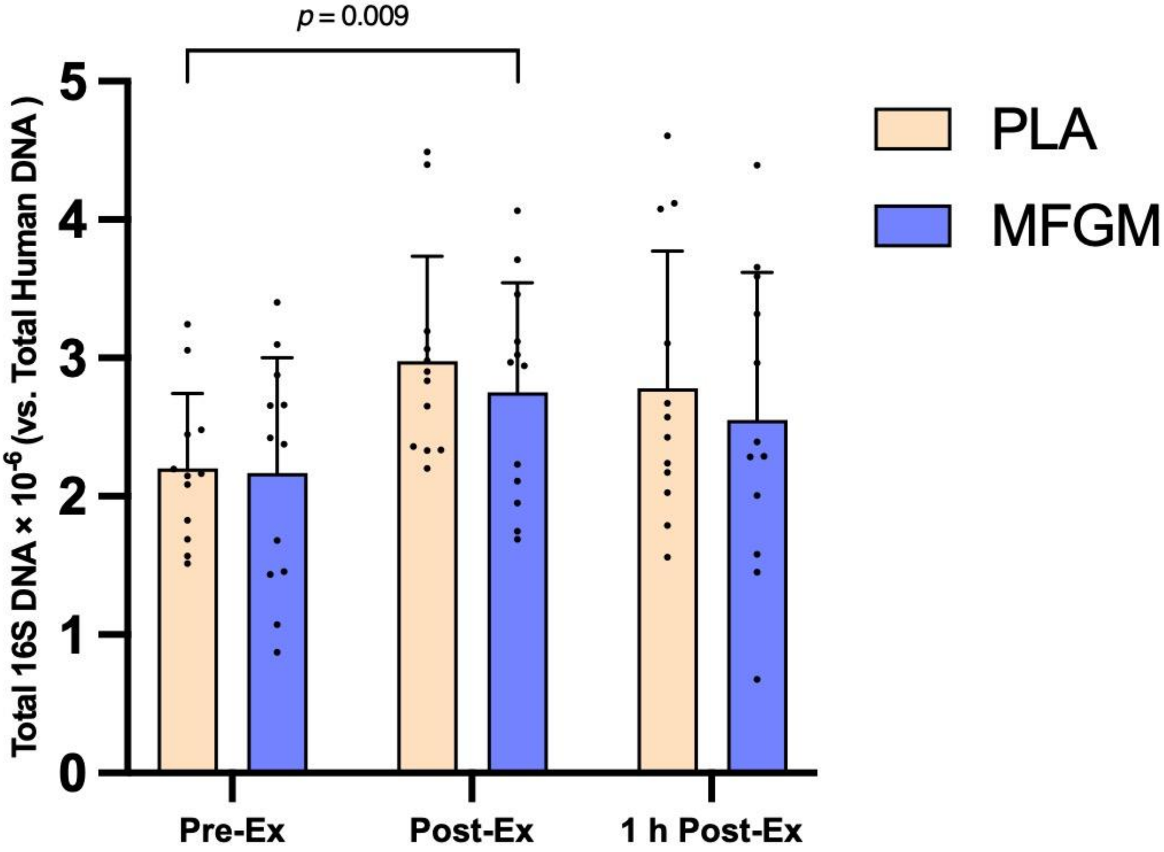

Figure 3.6. Bacterial load responses to strenuous exercise: Total 16S DNA/Total Human DNA ratio (× 10^{-6}). Two-way ANOVA main effects: p = 0.395 (trial); p = 0.009 (time); p = 0.907 (trial × time interaction). Significant increase from Pre-Ex to Post-Ex (p = 0.009; post-hoc analysis).

After QC and filtering, 42 (21 PLA. 21 MFGM) samples contained sufficient bacterial DNA for amplification. There was no difference in the proportion of samples present for bacterial DNA between PLA (58.3%, 21/36 samples) and MFGM (60%, 21/35 samples), χ^2 (1) = 0.020, p = 0.886. There was no difference in proportion of samples with bacterial DNA from Pre-Ex (54.2%, 13/24 samples) to Post-Ex (54.2%, 13/24 samples) and 1 h Post-Ex (69.6%, 16/23 samples), χ^2 (2) = 1.526, p = 0.466. The proportion of bacterial DNA present also did not change over time in PLA, χ^2 (2) = 2.743, p = 0.254 or MFGM, χ^2 (2) = 0.783, p = 0.676.

Bacterial composition in blood was measured using 16S rRNA gene sequencing at Pre-Ex, Post-Ex and 1 h Post-Ex, across both PLA and MFGM trials. At the genus level, the most abundant taxa were Escherichia-Shigella, Lactobacillus, Ruminococcus, Subdoligranulum and

Clostridium sensu stricto 1, all of which are known gut taxa (Figure 3.7). Changes in the relative abundance of these taxa can be seen over the different exercise timepoints (Figure 3.7), and PCoA analyses were performed to analyse whether there were any significant changes in bacterial composition.

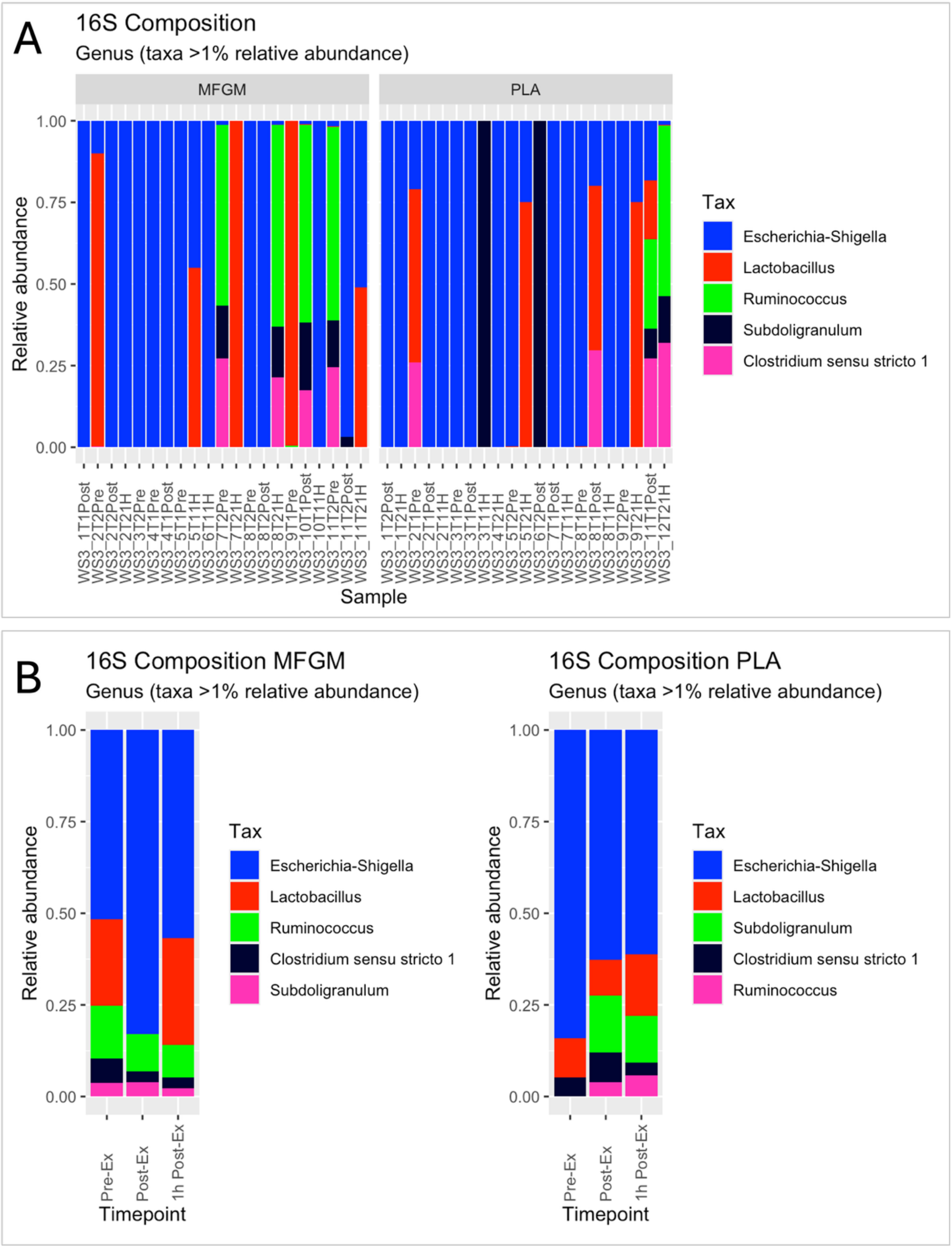

Figure 3.7. 16S relative abundance plots. A- 16S relative abundance for individual samples. B- Mean 16S relative abundance for time point.

To assess overall differences in bacterial composition in blood (beta-diversity), PCoA was performed on Bray-Curtis dissimilarity values for trial, time and trial × time interaction effects. PERMANOVA found no significant effect of trial, $R^2 = 0.020$, $F(1, 51) = 1.055$, $p = 0.267$, time, $R^2 = 0.028$, $F(2, 50) = 0.722$, $p = 0.971$, or trial × time interaction, $R^2 = 0.032$, $F(2, 47) = 0.830$, $p = 0.810$ on bacterial composition in blood (Figure 3.8).

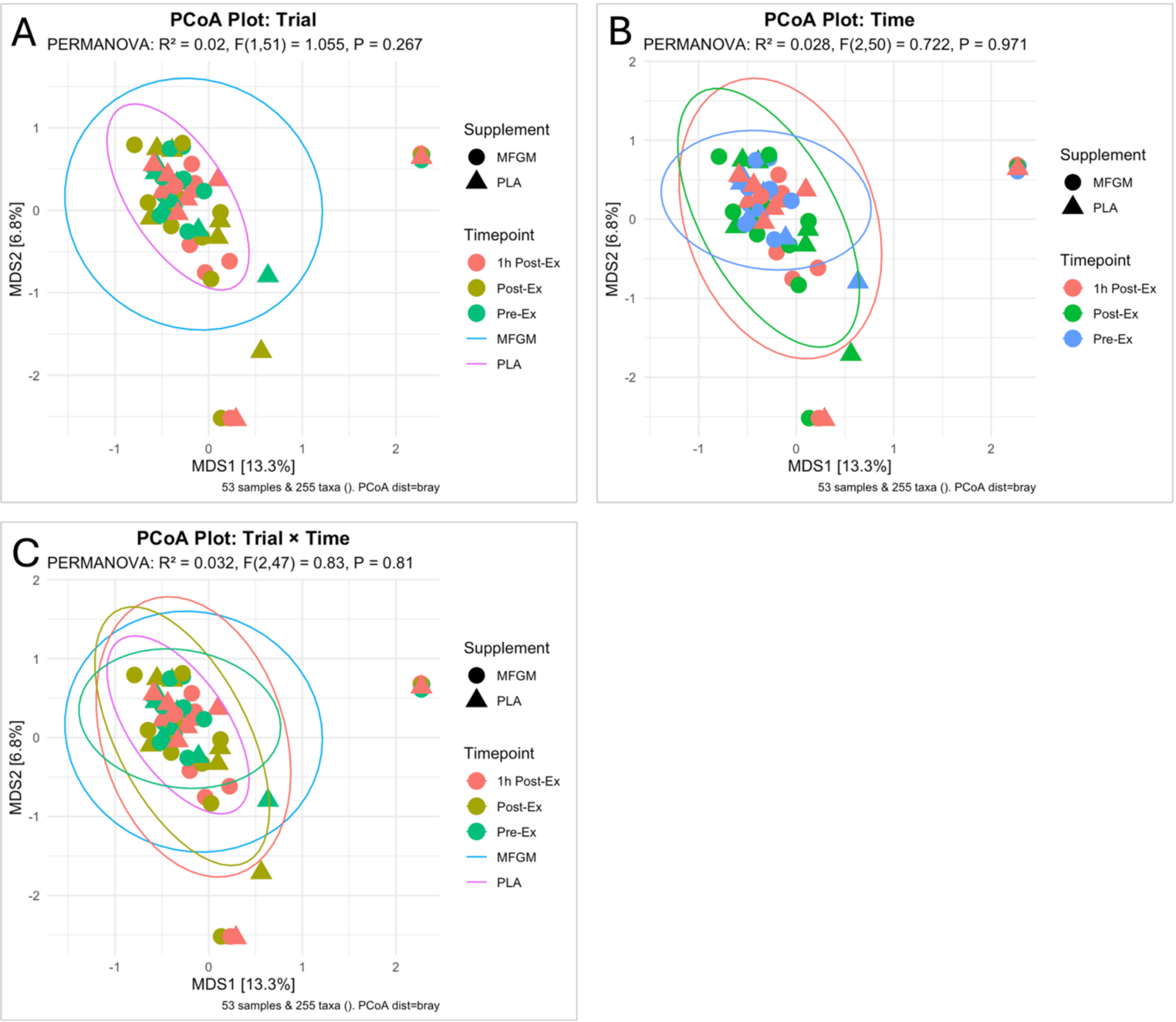

Figure 3.8. Principal coordinates analysis (PCoA). A- PCoA plot for trial effect. PERMANOVA results: $R^2 = 0.020$, F(1, 51) = 1.055, $p = 0.267$. B- PCoA plot for time effect. PERMANOVA results: $R^2 = 0.028$, F(2, 50) = 0.722, $p = 0.971$. C- PCoA plot for trial × time interaction. PERMANOVA results: $R^2 = 0.032$, F(2, 47) = 0.830, $p = 0.810$.

3.4.7 Gut symptoms

A two-way ANOVA showed a significant main effect of time, $F(2, 22) = 7.881$, $p = 0.003$ for weekly GSRS scores. However, there was no main effect of trial, $F(1, 11) = 0.102$, $p = 0.755$ or trial × time interaction, $F(2, 22) = 0.659$, $p = 0.527$ detected. Post-hoc Bonferroni-adjusted *t*-tests revealed a significant decrease from Day 7 to Day 14 ($p < 0.001$) and Day 21 ($p = 0.032$), but there was no change between Day 14 and Day 21 ($p > 0.05$) (Figure 3.9).

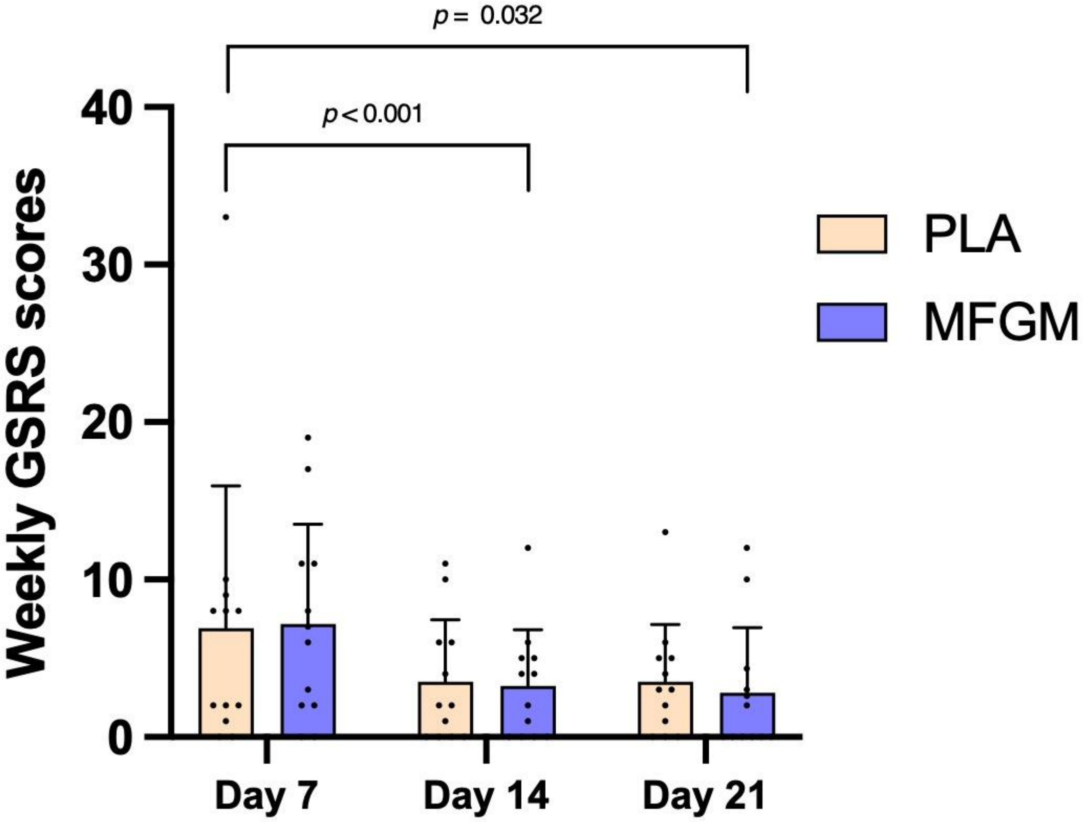

Figure 3.9. Weekly GSRS scores. Two-way ANOVA main effects: p = 0.755 (trial); p = 0.003 (time); p = 0.527 (trial × time interaction). Day 7 was significantly higher than Day 14 (p < 0.001) and Day 21 (p = 0.032; post-hoc analysis).

3.4.8 Adverse events reported

No adverse events or reactions were reported throughout the study.

3.5 Discussion

The findings of the present study show that two weeks of daily MFGM supplementation attenuated exercise-induced increases in gut damage and permeability markers. Despite the reductions in exercise-induced gut perturbations, these effects did not translate to significant changes in sCD14 concentrations, bacterial composition in blood or GI symptom incidence.

The exercise protocol used in this study was a running bout of 20 min at 80% $\dot{V}O_{2max}$, which is a model that has been utilised in previous studies to elicit increases in gut damage and permeability (Davison et al., 2016; March et al., 2017; Marchbank et al., 2011). To ensure the physiological stress imposed was consistent across both arms, $\dot{V}O_{2max}$ and thus the speed equivalent to 80% of $\dot{V}O_{2max}$ was determined within a week of each experimental trial. Furthermore, measurements of acute physiological stress; $\dot{V}O_2$, HR, RPE and total/differential leukocyte counts, displayed the expected responses to exercise but did not differ between trials, with the exception of N/L ratio which was higher in the PLA arm, suggesting that any positive effects of MFGM were likely unattributed to inconsistencies in exercise workload and stress imposed. The laboratory temperature was also kept as consistent as possible across trials to ensure that differences in body temperature would not interfere with supplement effects, although core temperature was not measured and could have provided valuable additional information.

The present study found that urinary L/R ratio significantly increased following exercise, but only in the PLA arm, suggesting that MFGM was able to blunt this response. As mentioned, due to variability in some samples, a combined mean for both baselines was calculated and used for trial comparisons. It is possible that such variability could be caused by analytical factors. However, a review of indirect markers of exercise-induced gut damage and permeability reported that many studies found no influence of exercise on the urinary L/R test, while others saw large influence (Chantler et al., 2021). In a healthy population, urinary lactulose concentrations will be very low and even with exercise-induced gut permeability, values likely remain near the lower end of the assay's standard curve or limit of detection/quantification. Measurements in this range would be more susceptible to proportional fluctuations, which may partly explain the variability observed in individual samples and between studies. These findings are in line with previous research using other dairy supplements, where bovine colostrum has been shown to provide considerable protection against exercise-induced increases in urinary L/R ratio, using similar protocols (Davison et al., 2016; March et al., 2017; Marchbank et al., 2011). However, where bovine colostrum is generally seen to truncate the exercise-induced increase in urinary L/R ratio, MFGM was able to fully prevent such a response in this case. The magnitude of change seen in the PLA trial (2.5-fold increase) was also similar with that seen following a clinically relevant dose of the NSAID indomethacin (Playford et al., 2001), which has been a proven cause of gut injury (Petruzzelli et al., 2007). Thus, indicating that MFGM could be beneficial to patients' GI integrity in a clinical context as well as in athletes, although this would require further research to confirm this.

Since damage to the gut epithelial cells and tight junction complexes is thought to be one of the key factors influencing elevations in gut permeability, measurements of I-FABP have been used to complement urinary L/R analyses, with a significant correlation between the two having been shown in some cases, but not always (March et al., 2017). Similarly to urinary L/R, the present study saw significant increases in plasma I-FABP from Pre-Ex to Post-Ex in the PLA trial, but not the MFGM trial. Moreover, the changes from Pre-Ex to Post-Ex and 1 h Post-Ex were significantly lower in the MFGM trial. To be consistent with previous research, I-FABP was expressed both in absolute values and as a percentage of Pre-Ex values (March et al., 2019, 2017; Pugh et al., 2017; van Wijck et al., 2014, 2013). This is due to the large inter-individual variability often seen in plasma I-FABP concentrations. In the case of the present study, statistical analyses did not differ between the two methods of reporting I-FABP, but the effects may be visually clearer in those reported as a percentage of Pre-Ex. Since I-FABP is specifically expressed within enterocytes (Lau et al., 2016), the elevations seen are indicative of gut epithelial cell damage experienced as a result of exercise. Similar findings have been seen with bovine colostrum, where exercise-induced increases in plasma I-FABP may be attenuated, indicating greater GI integrity in response to exercise (March et al., 2019, 2017). It should be noted that I-FABP is not a marker of gut permeability, rather it only indicates damage to enterocytes. However, I-FABP provides valuable information to accompany urinary L/R measurements and provides an insight to the association between gut damage and its downstream effects on permeability, and in this case, the role of MFGM in mediating both.

A change from Pre-Ex to Post-Ex was observed for plasma sCD14 concentrations, but there was no difference between PLA and MFGM. It has been suggested that plasma sCD14 may be a surrogate marker of systemic endotoxemia as it is a receptor for LPS (Costa et al., 2022b). Therefore, it could be suggested that elevated sCD14 may indicate increased levels of LPS, which may be a result of higher levels of bacterial translocation across the gut barrier into circulation. However, sCD14 is also an acute phase protein (APP), meaning it is also susceptible to increases following other causes of acute immune activation (Bas et al., 2004). Rather than solely increasing due to endotoxin exposure, sCD14 may also rise in association with other markers of acute physiological stress, such as leukocyte counts, reflecting exercise-induced tissue damage or injury instead of an exclusively infectious origin.

Despite the changes in gut damage and permeability markers observed, there were no changes in bacterial composition in blood either over time or between supplements. It has been previously demonstrated that elevated gut permeability following exercise may increase the translocation of luminal contents, including bacteria, into circulation (Henningsen et al., 2024;

March et al., 2019). Such occurrences could lead to elevated GI symptoms and acute inflammation, or in more extreme cases, cause severe endotoxemia, which could lead to sepsis, MOF, or in the most severe cases, death. It was hypothesised that if the gut barrier was impaired and overly permeable, then the bacterial community in blood may shift to a more gut taxa-dominant composition. However, since blood should generally be aseptic and does not possess a common microbiome (Tan et al., 2023), it is possible that most bacteria detected in blood is already of gut origin, thus explaining the lack of change detected following exercise. It should be noted that 16S rRNA gene sequencing is not used to quantify taxa, rather it is used to measure the composition of all bacteria in a sample. Therefore, the present study is unable to confirm whether there was any quantifiable change in bacterial presence, both for total bacteria and individual taxa.

There was also no difference between PLA and MFGM for weekly GI symptoms reported. Interestingly, both PLA and MFGM trials experienced higher GI symptom incidence following the first week. It is possible that the increased symptoms were a result of the $\dot{V}O_{2max}$ test performed in that week, which was a maximal exercise bout and may have caused some distress. It is also possible that the symptoms may have resulted from acclimating to the supplements, though, since no adverse events or reactions were reported during the study, this is unlikely to be the case. Alternatively, it is possible that the first week reflected the normal 'baseline' scores for these participants and both supplements had some benefit that was noticed after 1 week of use.

There are a few mechanisms thought to underpin exercise-induced gut damage and subsequent permeability. Firstly, heat is generated during exercise largely as a byproduct of metabolism, resulting in a rise in core temperature (Damatto et al., 2019). The resulting rise in core temperature, which can lead to a state of hyperthermia has shown to damage gut integrity through direct thermal damage to epithelial cells and denaturation of proteins within epithelial cell structure and those forming the tight junction complexes (Dokladny et al., 2016; Ghulam Mohyuddin et al., 2022; Walter et al., 2021b). These effects may lead to structural changes and impaired function of the gut barrier, creating a transient window of elevated permeability. In vitro/rodent studies have supported this, showing a reduction in transepithelial resistance after heating colon cells from 37 °C to 39 °C (Marchbank et al., 2011). In a more extreme scenario, a 34-fold increase in ^{51}Cr-EDTA translocation from the gut was observed after passively heating rats to 41.5 °C (Prosser et al., 2004). Another possible mechanism for the elevations in gut damage and permeability observed may be related to splanchnic hypoperfusion. The sympathetic nervous system is activated during exercise in an intensity and duration dependent manner, and prioritises blood flow to the heart, lungs and working skeletal muscles. The diversion of blood away from the gut can create a state of intestinal ischaemia and damage the components of the gut barrier via hypoxia (Ulluwishewa et al., 2024). Following the state of ischaemia, the gut must also be reperfused, which may also inflict further damage through oxidative and inflammatory stress (Grootjans et al., 2010; G. Li et al., 2022).

High impact sports such as running may also expose the body to repetitive mechanical forces stress that could cause microtrauma to components of the gut barrier, termed ‘gastric jostling’ (Costa et al., 2017). The occurrence of ‘gastric jostling’ may facilitate gut injury, especially if occurring alongside the other mechanisms mentioned. Such disruption to the components of the gut barrier may compromise gut function further, increasing the risk of translocation of undesirable contents into circulation (de Oliveira et al., 2014; Zuhl et al., 2014b). It has been proposed that increased translocation of luminal contents across the gut barrier is one of the factors influencing the increased incidence of GI symptoms experienced after some exercise bouts (de Oliveira et al., 2014). However, in the present study, increased gut permeability did not lead to increased GI symptom incidence.

MFGM possesses numerous bioactive components that could promote the integrity of the gut barrier. Lactadherin is a protein in particularly high abundance in bovine MFGM, with anti-inflammatory properties and is associated with the clearance of apoptotic cells (Ait-Oufella et al., 2007). Moreover, it has been demonstrated that lactadherin can promote the integrity of the gut barrier, where it has been suggested that alongside the removal of apoptotic epithelial cells, lactadherin may also restore the expression and localisation of tight junction and adherens junction proteins to optimal conditions (Shen et al., 2019). If cells in the gut barrier have been damaged to the extent of becoming apoptotic, they would create a weak link in the chain, possibly creating a window of opportunity for the passage of luminal contents across the barrier. Therefore, it is possible that lactadherin within the MFGM supplement may have facilitated the restoration of the gut barrier to more 'normal' condition. Lactadherin may also help restore the conditions of the tight/adherent junction proteins, which provide greater protection against contents relying on paracellular transport across the gut barrier. Phospholipids within MFGM have also shown to have promising effects on gut health and integrity. It has been demonstrated that MFGM phospholipids can increase the abundance of beneficial bacteria within the gut, including SCFA-producing bacteria (Feng et al., 2025; Gong et al., 2024; Kosmerl et al., 2021; Wu et al., 2021). SCFAs provide gut barrier support through various mechanisms, such as the upregulation of tight junction protein expression and reduction of pro-inflammatory cytokines (Liu et al., 2021). Therefore, it could be suggested that increased SCFA production associated with phospholipids within MFGM may result in these downstream effects, supporting the integrity of the gut barrier. There is also in vitro evidence to suggest that transepithelial electrical resistance and tight junction protein expression is increased following incubation with MFGM or its individual components, such as polar lipids or SM (Jiang et al., 2022; Milard et al., 2019).

3.5.1 Limitations

As with many other nutrition clinical trials, a large consideration of the present study was to ensure an appropriate placebo supplement was used, and in this case, the placebo used did not match the fat content of the MFGM supplement. When designing the placebo, it became evident that components used to match fat content, such as medium chain triglycerides (MCTs), may have effects on the GI system (Rial et al., 2016). Since protein was the primary constituent of the supplements, it was deemed a priority to ensure sufficient protein in the placebo. It was also essential that the total dose of both supplements was equal, to maintain blinding, so the missing fat components of the placebo was substituted with protein. This amount added was deemed insufficient to have any meaningful effect on study outcomes given its negligible contribution to daily fat intake. Another limitation of the present study was the lack of core temperature measurement. As highlighted, core temperature has been shown to be a driving factor in exercise-induced gut damage. Whilst the present study can refer to other studies using the same model (Davison et al., 2016) for expected changes in core temperature, the lack of measurement prevents any confirmation of temperature-related effects seen. There was considerable variation seen in baseline L/R measurements. As baseline samples were taken 2 weeks prior to the main trial, it was not possible to confirm whether this permeability remained consistent during this time. To account for the potential variability, the mean of both baseline measurements was calculated to provide a more robust control value, which was then used as a singular point of comparison for both experiential trials. The use of 16S rRNA gene sequencing has some limitations. Since blood is normally aseptic, when bacteria are present, it is usually in very low abundance. The result is that the low microbial biomass renders 16S sequencing more prone to contamination and index hopping. To address this, known contaminants were purged from the amplicon sequencing reads. Another limitation of 16S amplicon sequencing is its inability to provide bacterial quantification. Whilst compositional analysis provides valuable insights to shifts in the microbial community, future research should look to include qPCR analysis for quantification of specific gut taxa to complement 16S amplicon data. Lastly, during statistical analysis, no sensitivity checking took place and outliers were not considered. Although, for urinary L/R and I-FABP, the highest values were from the same individual, so it is unlikely that these would have affected the study outcomes.

3.5.2 Conclusion

This was the first study to assess the effects of MFGM on exercise-induced gut permeability. It was demonstrated that MFGM was able to prevent the increases in gut damage and permeability markers following exercise. Though, there was no change in bacterial composition in blood or GI symptom incidence. Alongside athletes, the findings of this study may be relevant to individuals working in hot environments, such as soldiers. Since the urinary L/R responses following exercise resemble those seen after clinical medication doses, MFGM may also provide valuable protection against gut permeability in a clinical setting, though further research is required to confirm this.

Chapter 4. Study 2 – Effects of two weeks of milk fat globule membrane supplementation on immune function and gut integrity following prolonged cycling.

4.1 Abstract

Research suggests that strenuous/prolonged exercise can depress several immune functions and inflict gut damage, which may contribute to illness susceptibility and incidence of GI symptoms. This study aims to assess whether short-term MFGM supplementation influences the effects of prolonged exercise on immune functions and gut integrity. Eleven healthy, active individuals (10 male, 1 female; age 36.9 ± 18.0 years; height 179.2 ± 6.7 cm; BM 74.9 ± 10.8 kg; $\dot{V}O_{2max}$ 54.9 ± 9.5 $mL \cdot kg^{-1} \cdot min^{-1}$) participated in a double-blind, crossover randomised controlled trial. Participants supplemented MFGM or a placebo daily for 2 weeks before completing a prolonged (3 h) cycling bout. Venous blood and saliva samples were collected at baseline (pre-supplementation), Pre-Ex, Post-Ex and 1 h Post-Ex, for analysis of neutrophil oxidative burst, mucosal immune factors, cortisol and markers of gut damage (I-FABP and bacterial translocation). MFGM significantly increased resting neutrophil oxidative burst ($p = 0.006$) compared to PLA and attenuated the drop in neutrophil oxidative burst at Post-Ex ($p = 0.015$) and 1 h Post-Ex ($p = 0.033$). MFGM also attenuated the increase in plasma I-FABP from Pre-Ex to Post-Ex ($p < 0.001$). However, there were no significant changes in salivary SIgA, lysozyme function, plasma cortisol or bacterial composition in blood. The findings of this study suggest that MFGM provides protection against exercise-induced depression of neutrophil functions and provides further evidence for the gut-protective effects of MFGM.

4.2 Introduction

It is well-documented that prolonged exercise, or periods of heavy training loads, can affect several immune functions and increase the risk of contracting infections, such as URTIs, compared to periods of lower training load and to less active, and sedentary individuals (Schwellnus et al., 2022). Whilst the mechanisms underpinning exercise-induced immune depression are not fully understood, it has been suggested that neutrophilia, which often occurs simultaneously with exercise-induced leukocytosis, may involve an influx of immature neutrophils into circulation. Immature neutrophils such as band cells have been shown to exhibit lesser granular content and NADPH oxidase activity compared to mature cells, which reduces their antimicrobial function and may contribute to the window of vulnerability to infection (Peake, 2002). Some mucosal immune functions have also seen similar depression following prolonged exercise, such as the secretion rate of SIgA (Li and Gleeson, 2004; Walsh et al., 2002). This is also a likely contributor to the increased risk of URTIs seen in those performing regular endurance exercise, with SIgA secretion rate providing a strong negative correlation with URTI incidence (Fahlman et al., 2017). Psychological factors may further modulate immune responses to exercise, with research having shown that psychological stress and anxiety can exacerbate exercise-induced immune depression and increase the risk of contracting URTIs (Cohen et al., 1993, 1991; Edwards et al., 2018a; Harrison et al., 2025). Since markers of physical stress such as inadequate recovery and sleep have also shown to negatively influence immune functions (Walsh, 2018), it is important to monitor psychological and physiological condition in immunological research, to ensure findings are not confounded by such factors.

Following from the work in study 1, another undesirable effect of exercise is the damage to epithelial cells and tight junction proteins within the gut. This generally occurs due to several mechanisms occurring simultaneously, including increased core temperature, reduced splanchnic blood flow and mechanical stress, although other factors such as post-exercise splanchnic reperfusion and oxidative stress also likely contribute to exercise-induced gut damage (Chantler et al., 2021). A consequence of damage to epithelial cells and tight junctions is that the gut barrier itself may become compromised, leading to elevations in undesirable luminal contents permeating the gut, causing various issues including systemic inflammation, immune responses and in extreme cases, sepsis (Michelena et al., 2015). An overly permeable gut may also impair fluid and nutrient absorption (van Wijck et al., 2013), which could inhibit athlete recovery and energy, but may also be implicated in dehydration and heat stroke in longer events (Fung et al., 2021).

As demonstrated in study 1, MFGM was able to provide protection against exercise-induced gut perturbations, potentially due to bioactive components such as lactadherin and phospholipids. Although there is little other research into the effects of MFGM on immune and gut functions in healthy adults, there are currently two contrasting studies that have investigated the effects of an MFGM-rich protein supplement on oral diarrheagenic *E. coli* challenges, with one demonstrating favourable effects of MFGM (Ten Bruggencate et al., 2016) and the other displaying no effect (Ulfman et al., 2022). However, there is promising research for MFGM and its relationship on early life immune function, as MFGM-rich infant formulas have exhibited numerous beneficial effects such as reduced incidence of otitis media (Timby et al., 2015), febrile episodes (Veereman-Wauters et al., 2012) and respiratory-associated adverse events (Li et al., 2019).

Given the comparable findings of MFGM on gut integrity with other bioactive dairy supplements, like bovine colostrum, it is plausible that MFGM may display other similarities. Bovine colostrum has shown to provide numerous immunomodulatory effects, where it has been shown to attenuate the exercise-induced depression of neutrophil functions, and enhance their recovery (Davison and Diment, 2010; Jones et al., 2015). Moreover, bovine colostrum has also been shown to aid other immune functions, such as preventing the decrease of salivary lysozyme following prolonged exercise (Davison and Diment, 2010), and increasing resting SIgA levels in some cases (Crooks et al., 2006; Mero et al., 2002), although there are contrasting findings for the latter (Crooks et al., 2010; Davison and Diment, 2010; Jones et al., 2014). Such effects have largely been accredited to the low molecular weight (< 10 kDa) components within bovine colostrum, as supported by Jensen et al. (2012), who found that a low molecular weight fraction of colostrum enhanced peripheral blood neutrophil phagocytic activity 1 and 2 h after consumption. Since MFGM and bovine colostrum possess some similar low molecular weight components, it is possible that MFGM may also exhibit benefits to immune function.

The present study aimed to assess the effects of 2 weeks of MFGM supplementation on cellular and mucosal immune functions, and gut damage and permeability following prolonged cycling. It was hypothesised that MFGM would attenuate the exercise-induced drop in neutrophil function and enhance mucosal immune functions. Moreover, following the results of study 1, it was hypothesised that MFGM would protect the gut from exercise-induced gut damage, as indicated by I-FABP responses. However, the previous findings indicated that reductions in gut damage at the magnitude observed may not necessarily translate to differences in translocation of gut bacteria.

4.3 Methods

4.3.1 Ethical approval

All procedures demonstrated in the study were approved by the University of Kent Sport, Exercise, and Rehabilitation Sciences REAG (Prop 26_20_23). The study was carried out in accordance with the Declaration of Helsinki principles. Prior to participation, all participants were required to provide their written and verbal informed consent. The trial was prospectively registered on the ISRCTN registry (ISRCTN56356024, https://doi.org/10.1186/ISRCTN56356024). ISRCTN is a WHO and ICMJE recognised primary clinical study registry.

4.3.2 Power calculation

This was the first study into MFGM using these measures, so no previous data was available for a preliminary power calculation. Since MFGM may share some mechanisms with bovine colostrum, a power calculation was performed (G*Power version 3.1.9.7, Kiel, Germany) using data from a similar study on bovine colostrum (Jones et al., 2015). For the primary outcome of the study, fMLP-stimulated neutrophil oxidative burst, bovine colostrum displayed a large effect size for the condition × time interaction (Cohen's $f = 0.44$). Following the results of Chapter 3, a slightly less conservative estimate was used for the MFGM effect size, set at 50% of that of bovine colostrum, Cohen's $f = 0.22$. Power calculations incorporating a correlation among repeated measures of 0.87 and an epsilon value of 0.72 indicated that a sample size of $n = 8$ would be required to detect a significant difference between supplements over time (trial × time interaction; ANOVA, repeated measures, within-subjects) at 80% power and alpha level 0.05. Accounting for a dropout rate of 20%, a target sample size of $n = 10$ was set.

4.3.3 Participants

Eleven individuals (10 male, 1 female) enrolled on the study, but 1 participant withdrew prior to the first main trial. As this participant withdrew early into the study an extra participant was recruited to ensure 10 participants were enrolled up to the first main trial, providing greater protection against dropouts. Ten healthy, recreationally active regular cyclists (9 male, 1 female; age 34.3 ± 16.6 years; height 179.4 ± 7.1 cm; BM 74.7 ± 11.1 kg; $\dot{V}O_{2max}$ 55.2 ± 10.8 $mL{\cdot}kg^{-1}{\cdot}min^{-1}$) completed the study. All individuals were free from injury or known medical conditions and were classified as 'healthy' via a standardised PAR-Q (see Appendix). Eligibility criteria included the absence of URTI symptoms for at least 4 weeks before enrolment, as well as no known allergies or hypersensitivities to the investigational products.

4.3.4 Study design

The present study was a double-blind, crossover, randomised controlled trial. Participants visited the laboratory on 6 occasions: at baseline, familiarisation, and main trial, across 2 crossover arms. At baseline, resting blood and saliva samples were taken for baseline measures of neutrophil oxidative burst and SIgA, and a $\dot{V}O_{2max}$ test was performed. Following this, participants were randomly assigned to either MFGM-rich whey protein concentrate or PLA for daily supplementation throughout that arm. Both supplements were consumed at 40 $g{\cdot}day^{-1}$ and all randomisations were performed using an online randomisation tool (https://www.graphpad.com). On approximately day 7, a familiarisation trial was performed in order to familiarise the participant with the protocol required for the upcoming main trial, and to ensure the work rate calculations were sufficient. On day 15 of each arm, the main trial was performed which included a 3 h cycling bout at 15% of the difference between the gas exchange threshold and $\dot{V}O_{2max}$ (15% Δ), which worked out to 55-60% of $\dot{V}O_{2max}$. This intensity was considered appropriate for a recreationally active population and sufficient for inducing exercise-induced depression of immune functions. Moreover, this protocol aligns with those used in previous studies investigating similar outcomes (Davison et al., 2009, 2007; Davison and Gleeson, 2006, 2005; Jones et al., 2015). Blood and saliva samples were taken Pre-Ex, Post-Ex and 1 h Post-Ex for analysis of cellular and mucosal immune functions, and markers of stress and gut damage. Before all visits, participants were instructed to abstain from exercise in the 24 hours prior, alcohol for the 48 hours prior and caffeine on the morning of the trial. Prior to the main trial, participants completed a 24 h food diary and were instructed to repeat this diet during the 24 h prior to the second main trial. Following the first arm, subjects underwent a 2-week washout period before beginning supplementation with the alternative supplement at Baseline in the second arm (Figure 4.1).

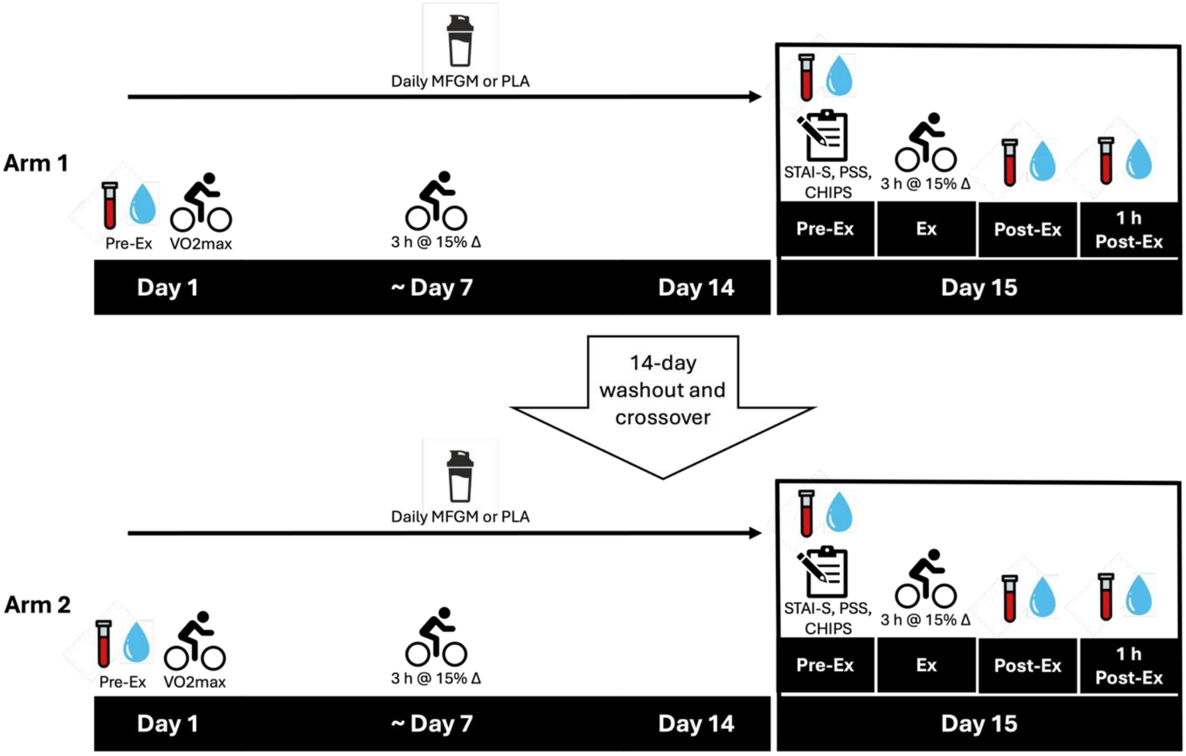

Figure 4.1. Schematic of Chapter 4 protocol. CHIPS, Cohen-Hoberman Inventory of Physical Symptoms. PSS, perceived stress scale. STAI-S, state aspect of the state-trait anxiety inventory.

4.3.5 Supplementation

The placebo (PLA) used in the trial was an isocaloric and near macronutrient-matched soy protein concentrate, flavoured similarly. Both supplements were consumed at 40 $g \cdot day^{-1}$ during the designated 2-week supplementation windows. Supplements were prepared in anonymous food-grade tubs and returned at the end of each arm; the remaining powder was inspected to confirm supplement compliance. Supplement compliance as per the daily log was 98.7%.

Table 4.1. Nutritional analysis of Chapter 4 supplements

	PLA	MFGM
Energy (Kcal/40 g)	170	182
Carbohydrates (%)	3.6	10.1
Protein (%)	67.4	66.2
Fat (%)	14.3	16.2
Total phospholipids (mg·g^{-1})	12.4	55.8
Sphingomyelin (mg·g^{-1})	Not detected	14.7
Phosphatidylcholine (mg·g^{-1})	3.8	15.5
Phosphatidylethanolamine (mg·g^{-1})	1.4	15.1
Phosphatidylinositol (mg·g^{-1})	3.2	3.7
Phosphatidylserine (mg·g^{-1})	Not detected	5.9
Other (mg·g^{-1})	0.6	0.8

4.3.6 Preliminary visits

Participants arrived at the laboratory between 08:00 and 10:00 following an overnight fast of at least 10 h. Following a period of 10 min seated rest, a venous blood and unstimulated saliva sample were collected. After which, $\dot{V}O_{2max}$ was calculated using an incremental ramp test (30 W·min^{-1} ramp rate) performed on an electronically braked cycle ergometer (Lode Excalibur; Groningen, The Netherlands). The handlebars and seat of the ergometer were adjusted for participant comfort, and if desired, participants were able to fit their own pedals to the cranks and use their own saddle. All equipment used, adjustments and fittings to their ergometer were kept consistent in all subsequent trials. Prior to beginning the ramp test, participants completed a 10-min warm up at 40 W and were instructed to find their preferred cadence during this time (between 70-90 rpm). After the warmup, participants were told to remain at their preferred cadence throughout the ramp test. During the ramp test, measurements of gas exchange were measured as described in Chapter 2.3 (General Methods). Verbal encouragement was given towards the final stages and the test was terminated when volitional exhaustion was reached, or when the cadence fell by 10 rpm below their target cadence for more than 10 s. $\dot{V}O_{2max}$ was determined by the highest 30 s average during the test, and the gas exchange threshold was estimated using the V-slope method (detecting the beginning of excess carbon dioxide [CO_2] output generated from the buffering of hydrogen ions) (Beaver et al., 1986).

For determination of familiarisation and main trial workloads, an exercise intensity was set to the power output relative to 15% Δ, which worked out to roughly 55% of $\dot{V}O_{2max}$ (54.7 ± 2.5 % of $\dot{V}O_{2max}$). The (submaximal) linear relationship between power output and $\dot{V}O_2$ was used to calculate a power equivalent of 15% Δ. This was then reduced by two thirds of the ramp rate (20 W) to account for lag in $\dot{V}O_{2max}$ responses.

4.3.7 Familiarisation

Familiarisation trials took place mid-supplementation, on approximately day 7, to familiarise the participant with the calculated workload and procedures required throughout the main trials. The timing of this trial was scheduled to be close enough to the main trial to avoid changes in fitness, but with sufficient recovery to prevent fatigue during the main trial. In addition, any effects on study outcomes (e.g. depression of immune functions) were expected to return to baseline within 24 hours (Walsh et al., 2011), thereby ensuring no carryover of effects to the main trial. Participants completed a 3 h cycling bout at 15% Δ on the same ergometer used in the $\dot{V}O_{2max}$ test, using the same saddle, pedals, and adjustments. Expired gas was analysed throughout the initial 10 min, and then for 1 min at the 30th, 60th, 90th, 120th, 150th, and 180th min. HR and RPE were measured at 30 min intervals. No saliva or venous blood samples were taken in this visit as it was intended to familiarise the participant with the exercise protocol required, and to ensure the calculated workload was correct.

4.3.8 Main trials

On day 15, participants arrived at the laboratory between 08:00 and 10:00 following an overnight fast of at least 10 h. After arrival, participants were instructed to remain seated with minimal movement for 10 min, prior to resting blood and saliva sample collection. Participants then completed form Y-1 (state aspect) of the state-trait anxiety inventory (STAI-S), the perceived stress scale (PSS) and the CHIPS. Following this, participants began the main exercise protocol, a 3 h cycling bout at 15% Δ. $\dot{V}O_2$ was recorded throughout the first 10 min to verify the correct work rate was achieved, and then for 1 min at the 30th, 60th, 90th, 120th, 150th and 180th minute of exercise. HR and RPE were also recorded at 30 min intervals. Blood and saliva samples were taken immediately after exercise cessation (Post-Ex), and then again after 1 h of seated rest (1 h Post-Ex).

4.3.9 Leukocyte counts

WBC differential counts were analysed at Baseline, Pre-Ex, Post-Ex and 1 h Post-Ex using the methods described in Chapter 2.4.2 (General Methods).

4.3.10 Neutrophil oxidative burst

In vitro neutrophil oxidative burst in response to fMLP was performed at Baseline, Pre-Ex, Post-Ex and 1 h Post-Ex using a commercially available chemiluminescence (CL) assay kit (ABEL Cell Activation Test Kit; Knight Scientific Ltd, Plymouth, UK). A volume of 10 µL

K_2EDTA whole blood was diluted with the provided blood dilution buffer at a ratio of 1:100. The contents of each microplate well were then prepared in duplicate with 20 µL of diluted blood, 20 µL of Adjuvant-K (enhances the luminescence of Pholasin during assays with diluted whole blood), 50 µL of Pholasin and 90 µL of assay buffer.

The microplate was then gently shaken and incubated in the luminometer (FLUOstar OPTIMA; BMG Labtech, Aylesbury, UK) at 37°C for 5 min. CL was measured as relative light units (RLU) every 1.36 s. At 37 s into the assay, 20 µL of fMLP (10 $\mu M \cdot L^{-1}$) was injected to the sample, bringing the total well volume to 200 µL and the final fMLP concentration to 1 µM, after which CL was continuously recorded for the remaining 263 s. Area under the CL curve was calculated for both the unstimulated (0-35 s) and stimulated (40-300 s) timepoints, then subtracting the average unstimulated CL from the stimulated area under the curve to account for any background assay noise or unstimulated oxidative burst activity.

Oxidative burst was reported on a per cell basis, and whilst there may be negligible oxidative burst activity from monocytes, as used in previous research, it is assumed that the CL measured is mostly attributable to neutrophils within the sample (Morozov et al., 2003). Therefore, fMLP-stimulated area under the CL curve was divided by the number of neutrophils in each well to provide a measurement of RLU per neutrophil. To assay inter-assay CV, 5 samples were analysed pre- and post-storage at -4 °C for 5 h, to replicate the storage of samples on main trial days. Inter-assay CV was 9.3%.

4.3.11 Saliva measures

Salivary SIgA and lysozyme activity were measured at Baseline, Pre-Ex, Post-Ex and 1 h Post-Ex, as outlined in Chapter 2.4.8 and 2.4.9 (General Methods).

4.3.12 Plasma Cortisol

Cortisol was measured in heparin-treated plasma taken at Pre-Ex, Post-Ex and 1 h Post-Ex using a commercially available ELISA kit (DRG Diagnostics, Marburg, Germany). All tests were performed in accordance with the manufacturer's instructions and samples were analysed in duplicate. Over 59 duplicate samples, intra-assay CV was 4.4%.

4.3.13 Plasma I-FABP

I-FABP was measured at Pre-Ex, Post-Ex and 1 h Post-Ex, as detailed in Chapter 2.4.3 (General Methods.

4.3.14 DNA extraction

DNA was extracted from lithium heparin-treated whole blood. Following the manufacturer’s guidelines, DNA was isolated from 100 µL whole blood using a Quick-DNA Miniprep Kit (D3024, Zymo Research, Irvine, California, USA). Prior to sample elution, the microcentrifuge tube used for sample storage was sterilised in an ultraviolet light cabinet (UVT-B-AR Economy PCR UV Cabinet, Grant Instruments, Cambridgeshire, UK). The eluted DNA was stored at -80 °C for later analysis.

4.3.15 Bacterial translocation

Bacterial load was measured in whole blood at Pre-Ex, Post-Ex and 1 h Post-Ex using qPCR, and 16S rRNA gene sequencing was performed to provide compositional insights into bacterial translocation, as outlined in Chapter 2.4.4 and 2.4.5 (General Methods).

4.3.16 Psychological and physiological wellbeing

Due to the potential influence of psychological and physiological factors on study outcomes, these factors were assessed on main trial days. All questionnaires were completed using an online survey platform (Qualtrics, Utah, USA). On the morning of all main trials, subjects completed the state aspect (Form Y-1) of the state-trait anxiety inventory (STAI-S). The STAI-S is a 20-item self-report questionnaire where subjects rate their current feelings of anxiety on a 4-point Likert scale, ranging from “not at all” to “very much so” (Not permitted to include questionnaire in appendices. For license, see Appendix E). In accordance with the authors’ instructions, the inventory includes a subset of positively worded items, so these items were reverse scored. Following the reverse coding where necessary, a total score for the items was calculated and used as an indication of state anxiety (Spielberger et al., 1983). Participants also completed the Perceived Stress Scale (PSS; see Appendix F) on the morning of all main trials. The PSS is a 10-item self-report questionnaire where subjects rate their feelings of stress on a 5-point Likert scale, ranging from “never” to “very often”. Positively worded items were reverse scored, and a cumulative score was added up as a measure of perceived stress (Cohen et al., 1983).

Physical wellbeing was assessed on the morning of all main trials using the CHIPS questionnaire, as detailed in Chapter 2.7 (General Methods).

4.3.17 Monitoring of illness and training load

URTI episodes/symptoms and training load were monitored daily throughout each crossover arm, and for 2 weeks following the main trial. Monitoring methods are described in Chapter 2.6 (General Methods).

4.3.18 Phospholipid quantification

Phospholipid content in red blood cells was quantified using the Stewart assay on MFGM baseline and main trial days. The Stewart assay methods are outlined in Chapter 2.4.6 (General Methods).

4.3.19 Statistical analysis

Before analysis was performed, all data was checked for normal distribution using a Shapiro-Wilk test and Z-scores for skewness and kurtosis. Data without a normal distribution were normalised using either a log or root transformation. Data unable to be normalised with log or root transformations were analysed using non-parametric tests. Missing data were imputed using estimation-maximisation and Little's test was performed to verify that data were missing completely at random (Grove and Cipher, 2024).

The following transformations were used to prepare data for parametric tests:

Log: neutrophil counts; saliva osmolality; training load and duration. Square root: N/L ratio; monocyte counts; saliva flow rate; salivary lysozyme activity.

Comparisons within and between trials were made using paired samples *t*-tests. Two-way (trial × time) repeated measures ANOVAs were used for analysis of total and differential leukocyte counts, neutrophil function, I-FABP, cortisol and SIgA. The proportion of samples with bacterial DNA present in each trial was analysed using chi-squared analysis.

Analysis of 16S rRNA gene sequencing data was performed as described in Chapter 2.4.4.2 (General Methods). Analysis of 16S rRNA gene sequencing was performed using R (version 4.5.1; R Core Team 2025). The following R packages were used: devtools, phyloseq, ggplot2, dplyr, ggpubr, BiocManager, microbiome, vegan, microViz, Polychrome, ape, corrplot, microbial and ggrepel.

4.4 Results

4.4.1 Exercise physiological and perceptual responses

A significant main effect of time, $F(1.730, 15.574) = 7.823$, $p = 0.006$ was observed for trial $\dot{V}O_2$ ($L \cdot min^{-1}$). There was no main effect of trial, $F(1, 9) = 0.770$, $p = 0.403$ or trial × interaction, $F(2.536, 22.822) = 1.494$, $p = 0.245$. Post-hoc t-tests showed that $\dot{V}O_2$ ($L \cdot min^{-1}$) significantly increased from 150 to 180 min ($p = 0.002$). A significant main effect of time, $F(2.089, 18.804) = 9.623$, $p = 0.001$ was found for trial $\dot{V}O_2$ (% of $\dot{V}O_{2max}$). Though, there was no main effect of trial, $F(1, 9) = 0.604$, $p = 0.457$ or trial × time interaction, $F(5, 45) = 1.420$, $p = 0.236$. Post-hoc Bonferroni-adjusted *t*-tests revealed a significant increase in $\dot{V}O_2$ (% of $\dot{V}O_{2max}$) from 150 min to 180 min ($p = 0.002$).

Due to a technical issue with HR monitors, HR could not be measured in one trial. Thus, HR was analysed for $n = 9$. There was a significant main effect of time, $F(2.105, 16.840) = 17.200$, $p < 0.001$ for trial HR. No main effect of trial, $F(1, 8) = 0.343$, $p = 0.574$ or trial × time interaction, $F(5, 40) = 0.622$, $p = 0.684$ was detected. Post-hoc Bonferroni-adjusted *t*-tests revealed a significant increase in HR from 150 to 180 min ($p < 0.001$).

There was a significant main effect of time, $F(1.694, 15.245) = 32.103$, $p < 0.001$ for trial RPE. There was no main effect of trial, $F(1, 9) = 0.375$, $p = 0.556$ or trial × time interaction, $F(5, 45) = 1.777$, $p = 0.137$. Post-hoc Bonferroni-adjusted *t*-tests found that RPE significantly increased from 30 min to 60 min ($p < 0.001$), 60 min to 90 min ($p = 0.020$) and 90 min to 120 min ($p = 0.010$).

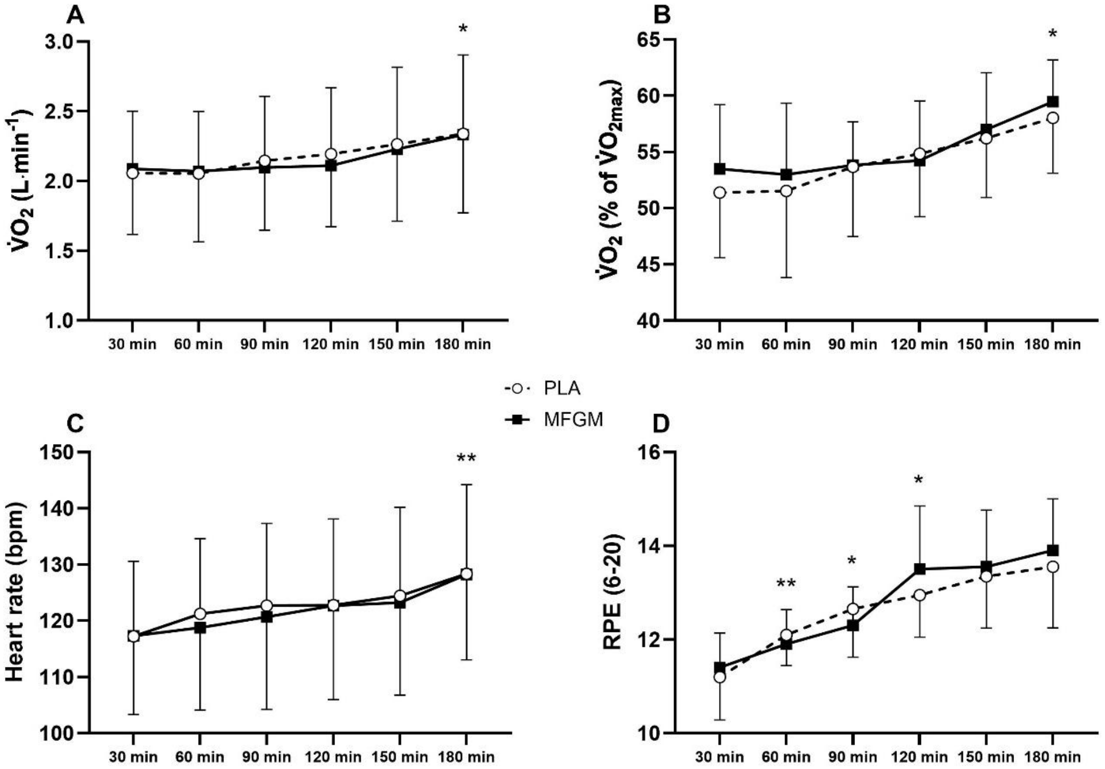

Figure 4.2. Exercise physiological and perceptual responses. A- $\dot{V}O_2$ (L·min^{-1}). Two-way ANOVA main effects: $p = 0.403$ (trial); $p = 0.006$ (time); $p = 0.245$ (trial × time interaction). B- $\dot{V}O_2$ (% of $\dot{V}O_{2max}$). Two-way ANOVA main effects: $p = 0.457$ (trial); $p = 0.001$ (time); $p = 0.236$ (trial × time interaction). C- Heart rate (bpm). Two-way ANOVA main effects: $p = 0.574$ (trial); $p < 0.001$ (time); $p = 0.684$ (trial × time interaction). D- RPE (6-20). Two-way ANOVA main effects: $p = 0.556$ (trial); $p < 0.001$ (time); $p = 0.137$. * Mean values were significantly different from previous time point ($p < 0.05$; post-hoc analysis). ** Mean values were significantly different from previous time point ($p < 0.001$; post-hoc analysis).

4.4.2 Leukocyte counts

There was a significant main effect of time, $F(1.268, 11.416) = 23.517$, $p < 0.001$ for total leukocyte count. However, there was no main effect of trial, $F(1, 9) = 0.000$, $p = 0.995$, or trial × time interaction, $F(3, 27) = 2.215$, $p = 0.109$. Post-hoc Bonferroni-adjusted *t*-tests showed a significant increase from Baseline to Post-Ex ($p = 0.001$) and 1 h Post-Ex ($p = 0.021$).

There was a main effect of time, $F(1.490, 13.407) = 53.991$, $p < 0.001$ for neutrophil count, but no main effect of trial, $F(1, 9) = 0.059$, $p = 0.906$ or trial × time interaction, $F(3, 27) = 1.168$, $p = 0.340$. Post-hoc Bonferroni-adjusted *t*-tests showed a significant increase from Baseline to

Post-Ex ($p < 0.001$) and 1 h Post-Ex ($p = 0.001$). There was a main effect of time, $F(2.069, 18.625) = 4.511$, $p = 0.024$ for lymphocyte count, but no main effect of trial, $F(1, 9) = 0.978$, $p = 0.348$ or trial × time interaction, $F(3, 27) = 0.866$, $p = 0.471$. There were no significant post-hoc comparisons detected. There was a main effect of time, $F(3, 27) = 19.253$, $p < 0.001$ for N/L ratio, but no main effect of trial, $F(1, 9) = 0.177$, $p = 0.684$ or trial × time interaction, $F(3,27) = 0.342$, $p = 0.795$. Post-hoc Bonferroni-adjusted *t*-tests showed a significant increase in N/L ratio from Baseline to Post-Ex ($p = 0.012$) and 1 h Post-Ex ($p = 0.009$).

There was a main effect of time, $F(3, 27) = 15.983$, $p < 0.001$ for monocyte counts, but there was no main effect of trial, $F(1, 9) = 0.129$, $p = 0.728$ or trial × time interaction, $F(3, 27) = 0.476$, $p = 0.702$. Post-hoc Bonferroni-adjusted *t*-tests revelated that monocyte counts significantly increased from Baseline to Post-Ex ($p = 0.001$) and 1 h Post-Ex ($p = 0.004$). No main effects of trial, $F(1, 9) = 3.630$, $p = 0.089$, time, $F(1.814, 16.33) = 1.514$, $p = 0.233$ or trial × time interaction, $F(3, 27) = 0.646$, $p = 0.593$ were detected for eosinophil count.

Table 4.2. Leukocyte count changes in response to prolonged cycling.

$10^9 \cdot L^{-1}$	Baseline	Pre-Ex	Post-Ex	1 h Post-Ex	*p* trial time interaction
Total leukocytes					0.995
PLA	4.06 ± 0.63	4.09 ± 0.97	8.33 ± 2.52	7.51 ± 2.32	< 0.001**
MFGM	4.54 ± 1.03	4.42 ± 1.22	8.17 ± 1.92	6.86 ± 2.80	0.109
Neutrophils					0.906
PLA	2.12 ± 0.43	1.97 ± 0.48	5.24 ± 1.67	4.98 ± 2.10	< 0.001**
MFGM	2.28 ± 0.56	2.06 ± 0.68	5.07 ± 1.23	4.71 ± 2.27	0.340
Lymphocytes					0.348
PLA	1.60 ± 0.39	1.78 ± 0.52	2.54 ± 0.93	1.85 ± 0.72	0.024*
MFGM	1.89 ± 0.57	1.96 ± 0.66	2.52 ± 1.08	1.87 ± 0.55	0.471
N/L ratio					0.684
PLA	1.39 ± 0.47	1.13 ± 0.17	2.17 ± 0.51	2.75 ± 0.99	< 0.001**
MFGM	1.28 ± 0.44	1.11 ± 0.41	2.31 ± 0.90	2.61 ± 1.37	0.795
Monocytes					0.728
PLA	0.18 ± 0.09	0.20 ± 0.09	0.34 ± 0.16	0.34 ± 0.21	< 0.001**
MFGM	0.20 ± 0.08	0.21 ± 0.07	0.31 ± 0.17	0.37 ± 0.18	0.702
Eosinophils					0.089
PLA	0.14 ± 0.08	0.15 ± 0.08	0.16 ± 0.11	0.13 ± 0.07	0.249
MFGM	0.17 ± 0.12	0.20 ± 0.09	0.17 ± 0.09	0.14 ± 0.10	0.593

* Significant main effect ($p < 0.05$). ** Significant main effect ($p < 0.001$).

4.4.3 Neutrophil function

A two-way ANOVA revealed a significant main effect of time, $F(1.277, 11.492) = 12.696$, $p = 0.003$ and trial × time interaction, $F(3, 27) = 4.402$, $p = 0.012$ for fMLP-stimulated CL per neutrophil (RLU). There was no main effect of trial, $F(1, 9) = 1.757$, $p = 0.218$. Bonferroni-adjusted post-hoc tests revealed that the changes from Baseline to Post-Ex ($p = 0.015$, smaller decrease in MFGM) and 1 h Post-Ex ($p = 0.033$, smaller decrease in MFGM) were significant different between trials. One-way ANOVAs performed on each arm showed a main effect of time, *F*(1.681, 15.130) = 11.325, $p = 0.001$ in the PLA arm. Bonferroni-adjusted post-hoc tests showed a significant decrease from Baseline to Post-Ex ($p = 0.009$) and 1 h Post-Ex ($p = 0.021$). In the MFGM arm, there was a significant main effect of time, *F*(1.234, 11.104) = 9.306, $p = 0.008$. Bonferroni-adjusted post-hoc tests showed a significant increase from Baseline to Pre-Ex ($p = 0.006$), but no significant decreases at Post-Ex ($p = 0.111$) or 1 h Post-Ex ($p = 0.135$) ().

To remain consistent with previous research (Jones et al., 2015, 2014), fMLP-stimulated neutrophil oxidative burst was also calculated as a percentage of Baseline. A two-way ANOVA showed a main effect of trial, *F*(1, 9) = 20.692, $p = 0.001$, time, *F*(3, 27) = 27.215, $p < 0.001$ and trial × time interaction, *F*(3, 27) = 4.475, $p = 0.011$ for fMLP-stimulated CL per neutrophil (% of Baseline). Bonferroni-adjusted post-hoc tests revealed that the change from Baseline to Post-Ex ($p = 0.015$, smaller decrease in MFGM) and 1 h Post-Ex ($p = 0.012$, smaller decrease in MFGM) were significantly different between groups. One-way ANOVAs performed on each arm showed a main effect of time, *F*(1.758, 15.818) = 18.260 in the PLA arm. Bonferroni-adjusted post-hoc *t*-tests showed a significant decrease from Baseline at Post-Ex ($p < 0.001$) and 1 h Post-Ex ($p < 0.001$). There was a significant main effect of time, *F*(3, 27) = 17.668, $p < 0.001$ in the MFGM arm. Bonferroni-adjusted post-hoc *t*-tests showed a significant increase from Baseline to Pre-Ex ($p = 0.021$), but no significant decreases at Post-Ex ($p = 0.072$) or 1 h Post-Ex ($p = 0.222$) .

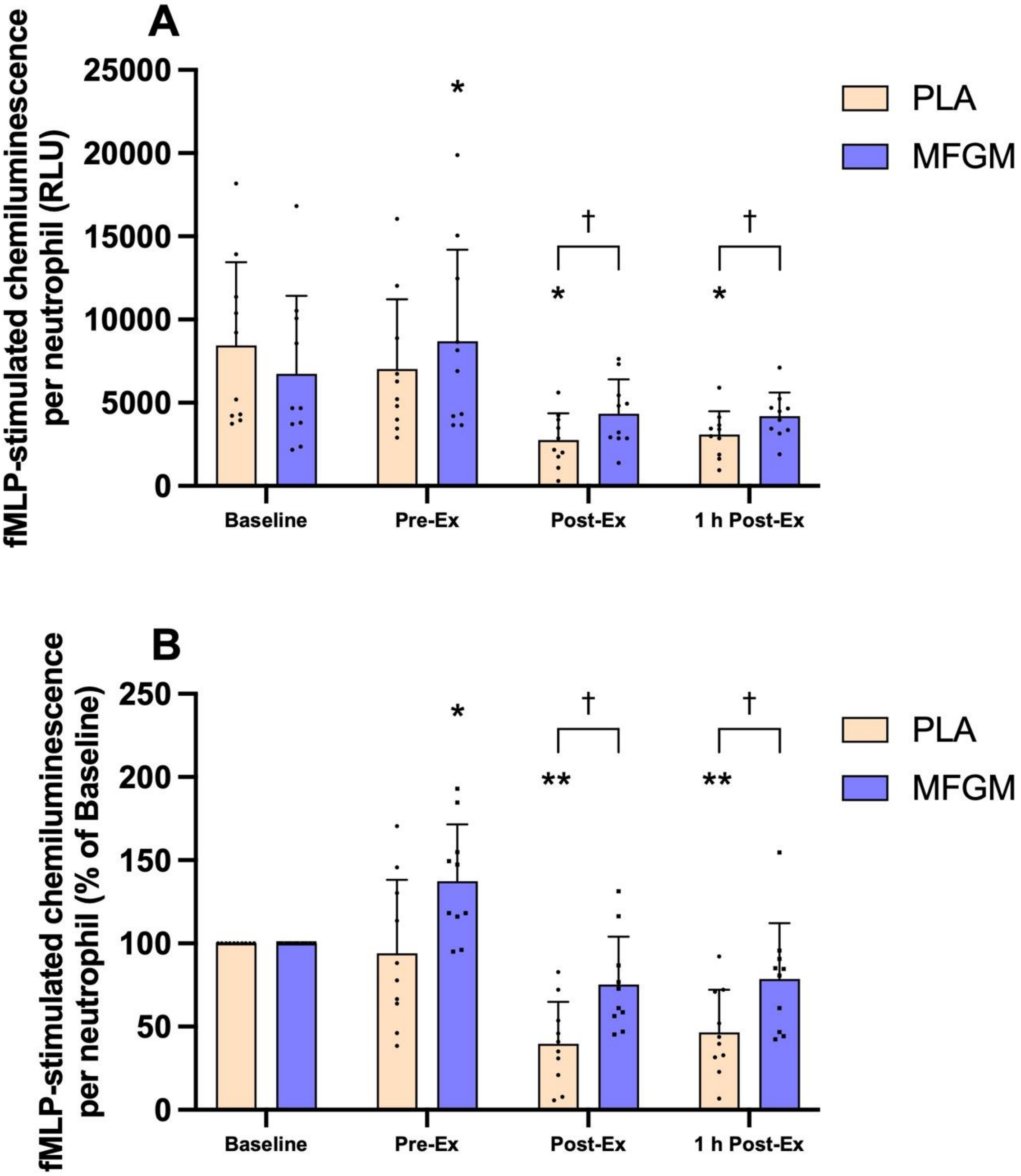

Figure 4.3. Neutrophil oxidative burst following prolonged cycling. A- fMLP-stimulated CL per neutrophil (RLU). Two-way ANOVA main effects: $p = 0.218$ (trial); $p = 0.003$ (time); $p = 0.012$ (trial × time interaction). B- fMLP-stimulated CL per neutrophil (% of Baseline). Two-way ANOVA main effects: $p = 0.001$ (trial); $p < 0.001$ (time); $p = 0.011$ (trial × time). * Mean values were significantly different from Baseline ($p < 0.05$; post-hoc analysis). ** Mean values were significantly different from Baseline ($p < 0.001$; post-hoc analysis). † Change from Baseline was significantly different between trials ($p < 0.05$; *post-hoc* analysis).

4.4.4 Saliva parameters

A significant main effect of time, *F*(3,27) = 5.335, *p* = 0.005 was detected for saliva osmolality. No significant main effect of trial, *F*(1, 9) = 0.214, *p* = 0.655, or trial × time interactions *F*(3,27) = 1.534, *p* = 0.228 were detected. Post-hoc tests did not show any significant comparisons.

A significant main effect of time, *F*(3,27) = 6.262, *p* = 0.002 was observed for saliva flow rate. Though, there was no main effect of trial, *F*(1,9) = 3.191, *p* = 0.108 or trial × time interaction, *F*(1.740, 15.663) = 2.260, *p* = 0.104. There were no significant post-hoc comparisons.

Table 4.3. Saliva parameters at baseline and on main trials.

Saliva parameter	Baseline	Pre-Ex	Post-Ex	1 h Post-Ex	*p* trial time interaction
Osmolality (mOsmol·kg^{-1})					0.655
PLA	62 ± 22	64 ± 20	77 ± 23	63 ± 13	0.005*
MFGM	67 ± 23	61 ± 18	83 ± 29	62 ± 11	0.228
Flow rate (mL·min^{-1})					0.108
PLA	0.50 ± 0.17	0.55 ± 0.16	0.41 ± 0.19	0.57 ± 0.16	0.002*
MFGM	0.51 ± 0.17	0.60 ± 0.56	0.54 ± 0.17	0.63 ± 0.17	0.104

* Significant main effect ($p < 0.05$).

4.4.5 Salivary SIgA

A significant main effect of time, *F*(3, 27) = 4.518, *p* = 0.011 was observed for salivary SIgA concentration. However, no significant post-hoc tests were detected. There was no significant main effect of trial, *F*(1, 9) = 0.002, *p* = 0.969 or significant trial × time interactions, *F*(3, 27) = 0.871, *p* = 0.468. No significant main effects of trial, *F*(1, 9) = 0.263, *p* = 0.620, time, *F*(3, 27) = 2.631, *p* = 0.070, or trial × time interaction, *F*(3, 27) = 0.913, *p* = 0.448 were observed for salivary SIgA:osmolality. No significant main effects of trial, *F*(1, 9) = 1.151, *p* = 0.311, time, $F(3, 27) = 2.137$, $p = 0.119$, or trial × time interaction $F(1.393, 12.534) = 1.567$, $p = 0.242$ were detected for salivary SIgA secretion rate (Figure 4.4).

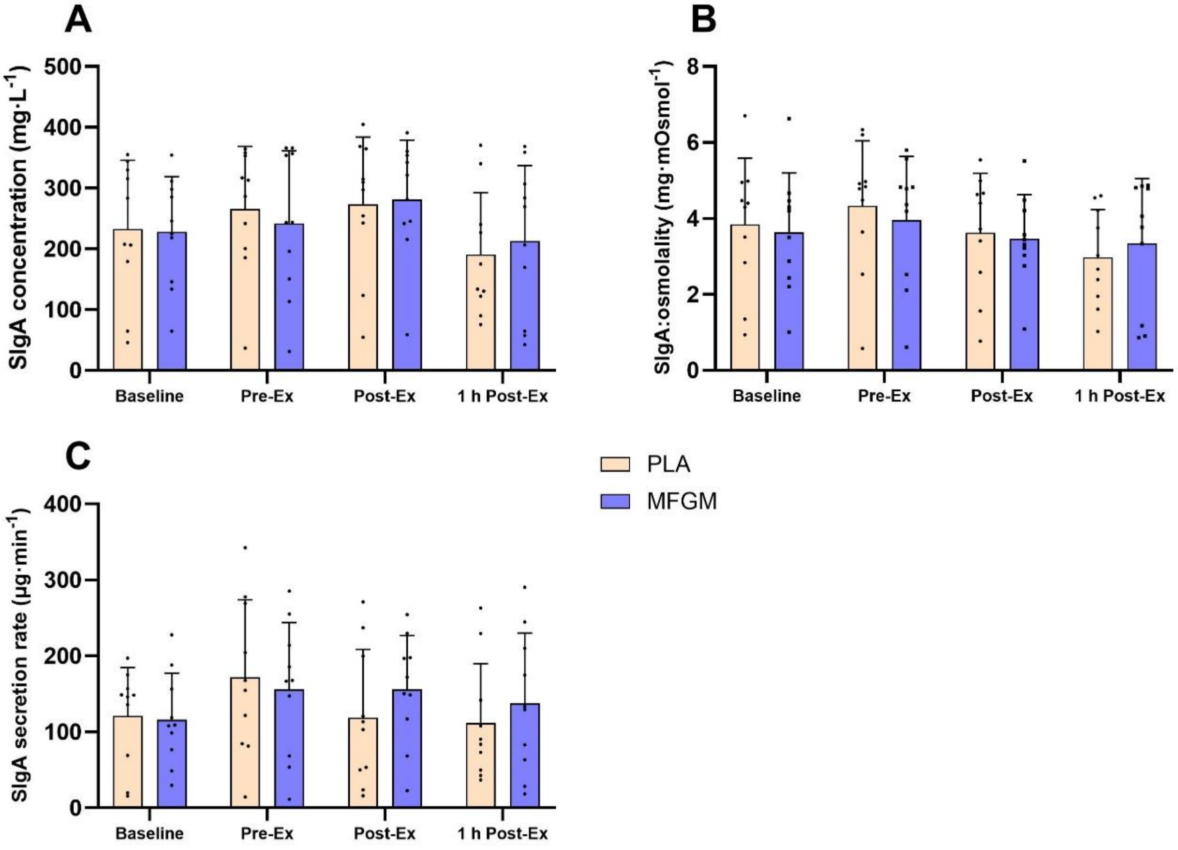

Figure 4.4. SIgA responses to prolonged cycling. A- SIgA concentration. Two-way ANOVA main effects: $p = 0.969$ (trial); $p = 0.011$ (time); $p = 0.468$ (trial × time interaction). B- SIgA:osmolality. Two-way ANOVA main effects: $p = 0.620$ (trial); $p = 0.070$ (time); $p = 0.448$ (trial × time interaction). C- SIgA secretion rate. Two-way ANOVA main effects: $p = 0.311$ (trial); $p = 0.119$ (time); $p = 0.242$ (trial × time interaction).

4.4.6 Salivary lysozyme activity

A two-way ANOVA showed no main effect of trial, $F(1, 8) = 2.368$, $p = 0.162$, time, $F(1.517, 12.139) = 3.651$, $p = 0.067$ or trial × time interaction, $F(3, 24) = 0.385$, $p = 0.765$ for salivary lysozyme activity (Figure 4.5).

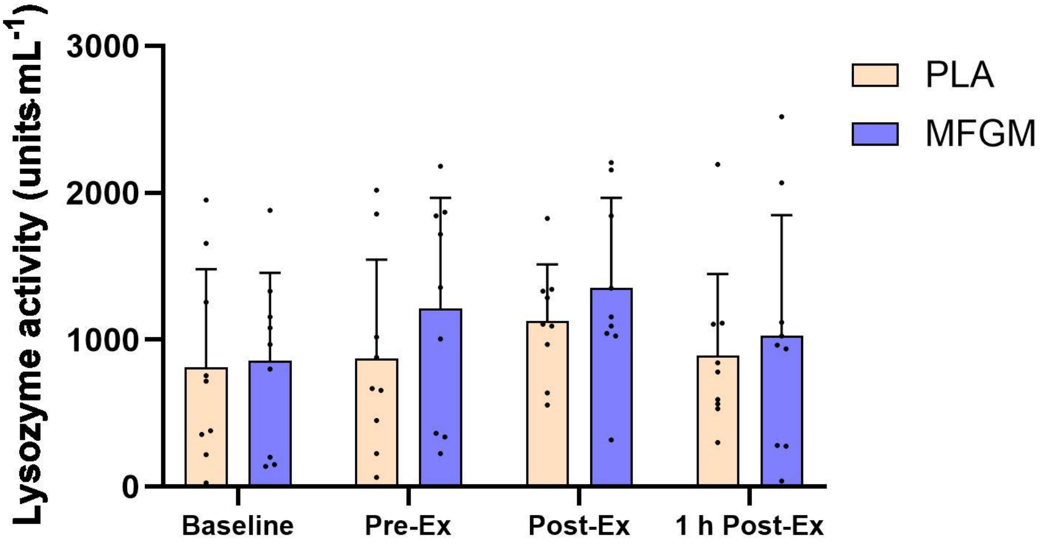

Figure 4.5. Salivary lysozyme activity following prolonged cycling. Lysozyme activity (units·mL^{-1}). Two-way ANOVA main effects: $p = 0.162$ (trial); $p = 0.067$ (time); $p = 0.765$ (trial × time interaction).

4.4.7 Plasma Cortisol

A two-way ANOVA showed no significant main effects of trial, $F(1, 9) = 0.504$, $p = 0.496$ or time, $F(2, 18) = 2.543$, $p = 0.106$ for plasma cortisol concentrations. There was no significant trial × time interaction, $F(2, 18) = 0.814$, $p = 0.459$ (Figure 4.6).

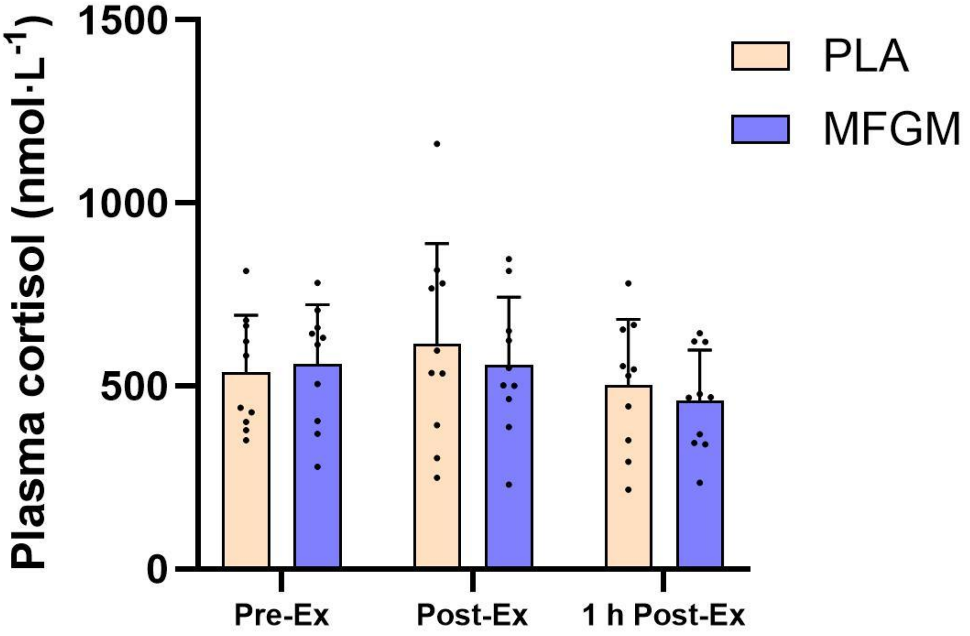

Figure 4.6. Cortisol responses to prolonged cycling. Plasma cortisol concentration (nmol·L^{-1}). Two-way ANOVA main effects: $p = 0.504$ (trial); $p = 0.106$ (time); $p = 0.459$ (trial × time interaction).

4.4.8 Plasma I-FABP

A two-way ANOVA showed no significant main effects of trial, $F(1, 9) = 0.003$, $p = 0.960$ or time, $F(2, 18) = 2.450$, $p = 0.115$ for absolute plasma I-FABP concentrations. However, a significant trial × time interaction was observed, $F(2, 18) = 20.392$, $p < 0.001$. Post-hoc contrasts revealed that the change in I-FABP concentrations from Pre-Ex to Post-Ex ($p < 0.001$) was significantly lower in the MFGM arm. One-way ANOVAs performed on each arm showed a significant main effect of time, $F(2, 18) = 5.773$, $p = 0.012$ in the PLA arm, with post-hoc tests revealing a significant increase from Pre-Ex to Post-Ex ($p = 0.034$). No main effect of time, $F(2, 18) = 1.054$, $p = 0.369$ was observed for the MFGM arm (Figure 4.7).

A two-way ANOVA revealed a significant main effect of trial, $F(1, 9) = 24.874$, $p < 0.001$ for I-FABP% change from Pre-Ex, with MFGM significantly lower than PLA. Though, no significant main effect of time, $F(2, 18) = 2.014$, $p = 0.162$ was observed. A significant trial ×

time interaction, *F*(2, 18) = 21.228, $p < 0.001$ was observed. Bonferroni-adjusted post-hoc tests revealed that the changes from Pre-Ex to Post-Ex ($p < 0.001$) and 1 h Post-Ex ($p = 0.024$) were significantly lower in the MFGM arm. One-way ANOVAs performed on each arm showed a significant main effect of time, *F*(2, 18) = 5.448, $p = 0.014$ in the PLA arm, and post-hoc contrasts revealed a significant increase from Pre-Ex to Post-Ex ($p = 0.034$). A main effect of time, *F*(2, 18) = 0.714, $p = 0.503$ was not observed for the MFGM arm (Figure 4.7).

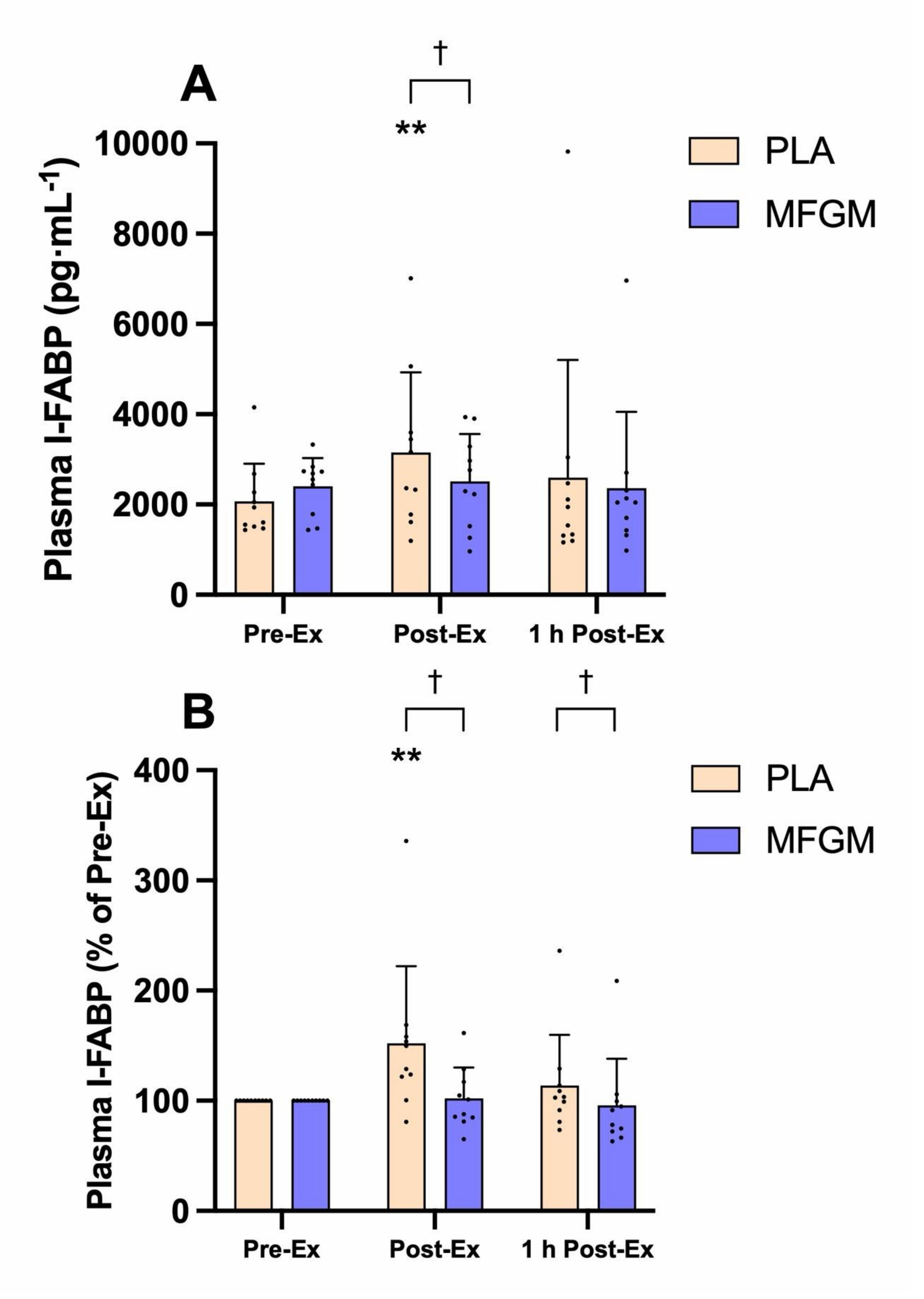

Figure 4.7. I-FABP responses to prolonged cycling. A- Plasma I-FABP concentration (pg·mL^{-1}). Two-way ANOVA main effects: $p = 0.960$ (trial); $p = 0.115$ (time); $p < 0.001$ (trial × time interaction). B- Plasma I-FABP% of mean values at Pre-Ex. Two-way ANOVA main effects: $p < 0.001$ (trial); $p = 0.162$ (time); $p < 0.001$ (trial × time interaction). * Mean values were significantly different from Baseline ($p < 0.05$; post-hoc analysis). † Change from Pre-Ex was significantly different between trials ($p < 0.05$; *post-hoc* analysis).

4.4.9 Bacterial translocation

There was a significant main effect of time, $F(2, 18) = 5.331$, $p = 0.015$ for blood bacterial load. There was no main effect of trial, $F(1, 9) = 3.966$, $p = 0.078$ or trial × time interaction, $F(2, 18) = 0.684$, $p = 0.517$. Bonferroni-adjusted post-hoc *t*-tests revealed a significant decrease from Post-Ex to 1 h Post-Ex ($p = 0.006$) (Figure 4.8).

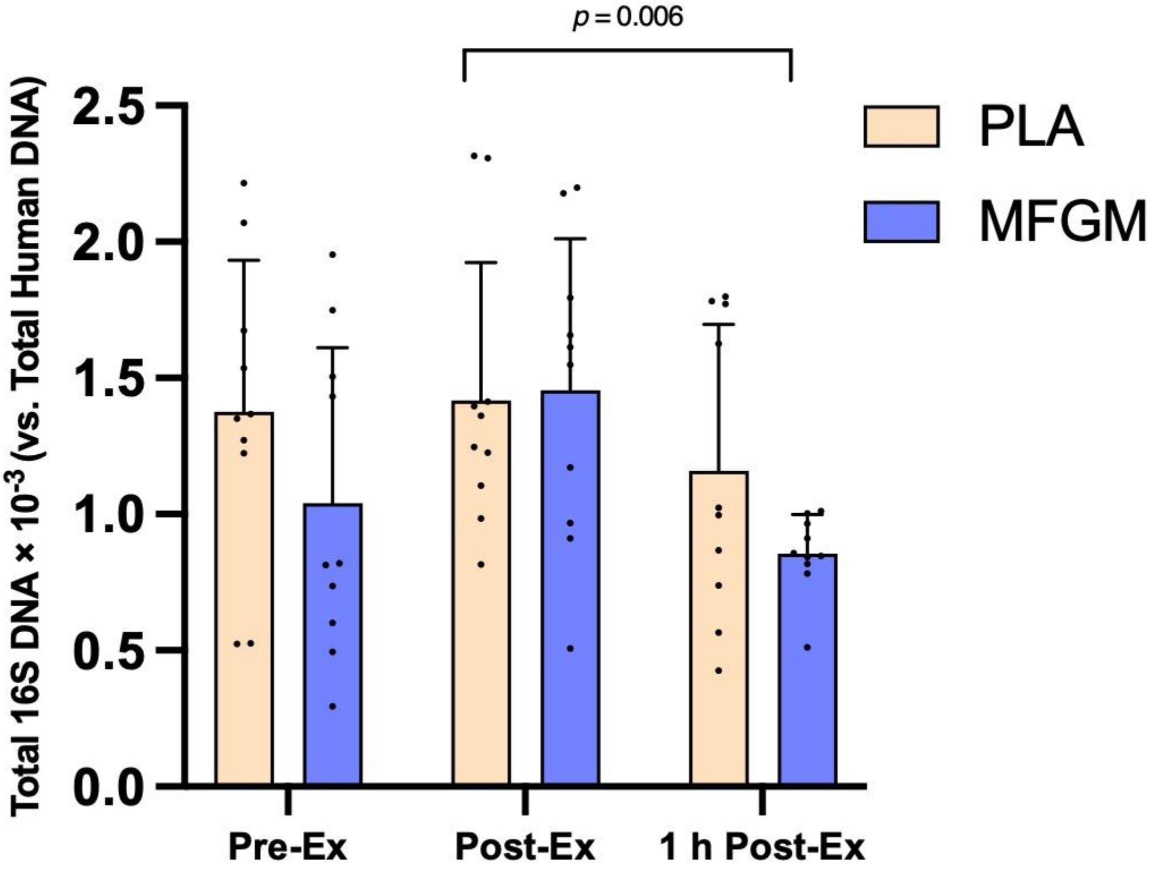

Figure 4.8. Bacterial load responses to prolonged cycling: Total 16S/Total Human DNA ratio ($\times 10^{-3}$). Two-way ANOVA main effects: $p = 0.078$ (trial); $p = 0.015$ (time); $p = 0.517$ (trial × time interaction). Significant decrease from Post-Ex to 1 h Post-Ex ($p = 0.006$; post-hoc analysis).

16S rRNA gene sequencing was used to assess bacterial composition in blood at Pre-Ex, Post-Ex and 1 h Post-Ex in both PLA and MFGM trials. After QC and filtering, 48 samples (28 PLA; 20 MFGM) contained sufficient bacterial DNA for amplification. There was a significantly lower proportion of samples with bacterial DNA present in MFGM (69%, 20/29 samples) compared to PLA (96.6%, 28/29 samples), χ^2 (1) = 7.733, p = 0.005. The proportion of samples present with bacterial DNA did not significantly change from Pre-Ex (80%, 16/20 samples) to Post-Ex (95%, 19/20 samples) and 1 h Post-Ex (77.8% 14/18 samples), χ^2 (2) = 2.611, p = 0.271. The proportion of samples with bacterial DNA present did not change over time in PLA, χ^2 (2) = 2.302, p = 0.316 or MFGM, χ^2 (2) = 2.468, p = 0.291.

At the genus level, a diverse bacterial composition was seen across subjects, of which the most abundant taxa were of gut origin; Escherichia-Shigella, Lactobacillus, Romboutsia, Clostridium sensu stricto 1 and a taxon of the Lactobacillaceae family that could not be classified to genus level (Figure 4.9A). Changes in the relative abundance of some genera can be seen over the different exercise timepoints in both PLA and MFGM. Visual increases in relative abundance from Pre-Ex levels can be seen for Escherichia-Shigella and the unclassified Lactobacillaceae genus in both trials, and a slight increase in Romboutsia can also be seen in the PLA arm. Though, these comparisons are only visual and PCoA analyses were performed to determine whether there were any significant shifts in bacterial compositions.

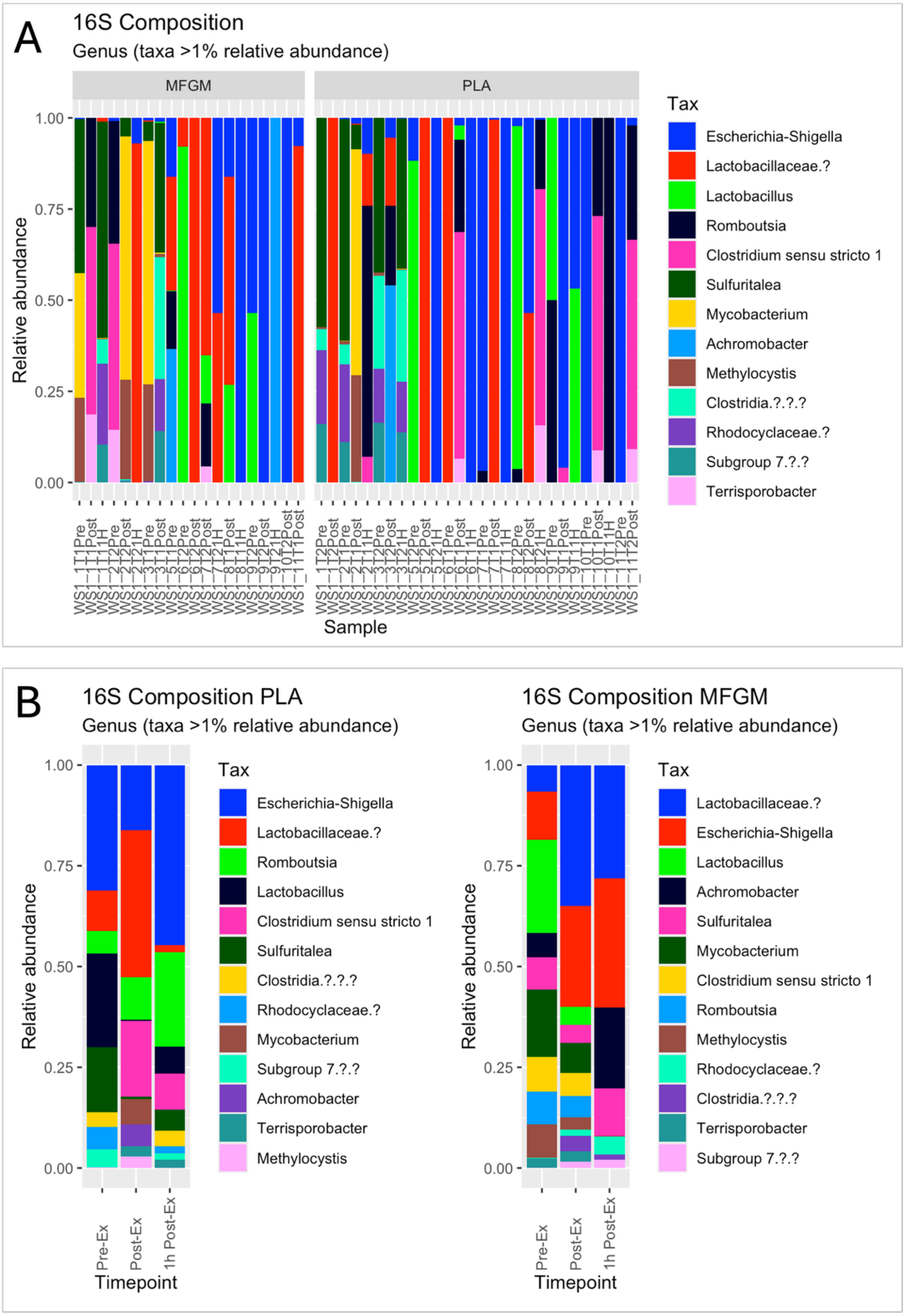

Figure 4.9. 16S relative abundance. A- 16S relative abundance for individual samples. B- Mean 16S relative abundance for time point. ?- classified to family level. ??- classified to order level. ???- classified to class level.

PCoA performed on Bray-Curtis dissimilarity values were used to assess overall differences in bacterial composition (beta-diversity) between supplements and over time. PERMANOVA showed no effect of trial, $R^2 = 0.014$, $F(1, 51) = 0.723$, $p = 0.796$, time, $R^2 = 0.043$, $F(2, 49) = 1.104$, $p = 0.153$, or trial × time interaction, $R^2 = 0.048$, $F(2, 46) = 1.221$, $p = 0.062$ for bacterial composition in blood (Figure 4.10).

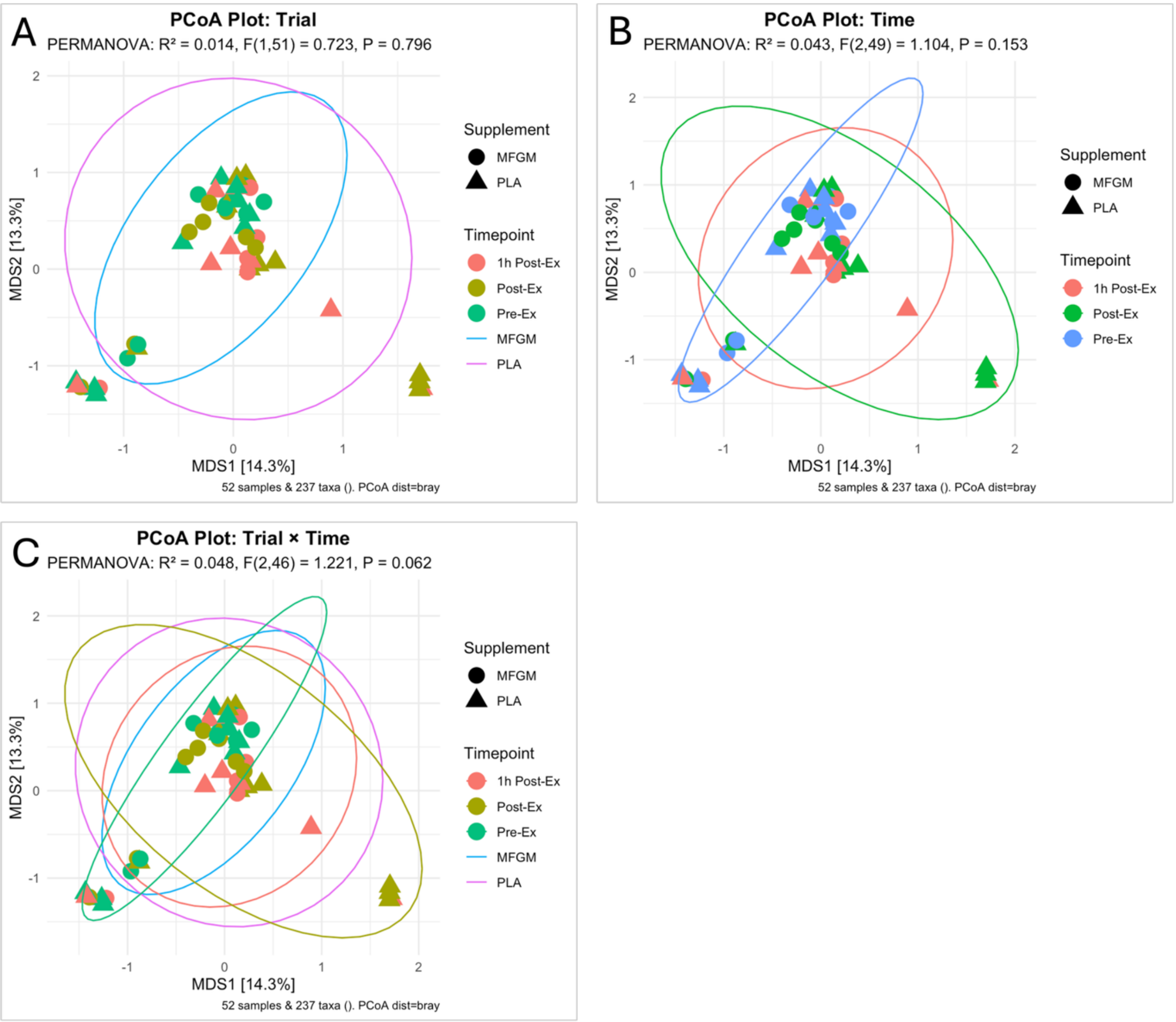

Figure 4.10. Principal coordinates analysis (PCoA). A- PCoA plot for trial effect. PERMANOVA results: $R^2 = 0.014$, $F(1, 51) = 0.723$, $p = 0.796$. B- PCoA plot for time effect. PERMANOVA results: $R^2 = 0.043$, $F(2, 49) = 1.104$, $p = 0.153$. C- PCoA plot for trial × time interaction. PERMANOVA results: $R^2 = 0.048$, $F(2, 46) = 1.221$, $p = 0.062$.

4.4.10 Psychological and physical wellbeing

A paired samples *t*-test revealed there was no significant difference in state anxiety on main trial days between MFGM (30.3 ± 6.9 STAI-S score) and PLA (31.2 ± 9.8 STAI-S score); *t*(9) = 0.380, *p* = 0.713. There was no significant difference in perceived stress between MFGM (10.6 ± 5.1 PSS score) and PLA (10.0 ± 3.0 PSS score) main trial days; *t*(9) = -0.56. *p* = 0.589. Lastly, there was no significant difference between physical wellbeing between MFGM (9.2 ± 7.7 CHIPS score) and PLA (3.7 ± 3.2 CHIPS score) arms; *t*(9) = -2.005, *p* = 0.076.

4.4.11 Training load

A paired samples *t*-test showed no difference in weekly training load (sRPE × duration) between MFGM (335 ± 107) and PLA (369 ± 160) ; *t*(7) = 0.731, *p* = 0.488. A paired samples *t*-test showed no difference in weekly training duration between MFGM (663 ± 491 min) and PLA (616 ± 461 min), *t*(7) = -0.150, *p* = 0.885.

4.4.12 Upper respiratory tract infections

No URTI cases reported met the Jackson criteria to be considered an URTI. No swabs were collected.

4.4.13 Phospholipid quantification

Due to issues with lipid extraction and subsequent sample availability, phospholipids could not be quantified for all participants. Red blood cell phospholipid content was quantified in a subgroup analysis of *n* = 5 (4 male, 1 female), at baseline and the main trial of the MFGM arm

(2 weeks of MFGM supplementation). A paired samples *t*-test revealed a significant increase in red blood cell phospholipid concentration from baseline (0.034 ± 0.010 $mg \cdot mL^{-1}$) to main trial (0.043 ± 0.004 $mg \cdot mL^{-1}$), $t(4) = -2.922$, $p = 0.043$.

4.4.14 Adverse events reported

No adverse events or adverse reactions were reported throughout the study.

4.5 Discussion

The present study demonstrated that 2 weeks of daily MFGM supplementation significantly increased fMLP-stimulated neutrophil oxidative burst and attenuated the increase in plasma I-FABP concentrations following prolonged cycling. However, there were no differences (compared to PLA) in the responses of plasma cortisol, salivary SIgA, or blood bacterial composition in response to exercise. This is the first study to examine the effects of MFGM on innate immune functions in healthy adults, so there is no direct literature to compare to. However, it has already been stated that MFGM draws some similarities to other bioactive dairy supplements, like bovine colostrum. Thus, comparisons to such products may still provide relevant insights into the mechanisms underpinning effects of MFGM immune functions. A subgroup analysis demonstrated that phospholipid content increased within red blood cells following MFGM supplementation. Since phospholipids are thought to be among the most bioactive components of MFGM, this indicates that positive effects in the MFGM trial *could* be related to the phospholipids within. However, phospholipid content was not measured in the PLA arm, so it is not possible to verify whether this increase was specific to the MFGM arm.

In line with similar studies in this field, significant increases in total leukocytes, neutrophils, lymphocytes, N/L ratio and monocytes were observed following the prolonged cycling bout (Davison and Diment, 2010; Jones et al., 2015). However, since there were no differences in total and differential leukocyte trafficking between PLA and MFGM, it is unlikely that the effects of MFGM on neutrophil function are underpinned by leukocytosis-related factors. Similarly, there were no differences between experimental arms for exercise physiological and perceptual responses, so any effects observed between supplements could not be attributed to inconsistencies in exercise workload. Furthermore, state anxiety, perceived stress, physical wellbeing and training load did not differ between arms, suggesting that the psychological and physical states of subjects were consistent across conditions.

The present study demonstrated that resting fMLP-stimulated neutrophil oxidative burst increased following 2 weeks of MFGM supplementation. MFGM also prevented a fall below Baseline levels following the prolonged exercise bout. These results align with those reported with bovine colostrum supplementation, where it has been shown to attenuate the decline in fMLP-stimulated oxidative burst following a prolonged cycling model (Jones et al., 2015). Bovine colostrum has also shown to enhance the recovery of neutrophil function post-exercise, however, the authors measured this using stimulated degranulation (Davison and Diment, 2010). Given the similarity in oxidative burst responses between MFGM and bovine colostrum supplementation, it is possible that such degranulation responses may also be similar between supplements and could provide a future opportunity for research.

If the enhanced neutrophil function demonstrated in the present study was not influenced by leukocytosis (via catecholamine or HPA activation), another possible mechanism that has been proposed may be the priming of neutrophils by various factors. It has been demonstrated that low molecular weight (< 10 kDa) fractions of bovine milk and colostrum possess neutrophil priming factors (Sugisawa et al., 2003). The lower molecular weight components within bovine milk and colostrum are not fully understood, but it has been suggested that proteose peptones may be a contributor to such effects (Merin et al., 2001; Sugisawa et al., 2003). Alternatively, neutrophils may enter a primed state following exposure to other agents such as pro-inflammatory lipids, cytokines and chemokines (El-Benna et al., 2008). Therefore, it is possible, albeit speculative at present, that MFGM may exhibit effects on these factors, leading to indirect priming of neutrophils and increased NADPH activation.

The present study observed no between-trial differences in plasma cortisol responses, which further enforces the notion that the benefits to neutrophil function seen were independent from HPA activation. These results differ from those found in similar research, which have typically observed increases in cortisol following prolonged cycling (Davison et al., 2007; Davison and Gleeson, 2006). Though, a likely explanation for the lack of cortisol changes seen in the present study is the timing of the exercise trials. All exercise trials in the present study took place around 09:00, with some trials taking place closer to 08:00. In comparison, the other studies started all exercise at 10:00 (Davison et al., 2007; Davison and Gleeson, 2006). Cortisol levels generally follow a consistent diurnal pattern, with levels high upon waking and surging briefly in the 30-40 min following waking, before dropping rapidly in the subsequent few hours, and then dropping at a slower rate leading to a low point around bedtime (Adam et al., 2017). If the cortisol response in the present study was plotted against the diurnal pattern, it is likely that the Pre-Ex sample aligned with high diurnal cortisol due to its close proximity to waking, and after a few hours at the Post-Ex and 1 h Post-Ex samples, diurnal cortisol would have dropped substantially. Therefore, since the expected diurnal decline in cortisol was blunted, this suggests that activation of the HPA axis occurred but was masked by the underlying diurnal rhythm. This highlights a limitation of the present study, as it may be prudent to time exercise trials for later in the day if cortisol is an important measure. As cortisol was not a primary outcome measure in the study, it was decided that this limitation was worth accepting. The lack of difference in cortisol responses between MFGM and PLA indicate that the significant effects observed, such as enhanced neutrophil function, are not a result of differing HPA activation between conditions.

Many studies have shown that exercise, of both shorter and prolonged nature, can elicit increases in plasma I-FABP concentrations, indicating intestinal epithelial cell damage (Davison et al., 2016; March et al., 2019, 2017; Marchbank et al., 2011; Walter et al., 2021a). Consistent with the previous research, the present study found that the prolonged exercise bout significantly increased plasma I-FABP at Post-Ex, but only in the PLA trial. Moreover, the changes from Pre-Ex to Post-Ex and 1 h Post-Ex were significantly lower in the MFGM arm. Further supporting the findings of study 1, these results demonstrate that MFGM was able to successfully prevent a significant increase in gut damage following exercise. As with the findings of study 1, the present study also saw no changes in bacterial composition in blood, despite the elevations in gut damage. The 16S compositions showed a more diverse range of taxa, and there were some visual changes in relative abundance for gut taxa, though this did not translate to any meaningful shifts in community composition.

A key component within MFGM that may support gut barrier integrity is lactadherin, which is largely implicated with the clearance of apoptotic cells (Ait-Oufella et al., 2007). Additionally, there is evidence to show that lactadherin may support the maintenance of tight junction and adherens junction proteins (Shen et al., 2019). Following exercise cessation, organs such as the gut must be reperfused, which can lead to elevated inflammation and oxidative stress (Chantler et al., 2021). Components of MFGM such as lactadherin have also shown to reduce NF-κβ and TNF-α activation, thus providing anti-inflammatory effects and reducing oxidative stress (Abe et al., 2014; Han et al., 2022). TNF-α activation can also initiate enterocyte apoptosis (Berghe et al., 2014; Woznicki et al., 2021), so suppression of this signalling pathway may also limit the extent of enterocyte apoptosis, maintaining the integrity of the gut barrier. The anti-inflammatory properties, alongside the clearing of apoptotic cells could help maintain the gut barrier and speed up repair in the case of epithelial injury.

Phospholipids, which are a major constituent of MFGM, have been shown to promote the production of SCFAs through their effects on SCFA-producing bacteria, like Bifidobacterium and LAB (Feng et al., 2025; Gong et al., 2024; Kosmerl et al., 2021; Wu et al., 2021). It is well known that SCFAs have beneficial effects on gut barrier function and integrity, which may be a result of their anti-inflammatory effects, which can reduce oxidative stress, alongside the maintenance of tight junction proteins (Liu et al., 2021). Phospholipids such as PC have been shown to integrate into the plasma membrane of enterocytes, influencing various functions (Küllenberg et al., 2012). Given the role of (phospho)lipids in maintaining cell integrity and stability (Ammendolia et al., 2021), it is possible that supplementation of dietary phospholipids and their incorporation into cellular membranes may help maintain or enhance cell integrity, and in this case, attenuate exercise-induced increases in enterocyte damage.

A main effect of time was observed for absolute SIgA concentrations, but there was no difference between PLA and MFGM. Despite a significant main effect of time for saliva osmolality and flow rate, SIgA:osmolality and SIgA secretion rate remained unchanged in both arms, thus highlighting that factors such as hydration were consistent across trials and did not have an effect. This is in line with some studies (Jones et al., 2015; Kunz et al., 2015; Laing et al., 2005; Li and Gleeson, 2004), but not all, as it has been documented that SIgA concentrations can experience significant drops following prolonged exercise (Li and Gleeson, 2004; Walsh et al., 2002). Likewise, there was no difference between PLA and MFGM for salivary lysozyme activity. This also follows the trends of previous research, with bovine colostrum having shown little to no effect on salivary lysozyme (Jones et al., 2015). It is worth noting that Jones and colleagues' analysed salivary lysozyme via ELISA, whereas the present study measured the function of lysozyme. Consequently, the findings of both studies may offer relevant context for the effects of bioactive dairy supplements on salivary lysozyme, they should not be taken as exact comparisons.

4.5.1 Limitations

A consideration of the present study was that the timing of the exercise trials was always between 08:00 and 10:00and were near awakening. Since cortisol follows a natural diurnal rhythm, characterised by high levels upon waking and a transient surge shortly thereafter, it is probable that cortisol levels were still higher at Pre-Ex (Adam et al., 2017). This may explain the lack of a cortisol response to exercise seen in the present study. Where it may have been expected that cortisol levels started lower and increased in response to exercise, the diurnal pattern would instead have started high and decreased over time, potentially masking any exercise-induced effect. If cortisol was a primary outcome measure, it would have been beneficial to start the exercise trials later in the day. However, since cortisol was a secondary measure, and participants were already required to remain fasted over an extended period, this limitation was considered acceptable. Another limitation of the present study was the use of an enzymatic assay to measure salivary lysozyme. While this method measures the functional activity of lysozyme, it does not quantify its absolute concentration. The use of ELISA for analysis of lysozyme is often considered the preferred method for accurately determining lysozyme concentration, allowing for direct comparison with other studies that have used the same techniques. However, measuring lysozyme activity through an enzymatic assay may provide a greater insight into its function as an antimicrobial agent, rather than solely its presence. Therefore, although the methodological difference limits its comparison with other studies using ELISA, it offers valuable insight into the relevance of lysozyme function under real-world conditions.

Similarly with study 1, it should also be highlighted that the use of 16S rRNA gene sequencing for bacterial translocation poses some limitations. The naturally low bacterial biomass in blood makes 16S amplicon sequencing more susceptible to contamination and index hopping. A series of filters were applied to the data to account for this, and known contaminants were purged from the dataset. 16S amplicon sequencing is also limited by its inability to provide absolute quantification of bacteria. 16S amplicon sequencing provides compositional analysis, giving insight into the bacterial community and any changes in taxa relative abundance, but qPCR would be required to provide quantifiable values of bacteria concentrations. Therefore, qPCR and 16S amplicon sequencing complement each other well and future research should look to include them both if possible.

4.5.2 Conclusion

This was the first study to assess the effects of MFGM on innate cellular and mucosal immune functions. MFGM significantly increased resting neutrophil function and prevented an exercise-induced drop below baseline levels. MFGM also prevented an increase in plasma I-FABP, indicating a lower extent of gut epithelial cell damage. There were no changes in mucosal immune functions, cortisol response or bacterial composition in blood between trials. The findings of the present study are promising, but as it the first to assess MFGM in this context, further research would be beneficial to understand the nutraceutical potential of MFGM.

Chapter 5. Study 3 – Effects of 12 weeks of milk fat globule membrane supplementation on immune function and upper respiratory illness in active people

5.1 Abstract

Following prolonged exercise, or during periods of heavy training load, components the immune system can become depressed and increase the risk of illnesses, like URTIs. Following from the work of Chapter 4, MFGM has shown to attenuate the depression of cellular immune function and may potentially reduce the risk of illness in these periods. The purpose of this study was to determine whether long-term (12 weeks) supplementation with MFGM reduces the incidence and/or burden of URTIs and affects mucosal immune functions in an active population. In total, 115 (77 male, 38 female; age 21.5 ± 5.3 years; height 175.5 ± 9.2 cm; BM 74.8 ± 16.2 kg) healthy, active individuals participated in a double-blind, parallel groups, randomised controlled trial. Participants consumed either MFGM or a placebo daily for 12 weeks. Illness/training logs were completed daily to monitor URTIs and training load, and URTIs were confirmed via pathogen detection using oral/nasal swabs. Salivary SIgA, salivary lysozyme activity and physical wellbeing was measured every 4 weeks. There were no differences in URTI incidence ($p = 0.304$), duration ($p = 0.433$), symptom ratings ($p < 0.05$), or viral/bacterial load ($p < 0.05$) between MFGM and PLA. There were also no differences in salivary SIgA ($p < 0.05$), salivary lysozyme activity ($p = 0.327$) or physical wellbeing ($p = 0.559$). Lastly, there were no differences in training load ($p = 0.601$), training days affected ($p = 0.265$), or training days missed ($p = 0.081$) due to URTIs. These findings suggest that the previously demonstrated benefits of MFGM may not translate to protection against URTIs. However, these findings may be influenced by issues pertaining to the placebo supplement used.

5.2 Introduction

URTIs are the most common illness seen in patient evaluations worldwide, accounting for a substantial proportion of primary care consultations and healthcare visits (Morbey et al., 2018). While moderate physical activity is generally accepted to aid immune functions and reduce illness susceptibility, athletes who engage in regular prolonged and/or strenuous exercise may be at risk of transient immune depression, which can lead to a temporary state of increased illness susceptibility. (Schwellnus et al., 2022). In fact, URTIs are one of the most frequent medical conditions seen in athlete clinics during the summer and winter Olympic games (Engebretsen et al., 2013, 2010). The risk of infection in some populations is influenced by numerous lifestyle factors, including nutrition and diet (Calder and Jackson, 2000), psychological stress (Cohen et al., 1991; Edwards et al., 2018b), sleep (Prather et al., 2015) and vaccination status (Davison et al., 2025a). Variations in these URTI risk factors mean there is no universal quantification for the increased risk of illness associated with prolonged/strenuous exercise. Though, seminal studies have consistently shown increased risks of URTI with heavy exercise load, with some reporting up to 6- to 10-fold increases in illness incidence compared with controls (Timpka et al. 2017; Konig et al; Spence et al; Nieman et al. 1990; Peters and bateman 1983; Svendsen et al).While URTIs are generally mild in nature and do not require extensive treatment or hospital admissions for generally healthy people, they may still have significant economic and social impacts (e.g. work absence, increased morbidity, reduced social interactions and reduced feelings of well-being/quality of life). URTIs may also have a severe impact on the performance of athletes, both directly (e.g. if occurs during or around competition) and indirectly (e.g. impacting training and/or recovery).

Due to the consequences associated with URTIs, especially to athletes, strategies of mitigating exercise-induced immune depression are of interest to researchers and practitioners. Following the results of studies 1 and 2, it is clear that MFGM may possess numerous health benefits, including those supporting the immune and GI systems. While the benefits seen to cellular immune functions provide a mechanistic insight into the immunomodulatory potential of MFGM, a more clinically relevant outcome would be to determine whether these effects translate to changes in illness susceptibility and/or burden (e.g. duration or severity). Bovine colostrum has been associated with a reduction in URTI incidence in athletes, an effect which is likely attributable to the diverse bioactive components contributing to a range of immunomodulatory effects (Davison, 2021; Jones et al., 2016). Given the compositional and possible functional connections previously identified between MFGM and bovine colostrum, it is reasonable to suggest that such similarities may extend to protection against URTIs.

This study aimed to assess the effects of 12 weeks of MFGM supplementation on mucosal immune functions and URTI incidence, duration, symptom ratings, viral/bacterial load and training affected (reduced or missed) due to URTIs. Following the previous findings of this thesis, it was hypothesised that MFGM would reduce URTI parameters, including incidence, duration and severity of symptoms. It was also hypothesised that MFGM would enhance mucosal immune functions.

5.3 Methods

5.3.1 Ethical approval

The study was approved by the University of Kent Sport, Exercise, and Rehabilitation Sciences REAG (Prop 40_20_23) and was conducted in accordance with the Declaration of Helsinki. All participants were informed, written and verbally, of all aspects of the study, including any risks, prior to giving their written consent to participate. The trial was prospectively registered on the ISRCTN registry (ISRCTN11636007, https://doi.org/10.1186/ISRCTN11636007). ISRCTN is a WHO and ICMJE recognised primary clinical study registry.

5.3.2 Power calculation

This is the first study of its type using MFGM, so no data was available to perform a preliminary power calculation. However, MFGM may share some mechanisms with bovine colostrum and the previous studies in this thesis successfully estimated MFGM effect sizes using bovine colostrum data, so a power calculation was performed (G*Power version 3.1.9.7, Kiel, Germany) using a bovine colostrum study with a similar design (Jones et al., 2014). Following the similar findings for MFGM and bovine colostrum across the previous two studies, an assumed effect size of MFGM was not used. For the primary outcome of this study, number of URTI episodes, Jones et al. (2014) reported an effect size of Cohen's $d = 0.57$. Using this data, it was calculated that a sample of $n = 100$ would be required to detect a significant difference in number of URTI episodes between MFGM and PLA at 80% power and alpha level 0.05 (two-tailed independent *t*-test). Since there was no conservative approach taken with MFGM effect size and to protect against potential dropouts, an effort was made to recruit beyond the target.

5.3.3 Participants

The present study recruited 115 (77 male, 38 female; age 21.5 ± 5.3 years; height 175.5 ± 9.2 cm; BM 74.8 ± 16.2 kg) individuals who regularly exercise - the inclusion criteria required all participants to exercise for at least 180 min per week at a moderate-to-vigorous intensity. Participants were excluded if on long-term medication, if a URTI or any URS had been experienced within the 4 weeks preceding the study, if taking any medication or supplements that might affect the immune system, if they had any allergies or intolerances to the supplements used in the study, and if they were not deemed 'healthy' by a pre-screening health questionnaire (see Appendix).

5.3.4 Study design

The study was a prospective, double-blind, parallel group, randomised controlled trial. Participants were monitored over a 12-week period, during which they consumed either MFGM or PLA daily. Treatments were randomised using an online randomisation tool (https://www.graphpad.com). Participants attended the laboratory at 4-week intervals: at Baseline, Week 4, Week 8 and Week 12 to provide a resting saliva sample used to measure mucosal immune factors. Throughout the 12 weeks, daily self-report training and illness logs were recorded, and oral/nasal swab samples were taken if a URTI episode occurred, for pathogen screening. The study took place in UK winter months, over 2 years. The first cohort were monitored between January and May 2024, and the second cohort between November 2024 and May 2025. The study was limited to winter and early spring months to account for seasonal variation in URTI risk, and to minimise the likelihood of allergies, such as hay fever, being misclassified as URTI symptoms.

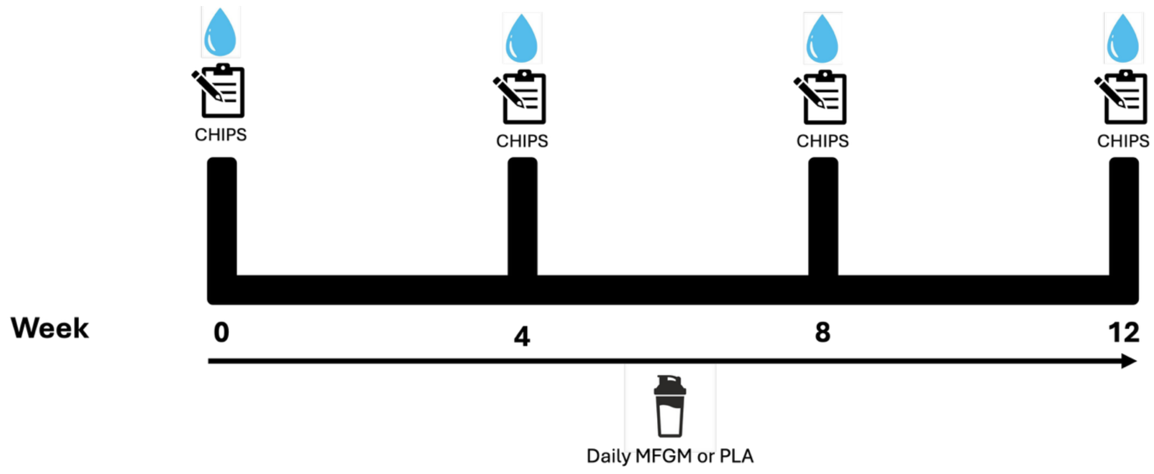

Figure 5.1. Schematic of Chapter 5 study protocol. CHIPS, Cohen-Hoberman Inventory of Physical Symptoms. MFGM, milk fat globule membrane. PLA, placebo.

5.3.5 Supplementation

MFGM was supplemented as an MFGM-rich whey protein concentrate, manufactured and supplied by Volac Whey Nutrition Ltd. An isocaloric and isomacronutrient pea protein concentrate was used for the placebo (PLA). Both supplements used in the study were consumed at 40 $g \cdot day^{-1}$ for 12 weeks. Due to manufacturing issues, the placebo unexpectedly contained phospholipids. This was only made apparent after the study had started, so could not be changed. Using the daily log, supplement compliance was 92.7%.

Table 5.1. Nutritional analysis of Chapter 5 supplements.

	Placebo	MFGM
Energy (Kcal/40 g)	159	157
Carbohydrates (%)	Not determined	Not determined
Protein (%)	70.2	70.44
Fat (%)	11.3	11.11
Total phospholipids (mg·g^{-1})	40.8	53.1
Sphingomyelin (mg·g^{-1})	0	14.0
Phosphatidylcholine (mg·g^{-1})	19.3	14.7
Phosphatidylethanolamine (mg·g^{-1})	5.8	13.9
Phosphatidylinositol (mg·g^{-1})	8.7	3.2
Phosphatidylserine (mg·g^{-1})	0	4.8
Other and minor species (mg·g^{-1})	9.9	2.7

5.3.6 Laboratory visits

Participants visited the laboratory every 4 weeks. All visits took place following an overnight fast of at least 10 h, whereby only water could be consumed. Participants were also instructed to abstain from vigorous exercise for 24 h and alcohol for 48 h before the visit. At Baseline, participants were screened for eligibility and completed all documentation required to enrol, and anthropometric data was collected (height and BM). Participants then completed the CHIPS and provided a resting unstimulated saliva sample. On all follow-up visits (at Week 4, 8 and 12), just the CHIPS and saliva sample were collected. These visits also served as an opportunity for participants to deliver any used URTI swabs and to receive extra supplements or swabs when necessary.

5.3.7 Monitoring of upper respiratory illness and training load

Self-report training logs and the Jackson common cold questionnaire (Jackson et al., 1958) were completed daily throughout the 12-week supplementation period, following the methods outlined in Chapter 2.5 (General Methods).

5.3.8 Pathogen confirmation

Participants were given an oral/nasal self-swab kit (DNA Genotek, Stittsville, Canada), containing 1 mL stabilisation buffer and were instructed to collect samples if they believed they were experiencing a URTI episode, on days 1 and 3 (where day 1 was first day of symptoms). All nucleic acid extraction, pre-amplification, reverse transcription and qPCR analysis were performed in an ISO 9001:2015 and GCLP-accredited facility (Central Biotechnology Services, Cardiff University, UK). Nucleic acids were extracted from the swab samples with a commercially available nucleic acid extraction kit (A42356, ThermoFisher Scientific, Roskilde, Denmark). Real-time qPCR was performed on the isolated nucleic acids using a commercially available array card (A49047, Thermo Fisher Scientific, Roskilde, Denmark), containing 41 known URTI-causing pathogens, on a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Roskilde, Denmark). Pathogens were normalised against RNase P (Guest et al., 2020; Miranda et al., 2021), using the Delta-Delta Ct method, to provide relative quantification (Rq).

5.3.9 Saliva sampling

Whole, unstimulated saliva was collected at rest on Baseline, Week 4, Week 8 and Week 12 using the methods described in Chapter 2.4.7 (General Methods). These samples were used for the analysis of salivary SIgA and lysozyme activity.

5.3.10 Physical wellbeing

The CHIPS was completed on every laboratory visit (every 4 weeks). Detailed methods are listed in Chapter 2.7 (General Methods).

5.3.11 Phospholipid quantification

Phospholipid content in red blood cells was quantified at Baseline and Week 4 using the Stewart assay in a subgroup analysis of n = 10 (4 PLA, 6 MFGM). Stewart assay methods are outlined in Chapter 2.4.6 (General Methods).

5.3.12 Statistical analysis

All statistical analyses were conducted using SPSS v.31.0 (IBM, Armonk, NY). Prior to analysis, all data were checked for normal distribution using a Shapiro-Wilk test and Z-scores for skewness and kurtosis. Non-normally distributed data were normalised using either a natural log or root (square or cube) transformation prior to analysis. Data that could not be normalised by log or root transformation was analysed using non-parametric tests. Missing data were imputed using estimation-maximisation and Little's test was performed to verify that data were missing completely at random (Grove and Cipher, 2024).

The following transformations were used to prepare data for parametric tests:

Log: URTI duration (swab and Jackson group); total Jackson score (Jackson only group) saliva osmolality. Root: Square- Saliva flow rate; salivary lysozyme activity; training load; training days affected and stopped by URTI (Jackson only group). Cube- SIgA concentration; SIgA secretion rate; CHIPS score.

The following variables could not be normalised using the transformations above, so were analysed with non-parametric tests: Number of URTI episodes per person (both groups); Jackson mean score per day (both groups); Mean Rq (day 1 and 3) for rhinovirus, *H. influenzae* and *S. aureus*.

Group comparisons were made using independent sample *t*-tests (normal distribution) and Mann-Whitney U tests (non-normal distribution). Two-way mixed (group [between] × time [within]) ANOVAs were used for analysis of SIgA, lysozyme activity, CHIPS and phospholipid concentration. Exploratory chi-squared analyses were performed on proportion of Jackson URTI days to whole study days and use of OTC medication.

5.4 Results

A total of 115 participants were enrolled (58 PLA; 57 MFGM), but 11 were lost to follow-up (*n* = 8 [5 PLA; 3 MFGM] due to voluntary withdrawal before completion; *n* = 2 [1 PLA; 1 MFGM] did not reply to communications; *n* = 1 [1 MFGM] due to surgery). Of the 11 lost to follow-up, 6 were lost prior to reaching the first checkpoint (week 4) and were not included in any analyses (3 PLA; 3 MFGM), whereas those that did reach the first checkpoint were included (3 PLA; 2 MFGM). One participant was excluded for poor study compliance (1 MFGM). Thus, analysis was completed on *n* = 108 (55 PLA; 53 MFGM).

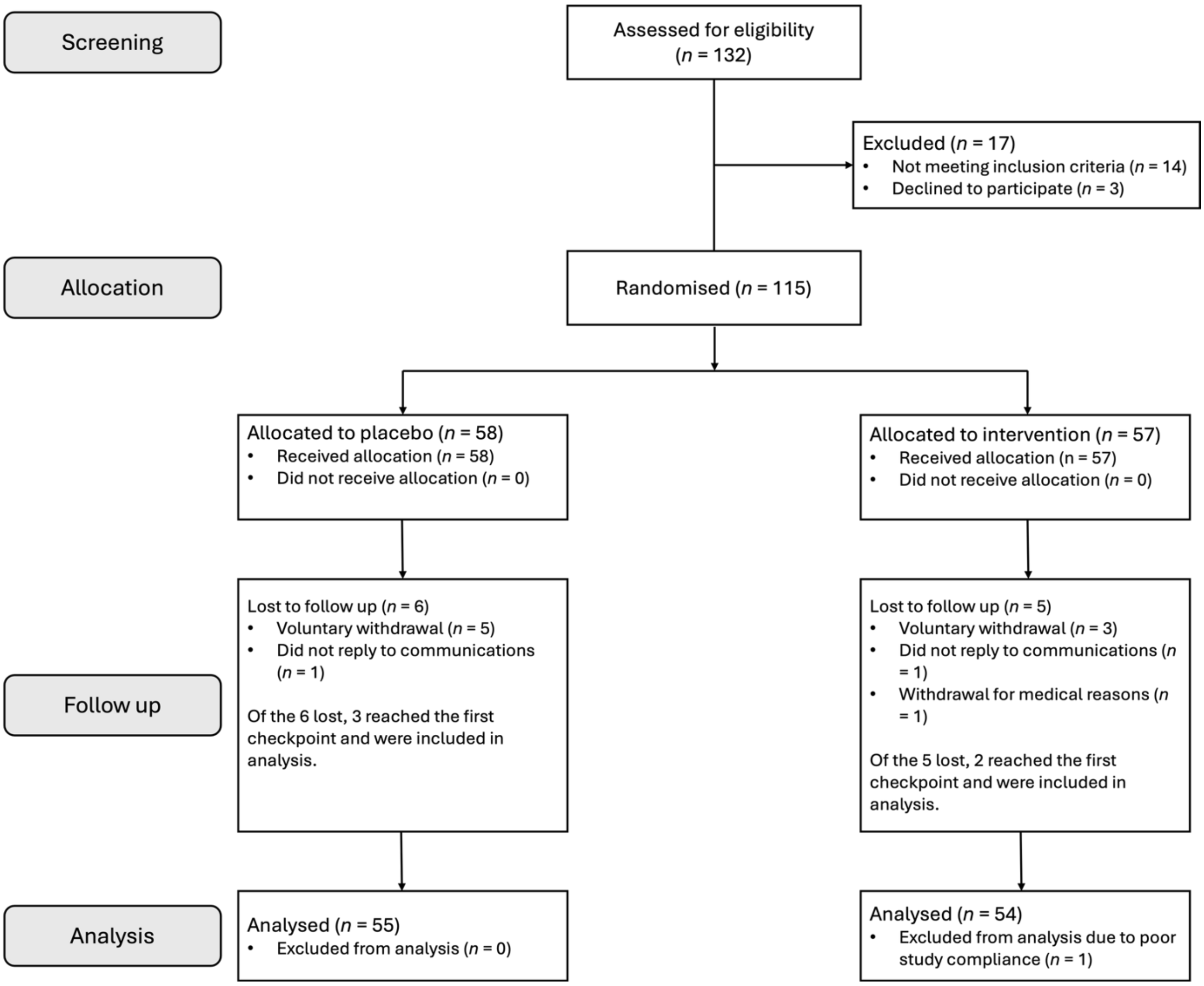

Figure 5.2. Consort diagram for participant recruitment and progression through study.

URTI analyses were performed on two groups: Jackson-only, which included all URTI episodes that met the Jackson criteria; Jackson- and swab-confirmed episodes, which only included URTI episodes that met the Jackson criteria and screened positive for known URTI-causing pathogens.

5.4.1 Jackson-verified upper respiratory tract infections

A total of 42 URTI episodes recorded met the Jackson criteria. There was no difference in number of episodes per person between MFGM and PLA, *p* = 0.304. There was also no difference in URTI episode duration between MFGM and PLA, *t*(40) = 0.791, *p* = 0.433. There were no significant differences in Jackson score for whole episode, $t(40) = 0.579$, $p = 0.566$, mean per day, $p = 0.814$, or episode peak, $p = 0.145$ (Table 5.2).

An exploratory analysis was performed, which found a significantly higher proportion of URTI days to non-URTI days in PLA (4.1%) compared to MFGM (2.6%), χ^2 (1) = 14.176, $p < 0.001$. Since this analysis was not pre-planned these results should be regarded as exploratory.

Table 5.2. Upper respiratory tract infection parameters using the Jackson questionnaire

Variable	PLA	MFGM	*P*
Number of URTI episodes per person			0.304
Mean ± SD	0.45 ± 0.69	0.32 ± 0.58	
Median (IQR)	0 (0-1)	0 (0-1)	
URTI episode duration (days)			0.433
Mean ± SD	6.7 ± 3.8	5.8 ± 3.3	
Median (interquartile range)	5 (4-8)	5 (3-7)	
Jackson score (whole episode)			0.566
Mean ± SD	37.4 ± 22.6	31.5 ± 13.2	
Median (interquartile range)	35.0 (18.0-51.0)	31.0 (19.0-39.0)	
Jackson score (mean per day)			0.814
Mean ± SD	5.6 ± 2.2	6.3 ± 3.0	
Median (interquartile range)	4.8 (4.0-5.8)	4.9 (3.6-7.8)	
Jackson score (episode peak)			0.145
Mean ± SD	8.4 ± 3.2	10.2 ± 4.0	
Median (interquartile range)	7.0 (6.0-10.0)	9.0 (7.0-15.0)	
Total URTI days			< 0.001**
% of total study days	4.1	2.6	

** Significant effect ($p < 0.001$).

5.4.2 Pathogen confirmation

Swabs were collected for 41 episodes, all of which were positive for at least one pathogen. In total, 27 pathogen strains were detected across 17 species (adenovirus, *Bacillus atrophaeus*, coronaviruses, enterovirus, Epstein-Barr virus, *Haemophilus influenzae*, human herpesvirus 6, influenza viruses, metapneumovirus, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Pneumocystis jirovecii*, respiratory syncytial virus, rhinovirus, *Staphylococcus aureus*, *Streptococcus pneumoniae* and SARS-CoV2), many of which were co-infections.

Of the 41 pathogen-positive swab cases, 29 also met the Jackson URTI criteria – a subgroup analysis was performed on these cases. There were no differences in number of swab- and Jackson-confirmed URTI episodes per person, $p = 0.510$, or URTI duration, $t(27) = 1.030$, $p = 0.312$. There were also no differences in Jackson score for whole episode, $t(27) = 0.771$, $p = 0.447$, mean score per day, $p = 0.744$, or episode peak, $t(27) = -1.799$, $p = 0.083$ (Table 5.3).

Table 5.3. Swab- and Jackson-confirmed upper respiratory tract infection parameters

Variable	PLA	MFGM	*p*
Number of URTI episodes per person			0.510
Mean ± SD	0.31 ± 0.60	0.23 ± 0.51	
Median (IQR)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	
URTI episode duration (days)			0.312
Mean ± SD	7.4 ± 4.3	5.9 ± 3.7	
Median (interquartile range)	5.0 (4.0-10.0)	5.0 (3.8-5.8)	
Jackson score (whole episode)			0.447
Mean ± SD	36.5 ± 21.3	31.0 ± 15.3	
Median (interquartile range)	35.0 (16.0-51.0)	30.0 (17.0-40.6)	
Jackson score (mean per day)			0.744
Mean ± SD	5.0 ± 1.4	5.9 ± 2.8	
Median (interquartile range)	4.8 (4.0-5.1)	5.5 (3.5-7.7)	
Jackson score (episode peak)			0.083
Mean ± SD	8.0 ± 2.7	10.2 ± 4.0	
Median (interquartile range)	7.0 (6.0-10.0)	9.5 (6.8-15.0)	

Due to the unexpected bioactive potential of the placebo used in the present study, an exploratory analysis was performed, comparing Jackson URTI parameters with those from another study taking place at the same time and in the same geographical location (Davison et al., 2025b). The subgroups included in this analysis were all endurance athletes and took part between January 2024 and May 2024 (tranche 1 for the present study; tranche 2 for Davison et al. 2025). As no significant results were detected between PLA and MFGM for Jackson URTI parameters, the treatments were pooled for analysis, thus *n* = 12 (7 PLA; 5 MFGM) for the present study, and *n* = 14 for the Davison and colleagues' study. The exploratory analysis revealed that URTI duration, *t*(19.039) = 3.288, *p* = 0.004 and score for whole episode, *t*(14.629) = 3.133, *p* = 0.007 were significantly lower in the present study. Mean score per day, *t*(24) = -0.675, *p* = 0.506 and episode peak, *t*(24) = 1.073, *p* = 0.294 were not significantly different between studies.

Table 5.4. Comparison of Jackson URTI parameters between present study and Davison et al. (2025).

Variable	Davison et al. (2025)	Tranche 1 (MFGM + PLA)	*p*
URTI episode duration (days)			0.004
Mean ± SD	16.2 ± 13.3	5.4 ± 2.5	
Median (interquartile range)	11.5 (7.0-24.5)	4.5 (4.0-6.0)	
Jackson score (whole episode)			0.007
Mean ± SD	90.9 ± 68.2	32.0 ± 15.9	
Median (interquartile range)	86.5 (29.5-137.8)	30.5 (18.3-41.0)	
Jackson score (mean per day)			0.506
Mean ± SD	5.9 ± 2.9	6.2 ± 2.5	
Median (interquartile range)	5.3 (4.0-8.1)	4.8 (4.0-8.1)	
Jackson score (episode peak)			0.294
Mean ± SD	11.4 ± 5.2	9.3 ± 4.3	
Median (interquartile range)	12.0 (9.0-15.8)	9.0 (5.8-12.8)	

5.4.3 Viral/bacterial load

Of the 17 different pathogen species, only rhinovirus, *H. influenzae*, and *S. aureus* were recorded in sufficient quantities to perform comparisons on viral and bacterial load between groups (Table 5.5). No significant difference was detected for rhinovirus viral load between PLA and MFGM, $p = 0.667$. There were also no differences in bacterial load for *H. influenzae*, $p = 0.094$ and *S. aureus*, $p = 0.737$ between PLA and MFGM (Table 5.5).

Table 5.5. Viral/bacterial load by relative quantification (Rq) method from array card analysis (mean of days 1 and 3)

Variable	PLA	MFGM	*P*
Rhinovirus viral load	*n* = 21	*n* = 21	0.667
×10-3 Rq (*vs.* RNase P) Mean			
Mean ± SD	42.9 ± 113.0	29.7 ± 66.7	
Median (IQR)	1 (0-7.5)	0.5 (0-13.5)	
***H. influenzae* bacterial load**	*n* = 14	*n* = 14	0.094
×10-3 Rq (*vs.* RNase P) Mean			
Mean ± SD	38.3 ± 87.1	3.6 ± 6.9	
Median (interquartile range)	0.8 (0.1-36.5)	0 (0-3.5)	
***S. aureus* bacterial load**	*n* = 18	*n* = 13	0.737
×10-3 Rq (*vs.* RNase P) Mean			
Mean ± SD	49.1 ± 123.1	13.2 ± 40.1	
Median (interquartile range)	0 (0-1.4)	0.5 (0-0.5)	

5.4.4 Salivary SIgA

There was a significant main effect of time, $F(2.266, 185.824) = 4.528$, $p = 0.009$ for saliva osmolality. Post-hoc tests showed that saliva osmolality was significantly higher at Week 12 ($p < 0.001$) compared to Week 0. However, no main effect of group, $F(1, 82) = 0.408$, $p = 0.525$

or group × time interaction, $F(2.266, 185.824) = 0.338$, $p = 0.798$. There was a significant main effect of time, $F(3, 246) = 3.625$, $p = 0.014$ for saliva flow rate. Post-hoc tests revealed that saliva flow rate was significantly higher at Week 12 compared to Week 0 ($p = 0.002$). There was no main effect of group, $F(1, 82) = 1.587$, $p = 0.211$ or group × time interaction, $F(3, 246) = 1.953$, $p = 0.122$ Table 5.6.

Table 5.6. Saliva osmolality and flow rate at Weeks 0, 4, 8 and 12.

Saliva parameter	Week 0	Week 4	Week 8	Week 12	*p* group time interaction
Osmolality (mOsmol·kg^{-1})					0.525
PLA	63 ± 23	62 ± 15	69 ± 25	70 ± 28	0.009*
MFGM	65 ± 24	68 ± 25	70 ± 30	73 ± 29	0.798
Flow rate (mL·min^{-1})					0.211
PLA	0.46 ± 0.19	0.53 ± 0.19	0.47 ± 0.19	0.54 ± 0.21	0.014*
MFGM	0.44 ± 0.2.0	0.43 ± 0.19	0.46 ± 0.20	0.49 ± 0.19	0.122

There was no main effect of group, $F(1, 82) = 2.721$, $p = 0.103$, time, $F(3, 246) = 1.907$, $p = 0.129$ or group × time interaction, $F(3, 246) = 1.771$, $p = 0.153$ for salivary SIgA concentration. No main effect of group, $F(1, 82) = 2.553$, $p = 0.114$, time, $F(3, 246) = 1.759$, $p = 0.156$ or group × time interaction, $F(3, 246) = 1.817$, $p = 0.145$ was detected for SIgA:osmolality. There was a significant main effect of time, $F(2.553, 209.321) = 3.714$, $p = 0.017$ for SIgA secretion rate. Though, there was no main effect of group, $F(1, 82) = 0.865$, $p = 0.355$ or group × time interaction, $F(2.553, 209.321) = 1.844$, $p = 0.149$. Post-hoc contrasts revealed that SIgA secretion rate was significantly higher at Week 12 compared to Week 0 ($p = 0.005$) (Figure 5.3).

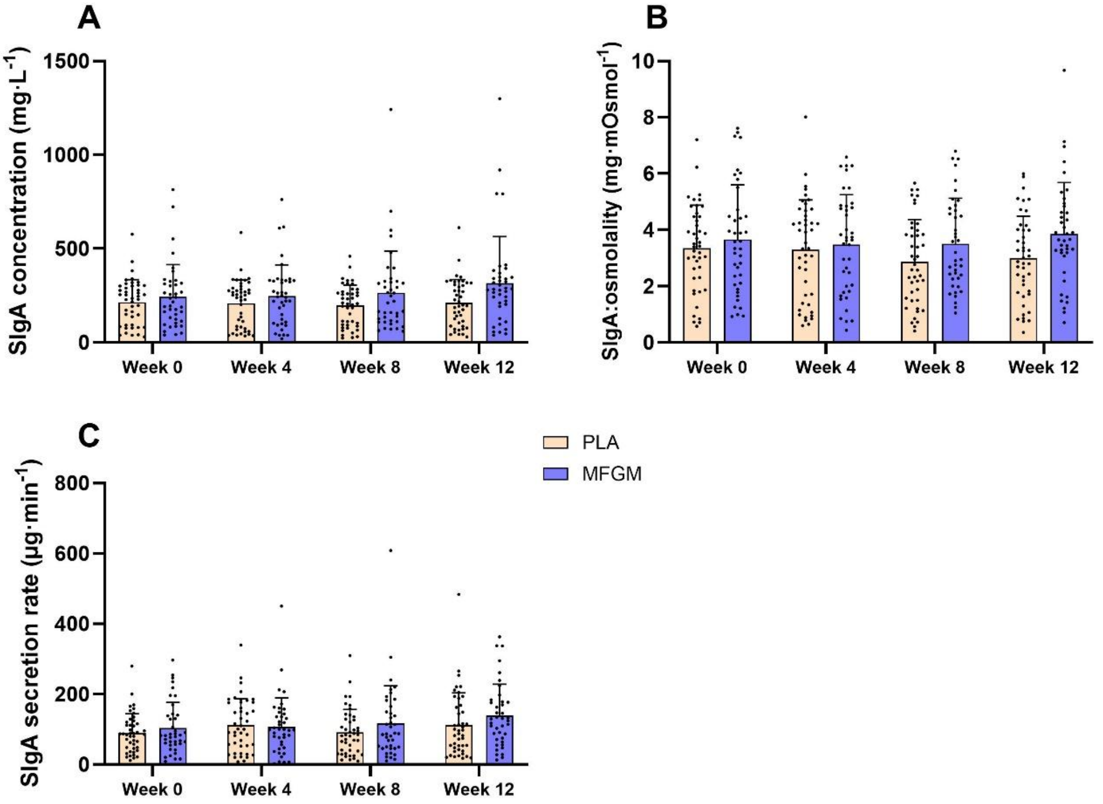

Figure 5.3. SIgA responses at Weeks 0, 4, 8 and 12. A- SIgA concentration. Two-way ANOVA main effects: p = 0.103 (group); p = 0.129 (time); p = 0.153 (group × time interaction). B- SIgA:osmolality. Two-way ANOVA main effects: p = 0.114 (group); p = 0.156 (time); p = 0.145 (group × time interaction). C- SIgA secretion rate. Two-way ANOVA main effects: p = 0.355 (group); p = 0.017 (time); p = 0.149 (group × time interaction).

5.4.5 Salivary lysozyme activity

A two-way mixed ANOVA showed no main effect of group, $F(1, 70) = 0.975$, $p = 0.327$, time, $F(3, 210) = 1.910$, $p = 0.129$ or group × time interaction, $F(3, 210) = 0.950$, $p = 0.418$ for salivary lysozyme activity (Figure 5.4).

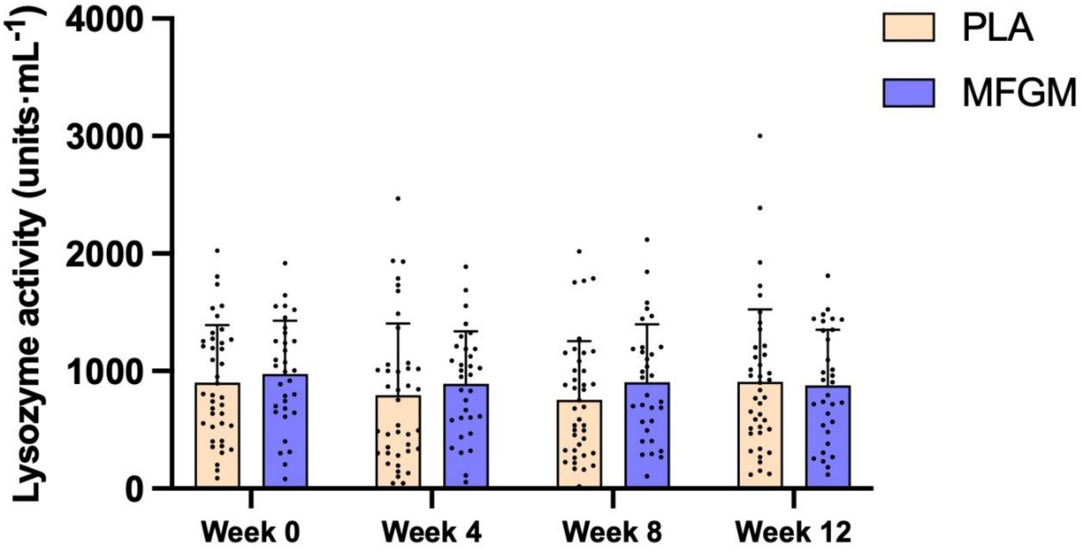

Figure 5.4. Salivary lysozyme activity at Weeks 0, 4, 8 and 12. Lysozyme activity (units·mL^{-1}). Two-way ANOVA main effects: p = 0.327 (group); p = 0.129 (time); 0.418 (group × time interaction).

5.4.6 Daily training logs

There was no significant difference in weekly training load between MFGM and PLA, *t*(102) = 0.525, *p* = 0.601. There was no significant difference in weekly training duration between MFGM and PLA, *p* = 0.156. There was also no significant difference in training days affected by Jackson URTI episodes, *t*(24) = 1.140, *p* = 0.265, or training days stopped by Jackson URTI episodes, *t*(24) = 1.821, *p* = 0.081 (Table 5.7).

Table 5.7. Training load and impact of upper respiratory tract infections using the Jackson questionnaire

Variable	PLA	MFGM	*p*
Weekly training load (sRPE × duration)			0.601
Mean ± SD	238.8 ± 148.2	226 ± 150.6	
Median (IQR)	230.5 (124.6-305.2)	202.1 (121.8-303.4)	
Weekly training duration (min)			0.156
Mean ± SD	272.6 ± 213.5	216.7 ± 163.1	
Median (IQR)	233.6 (175.6-328.9)	203.7 (85.6-288.2)	
Training affected by URTI (days)			0.265
Mean ± SD	4.0 ± 2.1	3.0 ± 2.0	
Median (IQR)	4.0 (2.0-6.0)	2.0 (2.0-3.5)	
Training stopped by URTI (days)			0.081
Mean ± SD	3.0 ± 1.7	1.7 ± 1.7	
Median (IQR)	2.0 (2.0-4.5)	1.0 (1.0-2.0)	

5.4.7 Use of OTC medication

There was no significant difference in the use of OTC medications during URTI episodes between MFGM (52.9% of cases: 9/17 episodes) and PLA (36% of cases: 9/25 episodes), χ^2 (1) = 1.186, $p = 0.276$. OTC medications used in the MFGM group consisted of analgesics only (paracetamol [acetaminophen] and/or ibuprofen) in 8 cases, and analgesics alongside cold and flu remedies (e.g. Lemsip, cough syrup, Night Nurse or similar) in 1 case. OTC medications used in the PLA group consisted of analgesics only in 4 cases, cold and flu remedies only in 2 cases, and a combination of both treatments in 4 cases.

5.4.8 Physical wellbeing

A two-way mixed ANOVA showed no main effect of group, $F(1, 86) = 0.344$, $p = 0.559$ or group × time interaction, $F(2.476, 212.943) = 0.214$, $p = 0.830$ for CHIPS score between MFGM and PLA. However, a main effect of time was detected, $F(2.476, 212.943) = 7.373$, *p*

< 0.001. Post-hoc Bonferroni-adjusted comparisons showed that physical wellbeing was significantly lower at Week 8 ($p = 0.009$) and Week 12 ($p = 0.001$) compared to Week 0 (Table 5.8).

Table 5.8. Physical wellbeing at Weeks 0, 4, 8 and 12.

Variable	Week 0	Week 4	Week 8	Week 12	*p* group time interaction
CHIPS Score					0.559
PLA	10.8 ± 14.0	9.9 ± 16.5	8.6 ± 14.3	8.1 ± 13.7	< 0.001**
MFGM	8.1 ± 10.2	6.9 ± 8.6	6.3 ± 9.0	6.1 ± 7.6	0.214

** Significant effect ($p < 0.001$).

5.4.9 Phospholipid quantification

Red blood cell phospholipid content was measured in a subgroup of $n = 10$ (4 PLA, 6 MFGM) at Week 0 and Week 4 (4 weeks of supplementation). There was a significant main effect of time, $F(1, 8) = 7.617$, $p = 0.025$. There was no significant main effect of group, $F(1, 8) = 0.422$, $p = 0.534$ or group × time interaction, $F(1, 8) = 0.159$, $p = 0.701$. Post-hoc Bonferroni-adjusted t-tests revealed a significant increase in phospholipid concentration from Week 0 to Week 4 ($p = 0.025$).

Table 5.9. Red blood cell phospholipid concentration at Baseline and Week 4.

Variable	Week 0	Week 4	*p* group time interaction
Phospholipid concentration ($mg \cdot mL^{-1}$)			0.422
PLA	0.024 ± 0.002	0.031 ± 0.002	0.025*
MFGM	0.026 ± 0.006	0.031 ± 0.005	0.701

* Significant effect ($p < 0.05$).

5.4.10 Adverse events reported

No adverse events or reactions were reported in the study.

5.5 Discussion

This was the first study to assess the effects of MFGM on URTIs and mucosal immunity. In the present study, MFGM did not reduce URTI parameters, including illness incidence, duration, severity and viral/bacterial load compared to the control group. Furthermore, MFGM did not affect mucosal immune functions, with neither SIgA nor lysozyme responses observing significant changes between conditions. However, the control supplement contained comparable phospholipid contents, which did not become apparent until after the study had already started. This is also supported by a subgroup analysis which saw similar increases in red blood cell phospholipid concentration between PLA and MFGM supplements. However, when comparing to a more appropriate control, which was derived from a placebo group from another relevant study in the same area, both supplements showed significantly lower Jackson URTI parameters.

Comparing study supplements, there were no differences between PLA and MFGM in the number of URTI episodes, episode duration, or severity of URTI symptoms using the Jackson questionnaire. The present study saw a 100% success rate in pathogen detection in swabs collected, there were no differences in URTI parameters between PLA and MFGM in the swab subgroup. Whilst there is no current research on the effects of MFGM on URTIs, the findings of the present study do not align with those seen in other dairy nutraceuticals, such as bovine colostrum. A systematic review of 5 trials (*n* = 152) has shown that bovine colostrum can reduce URS days and episodes (Jones et al., 2016). It has also been demonstrated that bovine colostrum may have beneficial effects on URS duration (Jones et al., 2014), but these results are less supported with varying outcomes in other studies, with some even finding that bovine colostrum increased duration of URS episodes (Shing et al., 2007a). Thus, the results of the present study do not align with previous findings of reduced URS/illness episodes and duration. An exploratory analysis showed that MFGM reduced the proportion of URTI days across the whole study, which is consistent with previous findings in this field (Jones et al., 2014). However, since this analysis was not pre-planned it remains exploratory.

A subgroup analysis showed that red blood cell phospholipid content increased after the first 4 weeks of supplementation in both groups, confirming the comparable phospholipid profiles between supplements. Since dietary phospholipids are the main proposed component of MFGM with health benefits, it is possible that the placebo group also received benefits to immunity, reducing the significance of any effects exhibited. This is supported by the exploratory analysis comparing Jackson parameters from tranche 1 from the present study with tranche 2 from a similar study taking place at the same time and in the same geographical location (Davison et al., 2025b). Where the present study saw no changes between PLA and MFGM for Jackson URTI parameters, the exploratory analysis revealed that Jackson URTI duration and total episode score were significantly lower in the present study. This implies that the placebo used in the present study provided a level of protection against URTIs, which could explain the lack of significant results observed. The placebo used was a pea-based protein concentrate intended to have minimal bioactivity. However, phospholipids were present in the supplement due to manufacturing issues, possibly due to unanticipated presence of lecithin, and we were not made aware of this by the manufacturers until the after the study had already started. Therefore, since the study was limited by time, and it was important that all participants were measured at the same time, there was no option but to continue the study with this placebo. Whilst this thesis has focused on the nutraceutical properties of milk-derived phospholipids, these properties may also extend to plant-based phospholipid sources, for which there is evidence of these effects. For example, soy lecithin, rich in phospholipids, has shown to enhance macrophage phagocytic activity and lymphocyte count in response to concanavalin A stimulation (Miranda et al., 2008). The authors accredited these results to PC within the soy lecithin, and the PC concentration in the present study was even higher in the placebo supplement than the MFGM supplement, thus highlighting its bioactive and potentially confounding potential.

Viral and bacterial load were only measured for rhinovirus, *H. influenzae*, and *S. aureus* due to no other pathogens being recorded in sufficient quantities. There were no differences in viral or bacterial load between PLA and MFGM. However, previous research has highlighted the importance of sample size in this analysis. For example, Davison et al., (2025) measured viral and bacterial load in rhinovirus and *H. influenzae*. They found a significant reduction in rhinovirus viral load in their treatment group, but not *H. influenzae* bacterial load. Whilst the magnitude of change in *H. influenzae* bacterial load was greater than that of the rhinovirus viral load, only the rhinovirus results were significant. The likely reason for this was the differing sample sizes between rhinovirus (n = 50) and *H. influenzae* (n = 30). Therefore, it is possible that the present study may have suffered from insufficient sample sizes in these analyses.

Saliva osmolality and flow rate were consistent across PLA and MFGM groups. The present study found no differences in absolute SIgA concentration, SIgA:osmolality, and SIgA secretion rate between PLA and MFGM. Although it has been reported that bovine colostrum can increase resting salivary SIgA levels (Crooks et al., 2006; Mero et al., 2002), there are several studies that have reported no effect on resting SIgA measures (Davison, 2021). Therefore, the study findings are generally consistent with much of the previous research, including the findings from study 2. There was also no difference observed between PLA and MFGM for salivary lysozyme activity. However, there is considerably less research on dairy supplements and antimicrobial proteins and peptides like lysozyme. Davison & Diment (2010) found that bovine colostrum truncated a decrease in salivary lysozyme following a prolonged exercise bout. In contrast, the findings of this study align with results seen in study 1 of this thesis, which found that MFGM had no effect on salivary lysozyme activity both at rest and following exercise. Additionally, other studies have reported that bovine colostrum has no effects on antimicrobial proteins such as lysozyme and lactoferrin both at rest and following exercise (Jones et al., 2015; McKenna et al., 2020). Whilst the lack of change between treatments in lysozyme activity is in agreement with some prior studies, the methodological differences in measurement techniques should be acknowledged. The aforementioned studies analysed lysozyme via ELISA, reporting as absolute concentration, normalised to osmolality and/or secretion rate. In comparison, the present study used an enzymatic assay to quantify lysozyme activity, which although could be considered to have more relevance to natural lysozyme functions (disruption of bacterial cell walls), ELISAs, which measure concentration rather than function, are widely considered more accurate and reliable in measurement. CHIPS scores and training load were consistent across PLA and MFGM groups, highlighting the similar physical stress experienced between groups over the study. There were also no differences in training days affected or stopped by URTIs between groups. It has been shown that training days affected and missed by URTIs may be consistent with other URTI parameters such as duration and severity (Davison et al., 2025b). Therefore, given the lack of difference between PLA and MFGM in the previous URTI parameters, it is understandable that there were no differences in training days affected or missed due to URTIs between groups.

Following the positive results seen in studies 1 and 2 in this thesis, MFGM may have been expected to provide some beneficial effects to host immunity. Furthermore, it has been demonstrated in previous research that bioactive components within MFGM can exhibit direct bactericidal activity (Sprong et al., 2001b) and inhibit pathogen virulence factors (Herrera-Insua et al., 2001). However, these factors did not translate into significant findings in the present study. A potential reason for the lack of significant results detected could have been the placebo used, which undesirably contained elevated levels of PC, PE and PI.

5.5.1 Limitations

Firstly, and most importantly, the phospholipid content in the placebo supplement is a significant limitation of this study. As previously mentioned, the potential bioactivity of the placebo could have reduced the significance of any effects exhibited by the MFGM supplement. This was an unexpected production error and only made apparent from a nutritional analysis performed after the first tranche had finished. However, it was decided that relevant comparisons could still be made as SM, a sphingolipid exclusive to animals, and PS were absent in the placebo. Given the bioactive potential of SM and PS (Venkat et al., 2024), it was possible that the MFGM supplement could still have various health benefits. In addition, comparisons between milk-based and plant-based phospholipids could also be a relevant area of research. Another limitation of the study was that the sample size target was based on number of participants rather than number of URTIs. A recent study demonstrated that it may be better to set a target number of URTIs for the study instead of a target number of participants (Davison et al., 2025b). Using data from their previous study (Davison et al., 2021), Davison and colleagues predicted that a sample size of ~114 participants would be sufficient to reach their target of ~88 URTIs. However, due to lower-than-expected incidence rates, a sample of ~150 participants were required to reach their target in the end. The present study was, therefore, subject to the same risks of lower incidence rates, especially considering that the first tranche of the present study and second tranche of Davison and colleagues' study took place at the same time and in similar geographical locations. To address this limitation, it would have been prudent to set a target number of URTIs instead of target number of participants, however, the present study was part of a PhD thesis and was limited by time. Therefore, whilst it was not possible to do this in the present study, future research should look to follow this guidance.

Another limitation of the study was that in studies of this nature, it is not possible to control the extent of pathogen exposure between individuals. In addition to the varying exposure to pathogens, the inclusion criteria of the study required all participants to engage in at least 3 h of moderate-to-intense exercise per week. This ensures that all participants meet a minimum threshold of physical stress, however, there was no upper limit. This means that some participants may have been under considerably more physical stress than others, though, consistencies across training load and CHIPS scores indicate that training load and incidence of physical symptoms were similar between treatment groups. Although, type of exercise could have also affected the results of the study, as endurance exercise has shown to elicit greater stress on the immune system than those taking part in other types of exercise, like resistance training (Schlagheck et al., 2020). Therefore, future studies may look to exclusively recruit endurance athletes, or non-endurance athletes.

5.5.2 Conclusion

To conclude, the present study found no effect of MFGM on URTI parameters or mucosal immune factors. Whilst this was the first study of its type using MFGM, the results do not align with those found using similar dairy supplements (Jones et al., 2014). It is speculated that these results could be partly due to bioactive components in the placebo supplement used, which contained comparable levels of some phospholipids (PC, PE and PI). Thus, it may be useful to use the present study as a guide for future research, addressing concerns relating to the placebo, infection rates and inclusion criteria.

Chapter 6. General Discussion

The overarching aims of this thesis were to characterise the effects of MFGM on GI and immune responses to exercise, and to determine whether these effects contributed to enhanced protection against URTIs. To comprehensively address these aims, a series of 3 randomised controlled trials (**Chapters 3-5**) were undertaken, each employing rigorous methodological approaches to evaluate specific mechanistic pathways and clinically relevant outcomes. **Chapter 3** was a prospective, double-blind, crossover randomised control trial (*n* = 12), which assessed the impact of short-term (2 weeks) MFGM supplementation on gut integrity, where it was demonstrated that MFGM was able to prevent increases in gut damage and permeability following strenuous exercise. Further examining the short-term effects of MFGM supplementation, **Chapter 4**, another prospective, double-blind, crossover randomised controlled trial (*n* = 11), investigated the effects of 2-week MFGM supplementation on cellular and mucosal immune functions, and gut integrity following prolonged (3 h) exercise. It was found that MFGM enhanced resting neutrophil function and prevented a fall below baseline levels following a prolonged cycling bout, although there were no effects on mucosal immune functions. MFGM also reduced gut damage following the prolonged exercise bout, thus demonstrating its ability to provide gut protection against exercise of both a high intensity and prolonged duration. Building on the findings of the previous two chapters, **Chapter 5** set out to determine whether short-term benefits of MFGM translated to long-term protection against URTIs. In a prospective, double-blind, parallel group randomised controlled trial, a cohort of 115 (*n* = 108 after drop outs) active individuals were supplemented with either MFGM or a placebo for 12 weeks during winter months. Over this time, URTI incidence and training logs were recorded, alongside regular (every 4 weeks) measurements of mucosal immune functions. Despite the positive findings of short-term MFGM supplementation, no benefits were observed for long-term supplementation. However, it is possible that these results could be explained by the placebo exhibiting unanticipated bioactive properties.

6.1 MFGM and gut integrity

In **Chapters 3** and **4**, short-term MFGM supplementation prevented the increase in plasma I-FABP, a marker of gut epithelial cell damage, following strenuous and prolonged exercise, respectively. Given the intensity dependent nature of I-FABP, these findings highlight the robust potential of MFGM as a gut-protecting nutraceutical. A common comparator throughout this thesis has been bovine colostrum due to the similarity in methods employed in much of its research and its similar bioactive composition, of which MFGM is a constituent. The findings of MFGM on plasma I-FABP are comparable with those of bovine colostrum, which has also shown to prevent increases in the marker following the same exercise model used in **Chapter 3** (March et al., 2017).

The effects of dairy nutraceuticals on I-FABP responses to prolonged exercise are notably less researched, although bovine colostrum supplementation for 7 d was shown not to influence I-FABP levels following a 90 min mixed exercise bout consisting of 15 min cycling, 60 min running and 15 min cycling (Morrison et al., 2014). Though, the findings from Morrison and colleagues are not a fully robust comparison to make as there are numerous significant methodological differences. These include a difference in exercise duration (3 h vs 1.5 h), supplementation length (14 days vs 7 days) and whether participants were fed or not – where it has been suggested that pre-exercise feeding may mitigate gut damage and permeability (Chantler et al., 2022). Nevertheless, the findings from **Chapter 4** complement those from **Chapter 3**, demonstrating that MFGM can protect the gut from damage associated with exercise of a strenuous or prolonged nature.

Damage imposed to the gut can compromise the barrier, resulting in a loss of function and a transient state of elevated permeability. The main measure of gut permeability in this thesis was in **Chapter 3**, which used urinary L/R following a strenuous exercise bout. The urinary L/R test is a robust technique that has been employed by other studies using the same exercise model (20 min run at 80% $\dot{V}O_{2\mathrm{max}}$) to assess gut permeability (Davison et al., 2016; March et al., 2017; Marchbank et al., 2011). To ensure comparability with these studies, and other exercise and clinical gastroenterology studies that have used this method, the same sugar probe composition was used to maintain consistency and osmolality: lactulose, rhamnose and mannitol (Davison et al., 2016; Mahmood et al., 2007; March et al., 2017; Marchbank et al., 2011, 2008; Playford et al., 2001).

In **Chapter 3**, it was demonstrated that while exercise increased urinary L/R, there was no increase in the MFGM arm. Moreover, urinary L/R was significantly lower in the MFGM trial compared to the placebo. These findings also allow for comparison with bovine colostrum, which is known for its efficacy in protecting the gut against exercise-induced perturbations, including urinary L/R (Davison et al., 2016; March et al., 2017; Marchbank et al., 2011). The simultaneous reductions in gut permeability and I-FABP levels with MFGM supplementation provide a mechanistic insight, indicating that its protective effect may occur as a result of reduced epithelial cell damage.

There are various consequences associated with higher-than-normal gut permeability, including increased GI symptoms, inflammation, impaired nutrient absorption (Costa et al., 2022b), and it may even contribute to exercise-related heat illness (Lim and Mackinnon, 2006). Despite clear reductions in gut damage and permeability following MFGM supplementation in **Chapter 3**, these effects did not affect GI symptom incidence. Elevated gut permeability has also been suggested to create a window of opportunity for the translocation of undesirable gut contents, such as bacteria and endotoxins, into systemic circulation (Costa et al., 2017). This translocation can lead endotoxemia and/or bacteremia, conditions that may provoke excessive activation of immune and inflammatory mediators (Selkirk et al., 2008). In severe cases, these conditions are implicated in SIRS, MOF, sepsis, and in the worst case, death (Michelena et al., 2015). Some have investigated exercise-induced changes in circulating LPS as an indication of gut permeability (Camus et al., 1998; Jeukendrup et al., 2000). However, the limulus amoebocyte lysate (LAL) assay is generally the benchmark for LPS measurement, and is posed with various limitations: reagent contamination, activity of plasma inhibitors (causing high inter-assay variability), and LPS is only present on Gram-negative bacteria which prevents the detection of Gram-positive bacteria (Kramski et al., 2011). Bacterial DNA analysis via qPCR of the 16S rRNA gene has been suggested to be an alternative method to the LAL for assessment of exercise-induced endotoxemia/bacteremia (Costa et al., 2022b).

Where some investigations have quantified bacterial DNA via 16S qPCR assays as an indication of exercise-induced gut permeability (March et al., 2019; Ogden et al., 2020), **Chapters 3** and **4** used 16S rRNA gene sequencing to measure changes in bacterial composition in whole blood. Until now, 16S amplicon sequencing had only been used in one other study to assess exercise-induced gut permeability, although it was performed on plasma, not whole blood (Henningsen et al., 2024). Despite increases in gut damage (**Chapters 3** and **4**) and permeability (**Chapter 3**), neither study observed any changes in bacterial composition in blood. The most abundant taxa in **Chapters 3** and **4** detected were predominantly gut taxa, such as Escherichia-Shigella and Lactobacillus. Despite this, there were slight differences between chapters, such as the samples in **Chapter 4** showing a more diverse taxonomy. This could be due to multiple factors, including differences in study protocol (e.g. intensity, duration) and DNA extraction kits. The extraction kit used in **Chapter 3** was a more robust fungal/bacterial DNA specific kit, which may have broken down hard-to-lyse bacteria that the kit in **Chapter 4** may not have been able to do. The differences in bacterial composition could also simply be due to having different individuals with different gut microbiomes. While no changes were observed for the composition of the bacterial communities, it is worth highlighting that 16S rRNA gene sequencing only provides compositional analysis and not absolute quantification. Despite also observing no significant compositional changes, Henningsen et al. (2024) saw an increase in microbial DNA concentration. It is possible that 16S rRNA gene sequencing could provide insights to if certain taxa translocated at higher rates than others, however, if translocation of all taxa increased, the composition would remain similar. Thus, qPCR assays for bacterial DNA quantification would have been beneficial to **Chapters 3** and **4** and provide direction for future research.

It has also been suggested that sCD14 may act as a surrogate marker of endotoxemia due to its role as a receptor for LPS (Costa et al., 2022b). **Chapter 3** saw an increase in sCD14 from Pre-Ex to Post-Ex, but no differences were detected between supplements. While it is true that sCD14 increases in response to elevated LPS in circulation (Landmann et al., 1996), it is also increased by immune and inflammatory mediators such as TNF-α and IFN-β (Kramski et al., 2011). Thus, sCD14 does not exclusively indicate endotoxin exposure and may have been influenced by other mediators – possibly exhibiting similar patterns to other markers of physiological stress, such as leukocyte counts.

The causes of exercise-induced gut damage, which can lead to losses of barrier function and elevated permeability, typically include elevations in core temperature (Dokladny et al., 2016; Ghulam Mohyuddin et al., 2022), reduced splanchnic blood flow (Ulluwishewa et al., 2024; van Wijck et al., 2012), and mechanical stress (Costa et al., 2017). For a more comprehensive review of these mechanisms, see **Chapter 1**.

MFGM has drawn interest as a potential nutraceutical due to its richness in bioactive components. This includes lactadherin, also known as MFG-E8, a protein of particularly high abundance in MFGM that possesses various anti-inflammatory properties and is implicated in the removal and clearance of apoptotic cells (Ait-Oufella et al., 2007). Cells in the intestinal barrier may become apoptotic as a result of disease or injury, occurring through stressors such as exercise or inflammatory conditions (Negroni et al., 2015). However, apoptotic cells create a ‘weak link’ in the gut barrier, resulting in a reduced barrier function and increased risk of undesirable contents gaining passage across the gut barrier. As such, the rapid clearance of these cells is a necessity for maintaining intestinal homeostasis, and lactadherin may play a significant role in this. Lactadherin has also been associated with the restoration of tight/adherens junction protein expression and localisation (Shen et al., 2019). Alongside the maintenance of intestinal cell homeostasis, the maintenance of tight/adherens junction proteins also provides greater protection against larger contents seeking paracellular passage across the gut barrier.

MFGM is rich in dietary phospholipids, which have also shown various gut-beneficial effects. It has been demonstrated that MFGM or individual components within, like polar lipids or SM, can increase epithelial barrier function (in vitro transepithelial resistance) and tight junction expression (Jiang et al., 2022; Milard et al., 2019). The mechanisms underpinning such effects are not yet fully elucidated, but it has been found that phospholipids in MFGM can promote the production of SCFA via their effects on SCFA-bacteria, such as Bifidobacterium and LAB (Feng et al., 2025; Gong et al., 2024; Kosmerl et al., 2021; Wu et al., 2021). SCFAs are known to possess various properties that benefit gut barrier integrity (Liu et al., 2021), so a link could be made between the phospholipid-mediated increase in SCFAs and the downstream effects on gut function and integrity.

Lastly, phospholipids are known as membrane lipids and are crucial components of all cell membranes. Phospholipids such as PC have shown to incorporate themselves in into the plasma membrane of enterocytes and can affect various functions (Küllenberg et al., 2012). Since phospholipids are implicated in cell structural stability and integrity (Ammendolia et al., 2021), it is plausible that the delivery of phospholipids to these structures, such as via MFGM supplementation, may promote the upkeep of these cells, thus maintaining cellular integrity and function.

To summarise, in **Chapters 3** and **4**, MFGM demonstrated its potential to mitigate exercise-induced increases in gut damage and permeability. These findings, likely driven by bioactive components such as lactadherin and dietary phospholipids, highlight the influence of MFGM on maintaining optimal gut integrity. Recognising the synergistic gut-immune relationship, **Chapters 4** and **5** of this thesis explored the influence of MFGM on immune function and illness susceptibility.

6.2 MFGM and immune function

The work in **Chapter 4** was designed to provide mechanistic insights into the immunomodulatory effects of MFGM, laying the foundation for the larger clinical trial presented in **Chapter 5**, which focused on more clinically relevant outcomes rather than mechanistic. The primary outcome measure in **Chapter 4** was fMLP-stimulated neutrophil oxidative burst, a proven marker of neutrophil function that has been used in previous exercise immunology studies (Davison and Gleeson, 2006; Jones et al., 2015, 2014). MFGM displayed positive effects on neutrophil function, increasing resting oxidative burst activity and preventing a drop below baseline levels following the prolonged cycling bout. These findings partially agree with those of bovine colostrum, which has been shown to mitigate exercise-induced depression of neutrophil function (Jones et al., 2015). Though, bovine colostrum has not shown an ability to influence resting neutrophil oxidative burst – highlighting a possible improvement with MFGM (Jones et al., 2015, 2014). Interestingly, bovine colostrum has demonstrated other benefits to neutrophil functions via degranulation responses (Davison and Diment, 2010), which may provide an idea for future investigations into the immune effects of MFGM.

A common mechanism proposed to influence neutrophil functions following exercise is the release of functionally immature neutrophils into circulation. However, **Chapter 4** saw no differences between treatment arms for total/differential leukocyte counts, so it could be assumed that the benefits seen to neutrophil function were not related to leukocytosis/neutrophilia. While the mechanisms underpinning the effects of dairy supplements on neutrophil functions are not elucidated, it has been suggested that low molecular weight components (< 10 kDa) components of bovine milk and colostrum, which could include proteose peptones (Merin et al., 2001), may possess neutrophil priming properties (Sugisawa et al., 2003). During neutrophil priming, components of the NADPH oxidase complex translocate towards the cell membrane. So, while this does not create ROS, it may sensitise the NADPH oxidase system, allowing for rapid assembly and activation once stimulated (Nguyen et al., 2017).

To accompany cellular immune functions, **Chapters 4** and **5** assessed mucosal immune parameters – salivary SIgA and lysozyme activity. Salivary SIgA is a common marker used in exercise immunology and, in some cases, has shown to experience depression following exercise of a prolonged or strenuous nature (Jones and Davison, 2018). Though, it should be noted that others have also found that salivary SIgA is not affected by exercise, including that of a long duration (Mackinnon and Hooper, 1994; Sari-Sarraf et al., 2007, 2006). Salivary SIgA is also a clinically relevant marker as it has been shown as a strong predictor of URTIs – lower SIgA levels or secretion can increase URTI susceptibility (Tiernan et al., 2020; Walsh et al., 2011). In **Chapter 4**, there was an effect of time for salivary SIgA concentration, but there were no differences between MFGM and the placebo. There were also no changes detected for

SIgA:osmolality and SIgA secretion rate. Likewise, **Chapter 5** also saw no differences between MFGM and placebo for salivary SIgA responses, despite an effect of time for SIgA secretion rate. It should be noted that saliva osmolality and flow rate were consistent between treatment groups in both **Chapters 4** and **5**, so they would not have influenced results. The findings of **Chapters 4** and **5** agree with some previous research, where dairy supplements failed to influence salivary SIgA responses (Crooks et al., 2010; Davison and Diment, 2010; Shing et al., 2007b), but not all, as some have observed increases in resting SIgA levels (Crooks et al., 2006; Mero et al., 2002).

Interestingly, one of the previous studies that observed an increase in resting SIgA levels following 2 weeks supplementation of bovine colostrum, was conducted with the dosage split over the day (Mero et al., 2002). This suggests that multiple doses may provide an additional effect, possibly through sustained exposure to the bioactive components and improving the absorption and utilisation of such components. However, a notable consideration when comparing with Mero et al. (2002) is the placebo used, which was neither energy- or macronutrient-matched, thus providing a poor control and potential confounder.

Salivary lysozyme is yet another crucial component of the immune system. Its primary mechanism of action is the cleaving of β-1, 4-glycosidic bonds between NAM and NAG in the polysaccharide section of peptidoglycans – which are a cell wall component of Gram-positive bacteria (Ferraboschi et al., 2021). Ultimately, this results in damage to or even destruction of the cell wall, effectively killing or significantly harming bacteria (Baron et al., 2016). **Chapters 4** and **5** measured salivary lysozyme activity through an enzymatic assay based on the work of Shugar (1952). Neither study detected any changes in lysozyme activity, both over time and between treatments. The literature for dairy nutraceuticals and lysozyme remains contested, for instance, while beneficial effects of bovine colostrum have been reported (Davison and Diment, 2010), others have found no significant impact (Jones et al., 2015). A consideration of these comparisons is the analytical method employed. Whereas the previous studies on bovine colostrum quantified salivary lysozyme concentration via ELISA (Davison and Diment, 2010; Jones et al., 2015), **Chapters 4** and **5** employed the aforementioned enzymatic assay. Consequently, comparisons to the previous studies are informative, but are not strictly direct.

It has been suggested that activation of the HPA axis and elevations in cortisol may influence various immune functions, including those related to neutrophils (Laing et al., 2008) and mucosal factors (Usui et al., 2011). For this reason, the cortisol response to prolonged exercise was measured in **Chapter 4**. There were no changes in plasma cortisol observed over time or between treatments, which was unanticipated given the increases in cortisol previously seen following similar exercise bouts (Davison et al., 2007; Davison and Gleeson, 2006). However, this almost certainly occurred due to timing of the exercise trials and sample collection. Most experimental trials in **Chapter 4** took place at 09:00, with some taking place at 08:00 – the timing of these trials were close to awakening. Cortisol follows a distinct diurnal rhythm – high levels upon wakening, a transient surge in the following 30-40 min and then a substantial decline over the following hours (Adam et al., 2017). Consequently, it is likely that Pre-Ex samples were taken during a period of naturally higher cortisol, and Post-Ex samples were taken when the diurnal levels had declined – effectively cancelling out the exercise response (see Figure 6.1). Previous studies have avoided this by scheduling trials for later in the morning (Davison et al., 2007; Davison and Gleeson, 2006). However, cortisol was not a primary measure for **Chapter 4** and the subjects were already required to fast for an extended period of time, it was deemed an acceptable limitation. Despite these results, the consistency in cortisol levels between MFGM and placebo arms suggests that the benefits seen for neutrophil functions were not a result of HPA activation.

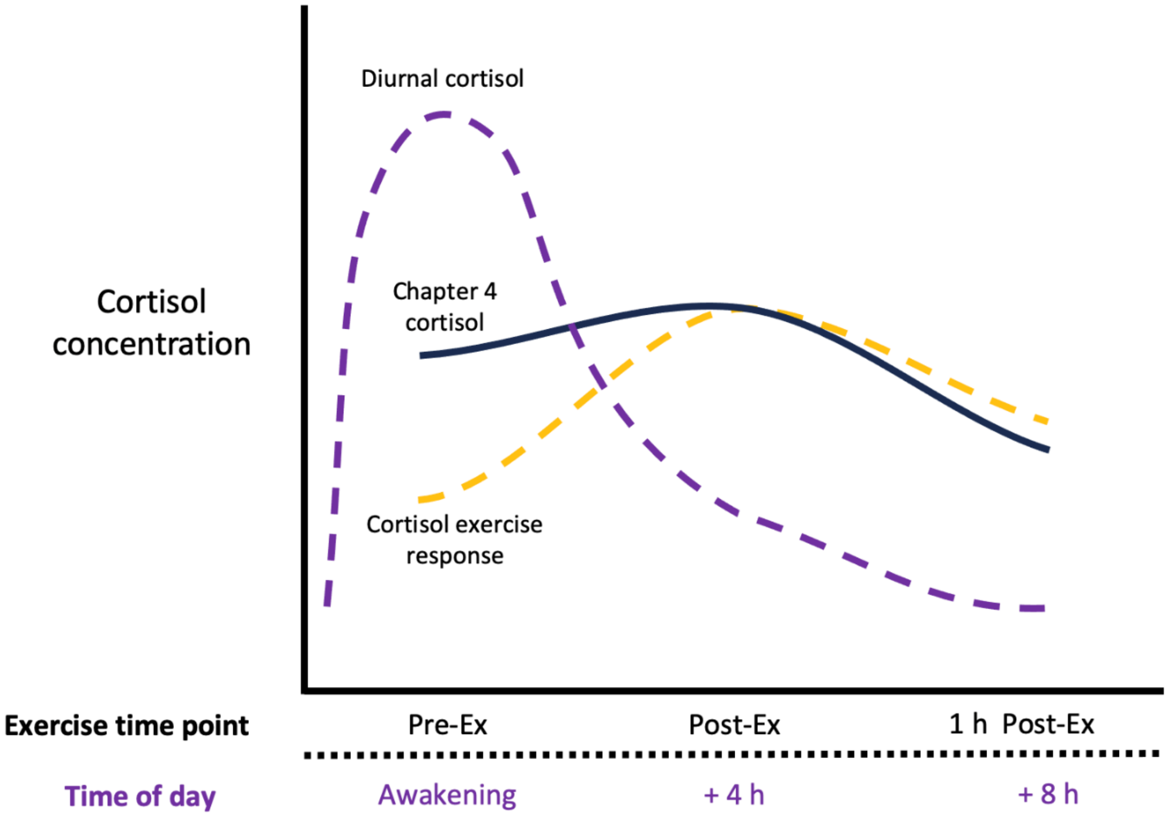

Figure 6.1. Schematic representation of Chapter 4 cortisol response, diurnal cortisol rhythm and typical cortisol response to exercise.

So far, this thesis has demonstrated that MFGM possesses several properties benefiting GI and immune functions. Yet, a more clinically relevant question is whether these effects translate to protection against illness. **Chapters 4** and **5** sought to address this question, measuring URTI incidence, duration and symptom severity, though this was a secondary measure of **Chapter 4** and no illnesses were recorded. In **Chapter 5**, URTIs were measured via the Jackson common cold questionnaire (Jackson et al., 1958) and pathogen detection in oral/nasal swab samples, which saw a detection rate of 100% in swab cases returned (n = 41). However, no differences in URTI incidence, duration or symptom ratings were observed between treatment groups, both for Jackson-verified URTIs (n = 42) and Jackson- and swab-verified URTIs (n = 29). There were also no differences in viral (rhinovirus, *n* = 42) and bacterial (*S. aureus*, *n* = 31; *H. influenzae*, *n* = 28) load between MFGM and the placebo.

These findings were unexpected following the beneficial properties of MFGM demonstrated in previous chapters and they contrast with prior research, where supplementation with bovine colostrum has been associated with reduced URTI incidence in a similar study design (Jones et al., 2014). While research directly investigating MFGM in an illness context remains scarce, infant studies provide supporting evidence, reporting reductions in acute otitis media (Timby et al., 2015), respiratory adverse events (Li et al., 2019) and febrile episodes (Veereman-Wauters et al., 2012).

The lack of findings in **Chapter 5** are likely explained by the placebo used, which contained elevated levels of PC, PE and PI due to manufacturing issues. This was confirmed with a subgroup analysis of red blood cell phospholipid content, that observed similar increases following 4 weeks of supplementation in both PLA and MFGM groups. Since phospholipids are proposed to be a key component of MFGM influencing immune responses, it is possible that the placebo also provided a level of protection and masked the effects of MFGM. To examine this further, an exploratory analysis was performed in **Chapter 5**, comparing Jackson URTI parameters with those of another study taking place in the same geographical location and at the same time (Davison et al., 2025b). A subgroup analysis of endurance athletes taking part from January 2024 to May 2024 revealed that Jackson URTI duration and total episode score were lower in the **Chapter 5** cohort, suggesting that the placebo itself did provide a degree of support. Following the positive findings in previous chapters, the deviation in placebo composition provides a logical explanation for the lack of findings in **Chapter 5** (see Table 6.1).

Table 6.1. Comparison of placebo supplements used throughout Chapters 3-5.

	Chapter 3	Chapter 4	Chapter 5
Energy (Kcal/40 g)	146	170	159
Carbohydrates (%)	2	3.6	Not determined
Protein (%)	92	67.4	70.2
Fat (%)	0.4	14.3	11.33
Total phospholipids ($mg \cdot g^{-1}$)	3.1	12.4	40.8
Sphingomyelin ($mg \cdot g^{-1}$)	0.9	0.0	0.0
Phosphatidylcholine ($mg \cdot g^{-1}$)	0.8	3.8	19.3
Phosphatidylethanolamine ($mg \cdot g^{-1}$)	0.7	1.4	5.8
Phosphatidylinositol ($mg \cdot g^{-1}$)	0.2	3.2	8.7
Phosphatidylserine ($mg \cdot g^{-1}$)	0.3	0.0	0.0
Other and minor species ($mg \cdot g^{-1}$)	0.1	4.0	9.9

6.3 Practical implications

This thesis conducted a series of investigations into the nutraceutical properties of MFGM, which had previously demonstrated promising effects to the GI and immune systems. This was tested over both short-term and long-term supplementation with MFGM and the following practical implications have been identified based on the collective findings.

1. Short-term supplementation with MFGM can help maintain GI integrity, attenuating increases in gut damage and permeability associated with strenuous and prolonged exercise. Due to the potentially harmful consequences of gut dysfunction on athlete performance and health, these findings may be relevant to athletes, coaches and other practitioners within sport.
    a. These findings were demonstrated within an exercise context. However, elevated gut permeability, commonly referred to as ‘leaky gut’, is also persistent

in various clinical conditions, particularly those involving chronic inflammation. The gut-protective effects of MFGM may have broader relevance for these populations as well, although further research will be required to confirm this potential application.

2. Short-term MFGM supplementation increases resting neutrophil function and helps mitigate the drop in neutrophil function following prolonged cycling. As periods of heavy training load are known to substantially increase the risk of illness, MFGM supplementation may hold practical relevance for individuals undergoing intensive training or leading up to competition, where maintenance of immune function is critical.
3. MFGM was delivered in the form of a whey protein concentrate, a type of supplement widely used in sport nutrition. This delivery method demonstrates that MFGM can be incorporated into the diet easily, making it readily available and translatable for athletes and healthy individuals. Sport nutrition companies may therefore consider incorporating MFGM into such products, providing additional GI and immune benefits in a way that maintains convenience and familiarity for their consumers.

6.4 Future directions

Following the positive results of MFGM on GI integrity in **Chapters 3** and **4**, it may be useful to examine whether these effects hold up under extreme stress. Gut damage and permeability are known to be temperature dependent, so future research could look to examine the effects of MFGM on maintaining GI integrity during prolonged exercise in the heat. Demonstrating such benefits would be particularly relevant to those competing in endurance events such as marathons and tour cycling and could further establish MFGM as a major gut nutraceutical.

Further investigation into the GI effects of MFGM could also include analysis of the gut microbiota. There is strong existing evidence to suggest that components within MFGM can influence the microbial community in the gut, leading to increases in SCFAs, which are known to exhibit numerous GI, immune and inflammatory. Therefore, research should investigate the long-term effects of MFGM on GI functions, allowing enough time for changes in the microbiota to occur.

Following the investigations in **Chapter 3** and **Chapter 4**, the importance of quantifying bacterial load is apparent. The use of 16S rRNA gene sequencing for bacterial translocation is relevant for providing insights into whether some bacteria translocated at greater rates than others. However, as seen in this thesis, 16S rRNA gene sequencing cannot detect other changes if the composition remains the same. Therefore, future research should look to couple 16S rRNA gene sequencing with qPCR quantification of specific taxa – this would provide compositional analysis of all bacteria and bacterial load quantification. In fact, it may be pragmatic to use the 16S compositional analysis to identify target taxa for qPCR quantification.

Chapter 4 demonstrated that MFGM possesses immunomodulatory effects, enhancing neutrophil oxidative burst. Given the comparisons with other supplements like bovine colostrum in this thesis, it may be prudent to investigate other similar markers that bovine colostrum has demonstrated favourable effects to, such as neutrophil degranulation.

As mentioned, the findings in **Chapter 5** were unanticipated and were likely influenced by the placebo providing a level of support. Therefore, given the clinical relevance of the study, it would be beneficial to conduct another investigation of the same nature, ensuring that the limitations are addressed. Logically, the first consideration would be to ensure an appropriate placebo, maintaining the energy and macronutrient profile but providing no additional bioactive effects. It would also be pragmatic to conduct the power analysis using target number of URTIs rather than number of participants, which has shown success in recent longitudinal URTI monitoring research. The final key consideration for a similar study would be stricter inclusion criteria, ensuring all participants experience similar physiological stress. For instance, **Chapter 5** required all participants to engage in moderate-vigorous exercise for ≥ 3 h a week, but this did not specify the type of exercise or have an upper limit. Therefore, it would be useful to conduct an investigation using exclusively endurance athletes due to their greater risk of illness, and if needed, employ an upper limit if necessary.

There is research to suggest that prior infection with latent herpesviruses, such EBV, may increase the risk of URTIs. Therefore, future immunological research could investigate the relationship between MFGM and EBV serostatus/reactivation, and whether this affects URTI incidence or severity. Another promising direction could be the application of in vivo immune markers. As highlighted, there is a demand for clinically relevant markers in exercise immunology, and the use in vivo markers, such as contact hypersensitivity responses to agents like DPCP, may provide an insight into the relationship between MFGM and real-time immune defence.

6.5 Conclusions

Previous research has indicated that MFGM may confer benefits to various aspects of health, particularly in relation to GI and immune function. Despite this, a clear gap has remained in the literature regarding the effects of MFGM in healthy adult populations, which are needed to confirm the potential effects under real-world conditions.

The collective findings of the thesis demonstrate that short-term MFGM supplementation protects the host against exercise-induced gut damage and mitigates the associated increase in gut permeability following strenuous exercise. **Chapters 3** and **4** were the first randomised control trials assessing the effects of MFGM on gut damage and permeability and have established MFGM as a promising nutraceutical in sport nutrition. The observed benefits to GI integrity are likely mediated by the bioactive components within MFGM, such as lactadherin and dietary phospholipids. Building on these findings, future research should aim to confirm the gut-protective properties of MFGM and extend investigation into other aspects of GI health and function, including its effects on the gut microbiota and GI integrity under further stress.

Chapter 4 provided a mechanistic insight into the immunological effects of MFGM, observing significant increases in neutrophil oxidative burst at rest, and attenuating the decline in neutrophil function following prolonged cycling. However, MFGM did not affect mucosal immune functions, which was also evident in **Chapter 5**. Despite the positive effects of MFGM on GI and cellular immune functions, this did not translate to protection against URTIs, as **Chapter 5** observed no changes in URTI incidence, duration or symptom ratings. However, the lack of findings in **Chapter 5** may possibly be explained by issues pertaining to the placebo supplement used, although further research is required to confirm this.

To summarise, this thesis presents the first series of investigations on MFGM within an exercise context and assessing these outcomes. These findings contribute to the growing body of evidence the health benefits of MFGM and highlight opportunities for future research to examine its wider roles in GI and immune function.

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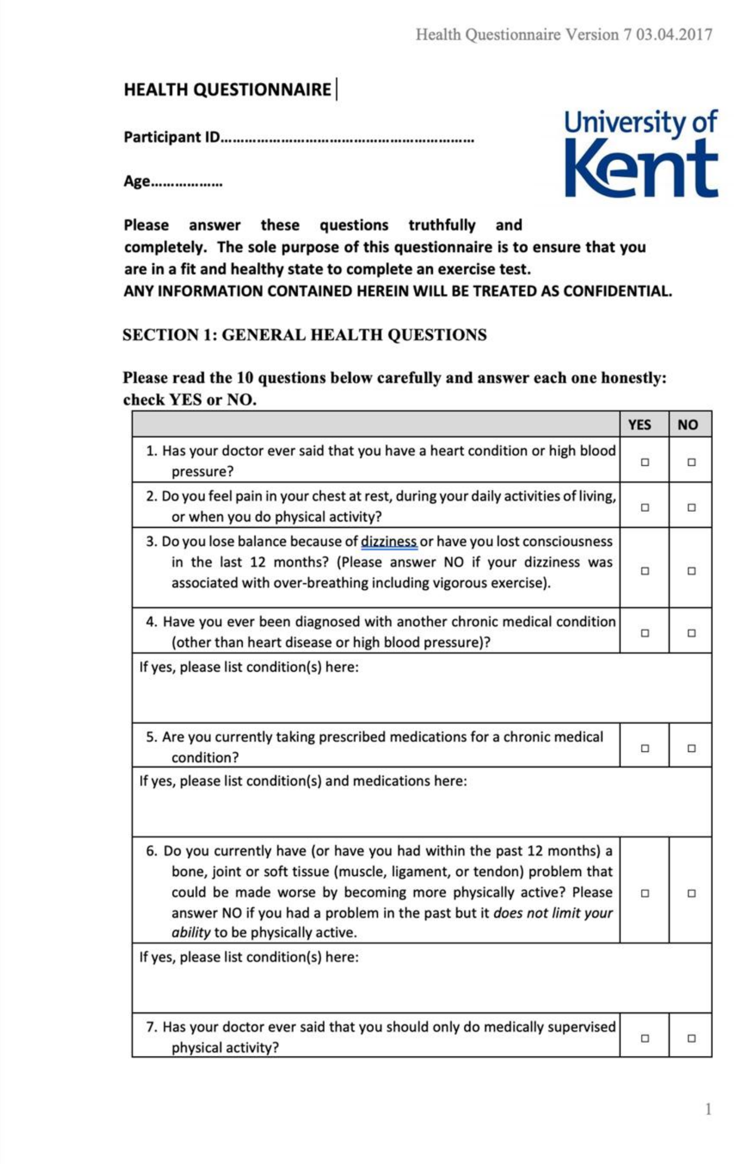
Appendix A



Health Questionnaire Version 7 03.04.2017

HEALTH QUESTIONNAIRE

Participant ID……………………………………………………

Age………………

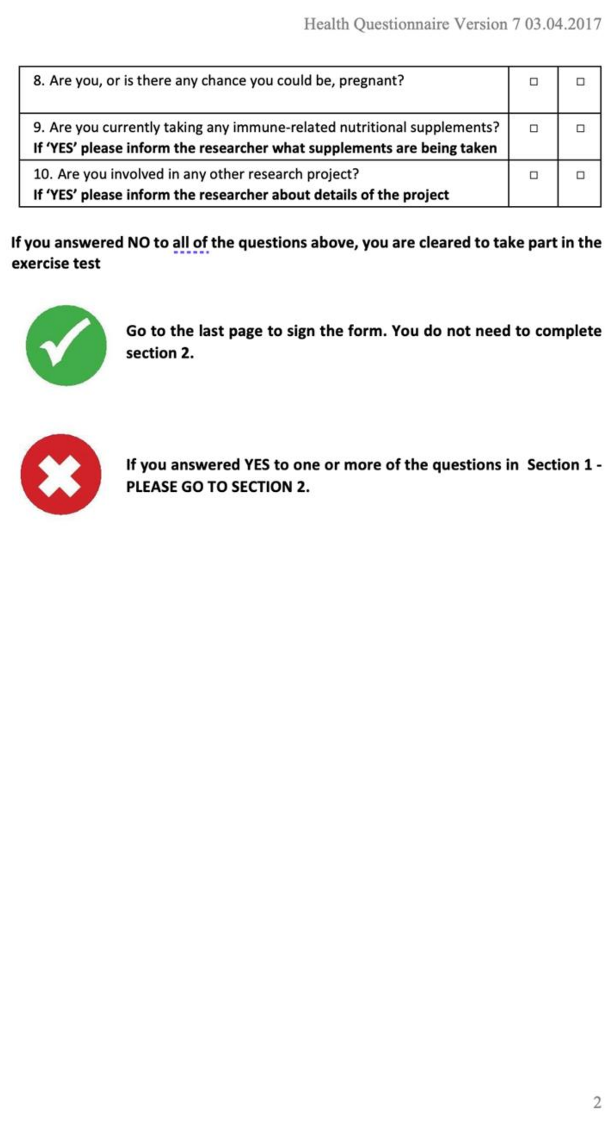
Please answer these questions truthfully and completely. The sole purpose of this questionnaire is to ensure that you are in a fit and healthy state to complete an exercise test.
ANY INFORMATION CONTAINED HEREIN WILL BE TREATED AS CONFIDENTIAL.

SECTION 1: GENERAL HEALTH QUESTIONS

Please read the 10 questions below carefully and answer each one honestly: check YES or NO.

	YES	**NO**
1. Has your doctor ever said that you have a heart condition or high blood pressure?	☐	☐
2. Do you feel pain in your chest at rest, during your daily activities of living, or when you do physical activity?	☐	☐
3. Do you lose balance because of dizziness or have you lost consciousness in the last 12 months? (Please answer NO if your dizziness was associated with over-breathing including vigorous exercise).	☐	☐
4. Have you ever been diagnosed with another chronic medical condition (other than heart disease or high blood pressure)?	☐	☐
If yes, please list condition(s) here:		
5. Are you currently taking prescribed medications for a chronic medical condition?	☐	☐
If yes, please list condition(s) and medications here:		
6. Do you currently have (or have you had within the past 12 months) a bone, joint or soft tissue (muscle, ligament, or tendon) problem that could be made worse by becoming more physically active? Please answer NO if you had a problem in the past but it *does not limit your ability* to be physically active.	☐	☐
If yes, please list condition(s) here:		
7. Has your doctor ever said that you should only do medically supervised physical activity?	☐	☐

1



Health Questionnaire Version 7 03.04.2017

8. Are you, or is there any chance you could be, pregnant?	☐	☐
9. Are you currently taking any immune-related nutritional supplements? **If 'YES' please inform the researcher what supplements are being taken**	☐	☐
10. Are you involved in any other research project? **If 'YES' please inform the researcher about details of the project**	☐	☐

If you answered NO to all of the questions above, you are cleared to take part in the exercise test

✓ **Go to the last page to sign the form. You do not need to complete section 2.**

✖ **If you answered YES to one or more of the questions in Section 1 - PLEASE GO TO SECTION 2.**

2

SECTION 2: CHRONIC MEDICAL CONDITIONS

Please read the questions below carefully and answer each one honestly: check YES or NO.

		YES	NO
1.	**Do you have arthritis, osteoporosis, or back problems?** If YES answer questions 1a-1c. If NO go to Question 2.	☐	☐
1a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	☐	☐
1b.	Do you have joint problems causing pain, a recent fracture or fracture caused by osteoporosis or cancer, displaced vertebrae (e.g. spondylolisthesis), and/or spondyloysis/pars defect (a crack in the bony ring on the back of the spinal column)?	☐	☐
1c.	Have you had steroid injections or taken steroid tablets regularly for more than 3 months?	☐	☐
2.	**Do you have cancer of any kind?** If YES answer questions 2a-2b. If NO, go to Question 3.	☐	☐
2a.	Does your cancer diagnosis include any of the following types: lung/bronchogenic, multiple myeloma (cancer of plasma cells), head and neck?	☐	☐
2b.	Are you currently receiving cancer therapy (such as chemotherapy or radiotherapy)?	☐	☐
3.	**Do you have heart disease or cardiovascular disease? This includes coronary artery disease, high blood pressure, heart failure, diagnosed abnormality or heart rhythm.** If YES answer questions 3a-3e. If NO go to Question 4.	☐	☐
3a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	☐	☐
3b.	Do you have an irregular heartbeat that requires medical management? (e.g. atrial fibrillation, premature ventricular contraction)	☐	☐
3c.	Do you have chronic heart failure?	☐	☐
3d.	Do you have a resting blood pressure equal to or greater than 160/90mmHg with or without medication? Answer YES if you do not know your resting blood pressure.	☐	☐
3e.	Do you have diagnosed coronary artery (cardiovascular) disease and have not participated in regular physical activity in the last 2 months?	☐	☐

		YES	NO

4.	**Do you have any metabolic conditions? This includes Type 1 Diabetes, Type 2 Diabetes and Pre-Diabetes.** If YES answer questions 4a-4c. If NO, go to Question 5.	☐	☐
4a.	Is your blood sugar often above 13mmol/L? (Answer YES if you are not sure).	☐	☐
4b.	Do you have any signs or symptoms of diabetes complications such as heart or vascular disease and/or complications affecting your eyes, kidneys, OR the sensation in your toes and feet?	☐	☐
4c.	Do you have other metabolic conditions (such as thyroid disorders, current pregnancy related diabetes, chronic kidney disease, or liver problems)?	☐	☐
5.	**Do you have any mental health problems or learning difficulties?** This includes Alzheimer's, dementia, depression, anxiety disorder, eating disorder, psychotic disorder, intellectual disability and down syndrome. If YES answer questions 5a-5b. If NO go to Question 6.	☐	☐
5a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	☐	☐
5b.	Do you also have back problems affecting nerves or muscles?	☐	☐
6.	**Do you have a respiratory disease?** This includes chronic obstructive pulmonary disease, asthma, pulmonary high blood pressure. If YES answer questions 6a-6d. If NO, go to Question 7.	☐	☐
6a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	☐	☐
6b.	Has your doctor ever said you blood oxygen level is low at rest or during exercise and/or that you require supplemental oxygen therapy?	☐	☐
6c.	If asthmatic, do you currently have symptoms of chest tightness, wheezing, laboured breathing, consistent cough (more than 2 days/week), or have you used your rescue medication more than twice in the last week?	☐	☐
6d.	Has your doctor ever said you have high blood pressure in the blood vessels of your lungs?	☐	☐
7.	**Do you have a spinal cord injury?** This includes tetraplegia and paraplegia. If YES answer questions 7a-7c. If NO, go to Question 8.	☐	☐
7a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	☐	☐
7b.	Do you commonly exhibit low resting blood pressure significant enough to cause dizziness, light-headedness, and/or fainting?	☐	☐

7c.	Has your physician indicated that you exhibit sudden bouts of high blood pressure (known as autonomic dysreflexia)?	☐	☐

		YES	NO
8.	**Have you had a stroke?** This includes transient ischemic attack (TIA) or cerebrovascular event. If YES answer questions 8a-8c. If NO go to Question 9.	☐	☐
8a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	☐	☐
8b.	Do you have any impairment in walking or mobility?	☐	☐
8c.	Have you experienced a stroke or impairment in nerves or muscles in the past 6 months?	☐	☐
9.	**Do you have any other medical condition which is not listed above or do you have two or more medical conditions?** If you have other medical conditions, answer questions 9a-9c. If NO go to Question 10.	☐	☐
9a.	Have you experienced a blackout, fainted, or lost consciousness as a result of a head injury within the last 12 months OR have you had a diagnosed concussion within the last 12 months?	☐	☐
9b.	Do you have a medical condition that is not listed (such as epilepsy, neurological conditions, and kidney problems)?	☐	☐
9c.	Do you currently live with two or more medical conditions?	☐	☐
	Please list your medical condition(s) and any related medications here:		
10.	**Have you had a viral infection in the last 2 weeks (cough, cold, sore throat, etc.)?** If YES please provide details below:	☐	☐
11.	**Is there any other reason why you cannot take part in this exercise test?** If YES please provide details below:	☐	☐

12. **Please provide brief details of your current weekly levels of physical activity (sport, physical fitness or conditioning activities), using the following classification for exertion level:**

L = light (slightly breathless)
M = moderate (breathless)
V = vigorous (very breathless)

	Activity	**Duration (mins.)**	**Level (L/M/V)**
Monday			
Tuesday			
Wednesday			
Thursday			
Friday			
Saturday			
Sunday			

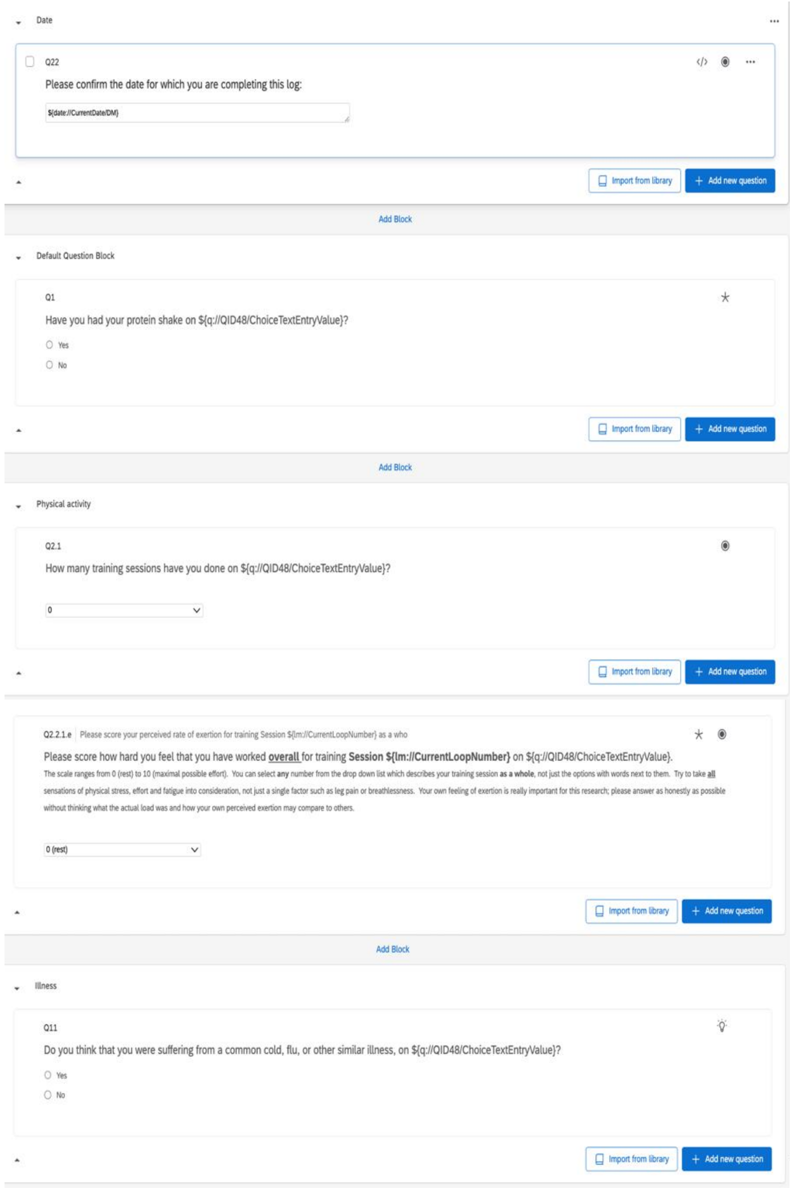
Please see below for recommendations for your current medical condition and sign this document:

✓ **If you answered NO to all of the follow-up questions about your medical condition, you are cleared to take part in the exercise test.**

✗ **If you answered YES to one or more of the follow-up questions about your medical condition it is strongly advised that you should seek further advice from a medical professional before taking part in the exercise test.**

This health questionnaire is based around the PAR-Q+, which was developed by the Canadian Society for Exercise Physiology www.csep.ca

Appendix B



Q22

Please confirm the date for which you are completing this log:

${date://CurrentDate/DM}

Q1

Have you had your protein shake on ${q://QID48/ChoiceTextEntryValue}?

○ Yes

○ No

Q2.1

How many training sessions have you done on ${q://QID48/ChoiceTextEntryValue}?

0

Q2.2.1.e Please score your perceived rate of exertion for training Session ${lm://CurrentLoopNumber} as a who

Please score how hard you feel that you have worked <u>overall</u> for training **Session ${lm://CurrentLoopNumber}** on ${q://QID48/ChoiceTextEntryValue}.

The scale ranges from 0 (rest) to 10 (maximal possible effort). You can select **any** number from the drop down list which describes your training session **as a whole**, not just the options with words next to them. Try to take <u>all</u> sensations of physical stress, effort and fatigue into consideration, not just a single factor such as leg pain or breathlessness. Your own feeling of exertion is really important for this research; please answer as honestly as possible without thinking what the actual load was and how your own perceived exertion may compare to others.

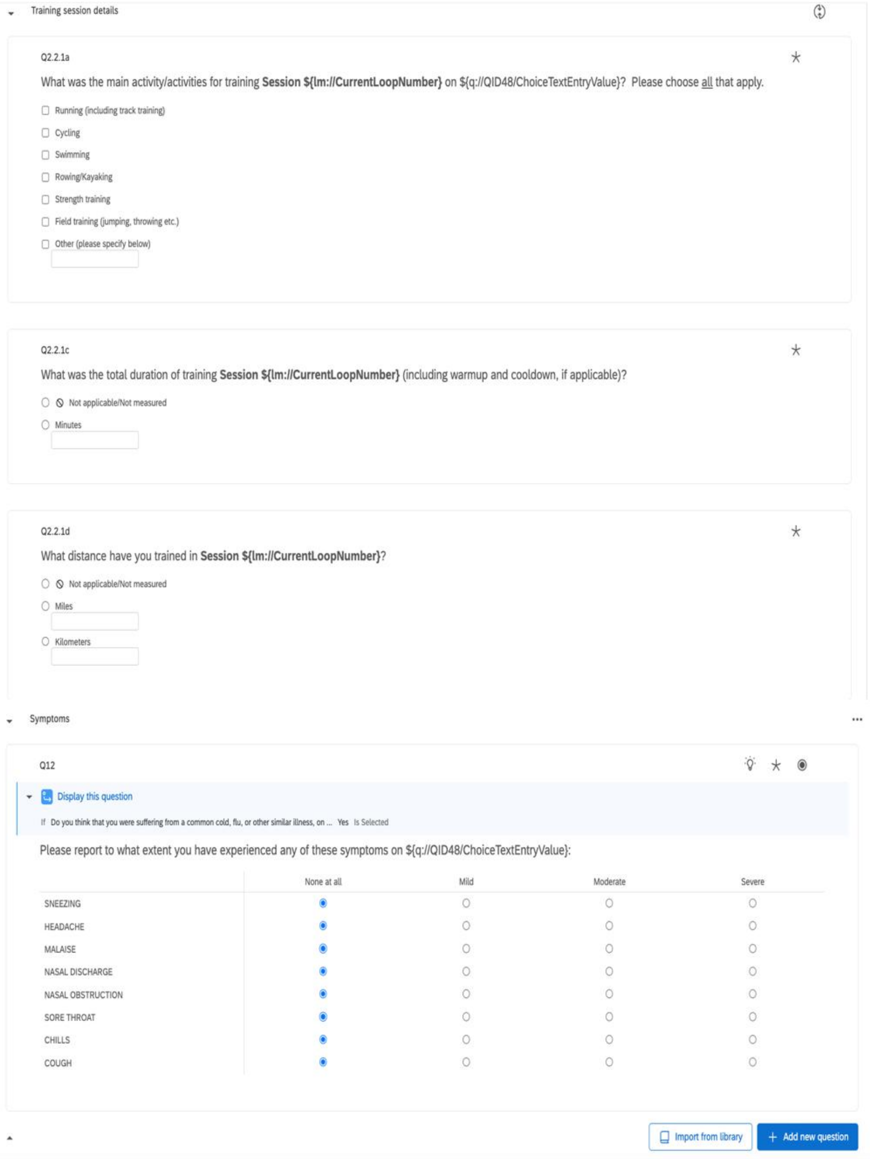
0 (rest)

Q11

Do you think that you were suffering from a common cold, flu, or other similar illness, on ${q://QID48/ChoiceTextEntryValue}?

○ Yes

○ No



Q2.2.1a

What was the main activity/activities for training **Session ${lm://CurrentLoopNumber}** on ${q://QID48/ChoiceTextEntryValue}? Please choose <u>all</u> that apply.

- ☐ Running (including track training)
- ☐ Cycling
- ☐ Swimming
- ☐ Rowing/Kayaking
- ☐ Strength training
- ☐ Field training (jumping, throwing etc.)
- ☐ Other (please specify below)

Q2.2.1c

What was the total duration of training **Session ${lm://CurrentLoopNumber}** (including warmup and cooldown, if applicable)?

○ ⦸ Not applicable/Not measured

○ Minutes

Q2.2.1d

What distance have you trained in **Session ${lm://CurrentLoopNumber}**?

○ ⦸ Not applicable/Not measured

○ Miles

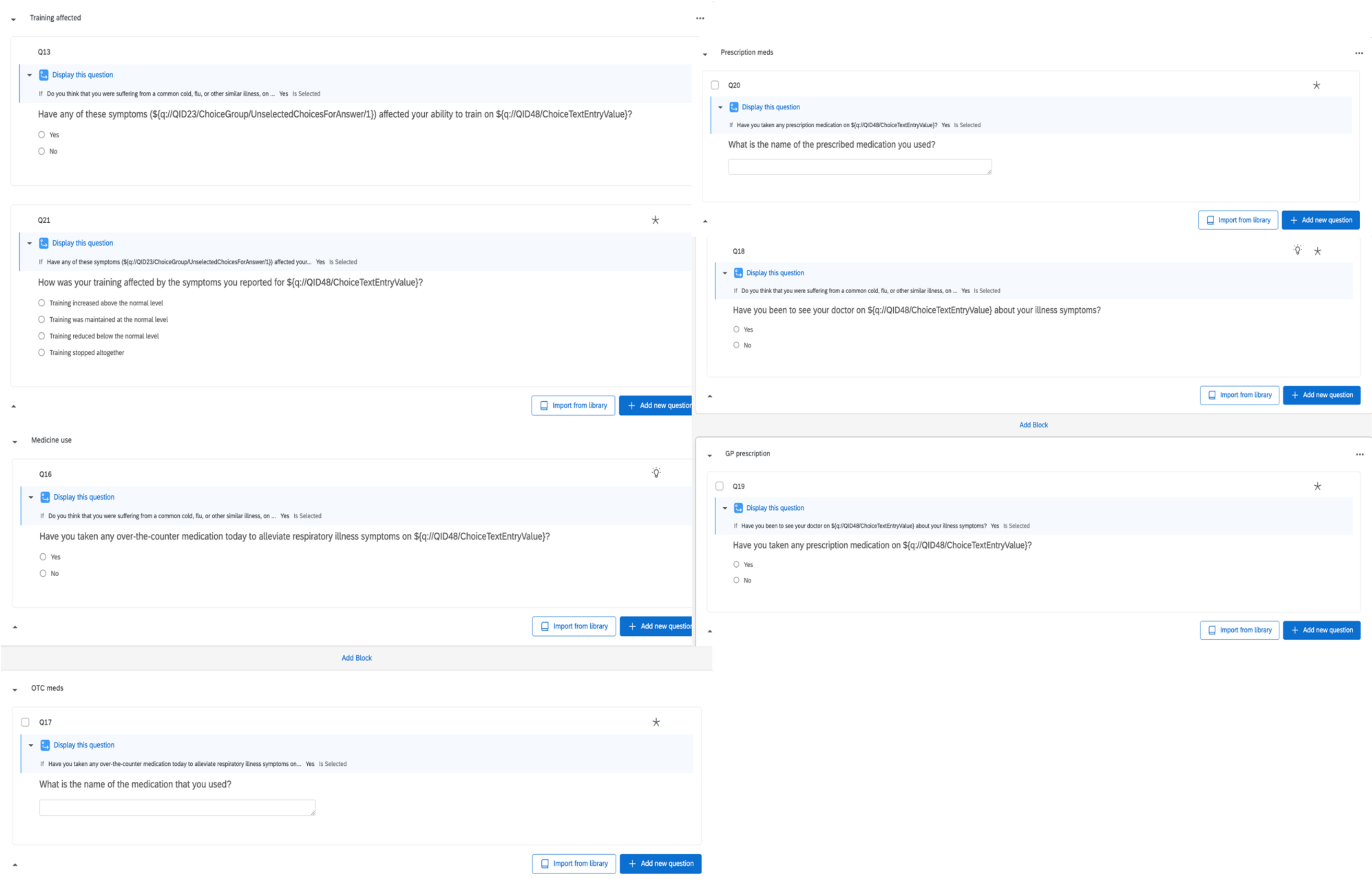
○ Kilometers

Q12

If Do you think that you were suffering from a common cold, flu, or other similar illness, on ... Yes Is Selected

Please report to what extent you have experienced any of these symptoms on ${q://QID48/ChoiceTextEntryValue}:

	None at all	Mild	Moderate	Severe
SNEEZING	●	○	○	○
HEADACHE	●	○	○	○
MALAISE	●	○	○	○
NASAL DISCHARGE	●	○	○	○
NASAL OBSTRUCTION	●	○	○	○
SORE THROAT	●	○	○	○
CHILLS	●	○	○	○
COUGH	●	○	○	○



Training affected

Q13

Display this question

If Do you think that you were suffering from a common cold, flu, or other similar illness, on ... Yes Is Selected

Have any of these symptoms (${q://QID23/ChoiceGroup/UnselectedChoicesForAnswer/1}) affected your ability to train on ${q://QID48/ChoiceTextEntryValue}?

○ Yes
○ No

Q21

Display this question

If Have any of these symptoms (${q://QID23/ChoiceGroup/UnselectedChoicesForAnswer/1}) affected your... Yes Is Selected

How was your training affected by the symptoms you reported for ${q://QID48/ChoiceTextEntryValue}?

○ Training increased above the normal level
○ Training was maintained at the normal level
○ Training reduced below the normal level
○ Training stopped altogether

Medicine use

Q16

Display this question

If Do you think that you were suffering from a common cold, flu, or other similar illness, on ... Yes Is Selected

Have you taken any over-the-counter medication today to alleviate respiratory illness symptoms on ${q://QID48/ChoiceTextEntryValue}?

○ Yes
○ No

OTC meds

☐ Q17

Display this question

If Have you taken any over-the-counter medication today to alleviate respiratory illness symptoms on... Yes Is Selected

What is the name of the medication that you used?

Prescription meds

☐ Q20

Display this question

If Have you taken any prescription medication on ${q://QID48/ChoiceTextEntryValue}? Yes Is Selected

What is the name of the prescribed medication you used?

Q18

Display this question

If Do you think that you were suffering from a common cold, flu, or other similar illness, on ... Yes Is Selected

Have you been to see your doctor on ${q://QID48/ChoiceTextEntryValue} about your illness symptoms?

○ Yes
○ No

GP prescription

☐ Q19

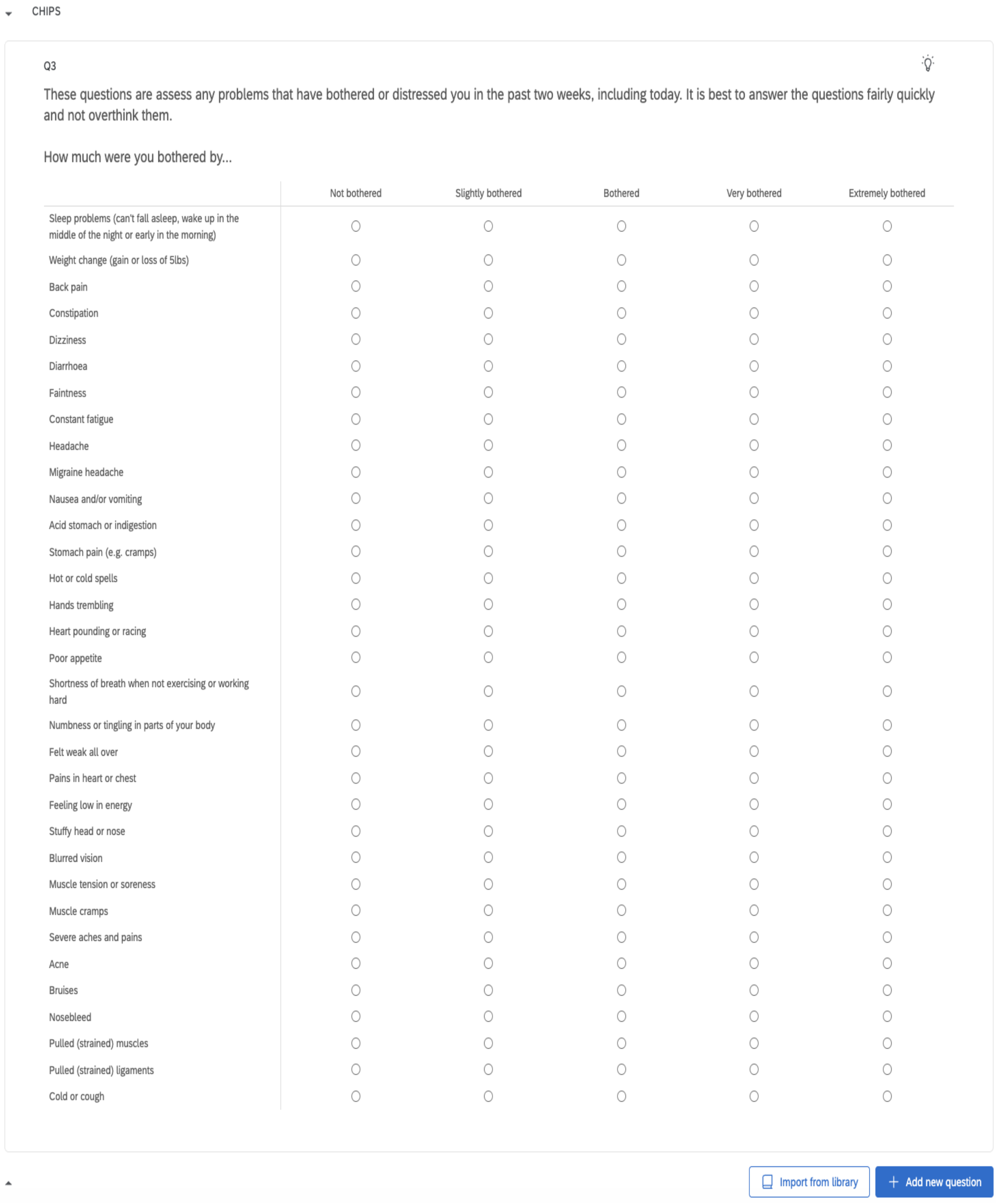
Display this question

If Have you been to see your doctor on ${q://QID48/ChoiceTextEntryValue} about your illness symptoms? Yes Is Selected

Have you taken any prescription medication on ${q://QID48/ChoiceTextEntryValue}?

○ Yes
○ No

Appendix C

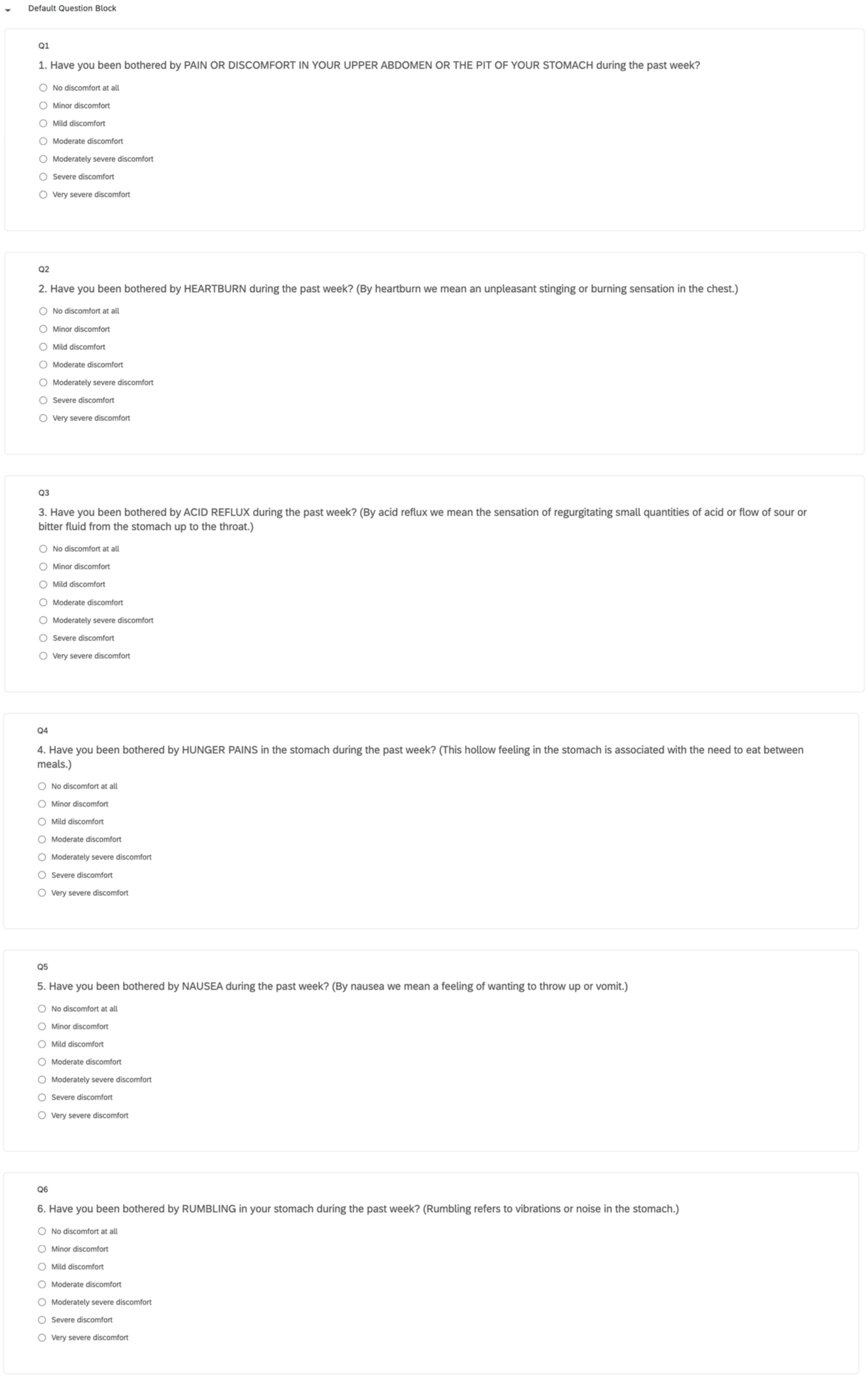


These questions are assess any problems that have bothered or distressed you in the past two weeks, including today. It is best to answer the questions fairly quickly and not overthink them.

How much were you bothered by...

	Not bothered	Slightly bothered	Bothered	Very bothered	Extremely bothered
Sleep problems (can't fall asleep, wake up in the middle of the night or early in the morning)	○	○	○	○	○
Weight change (gain or loss of 5lbs)	○	○	○	○	○
Back pain	○	○	○	○	○
Constipation	○	○	○	○	○
Dizziness	○	○	○	○	○
Diarrhoea	○	○	○	○	○
Faintness	○	○	○	○	○
Constant fatigue	○	○	○	○	○
Headache	○	○	○	○	○
Migraine headache	○	○	○	○	○
Nausea and/or vomiting	○	○	○	○	○
Acid stomach or indigestion	○	○	○	○	○
Stomach pain (e.g. cramps)	○	○	○	○	○
Hot or cold spells	○	○	○	○	○
Hands trembling	○	○	○	○	○
Heart pounding or racing	○	○	○	○	○
Poor appetite	○	○	○	○	○
Shortness of breath when not exercising or working hard	○	○	○	○	○
Numbness or tingling in parts of your body	○	○	○	○	○
Felt weak all over	○	○	○	○	○
Pains in heart or chest	○	○	○	○	○
Feeling low in energy	○	○	○	○	○
Stuffy head or nose	○	○	○	○	○
Blurred vision	○	○	○	○	○
Muscle tension or soreness	○	○	○	○	○
Muscle cramps	○	○	○	○	○
Severe aches and pains	○	○	○	○	○
Acne	○	○	○	○	○
Bruises	○	○	○	○	○
Nosebleed	○	○	○	○	○
Pulled (strained) muscles	○	○	○	○	○
Pulled (strained) ligaments	○	○	○	○	○
Cold or cough	○	○	○	○	○

Appendix D



Default Question Block

Q1

1. Have you been bothered by PAIN OR DISCOMFORT IN YOUR UPPER ABDOMEN OR THE PIT OF YOUR STOMACH during the past week?

○ No discomfort at all
○ Minor discomfort
○ Mild discomfort
○ Moderate discomfort
○ Moderately severe discomfort
○ Severe discomfort
○ Very severe discomfort

Q2

2. Have you been bothered by HEARTBURN during the past week? (By heartburn we mean an unpleasant stinging or burning sensation in the chest.)

○ No discomfort at all
○ Minor discomfort
○ Mild discomfort
○ Moderate discomfort
○ Moderately severe discomfort
○ Severe discomfort
○ Very severe discomfort

Q3

3. Have you been bothered by ACID REFLUX during the past week? (By acid reflux we mean the sensation of regurgitating small quantities of acid or flow of sour or bitter fluid from the stomach up to the throat.)

○ No discomfort at all
○ Minor discomfort
○ Mild discomfort
○ Moderate discomfort
○ Moderately severe discomfort
○ Severe discomfort
○ Very severe discomfort

Q4

4. Have you been bothered by HUNGER PAINS in the stomach during the past week? (This hollow feeling in the stomach is associated with the need to eat between meals.)

○ No discomfort at all
○ Minor discomfort
○ Mild discomfort
○ Moderate discomfort
○ Moderately severe discomfort
○ Severe discomfort
○ Very severe discomfort

Q5

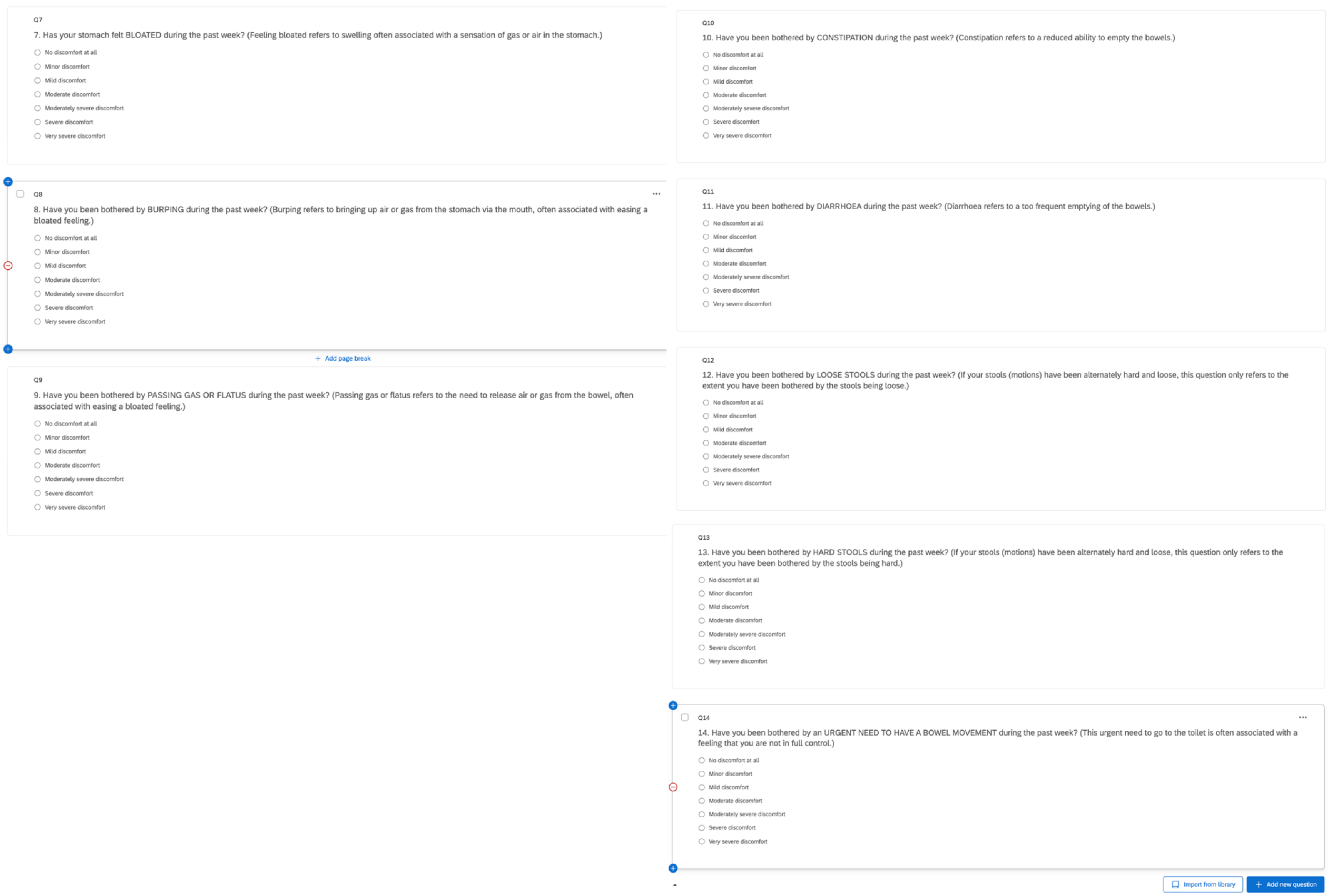
5. Have you been bothered by NAUSEA during the past week? (By nausea we mean a feeling of wanting to throw up or vomit.)

○ No discomfort at all
○ Minor discomfort
○ Mild discomfort
○ Moderate discomfort
○ Moderately severe discomfort
○ Severe discomfort
○ Very severe discomfort

Q6

6. Have you been bothered by RUMBLING in your stomach during the past week? (Rumbling refers to vibrations or noise in the stomach.)

○ No discomfort at all
○ Minor discomfort
○ Mild discomfort
○ Moderate discomfort
○ Moderately severe discomfort
○ Severe discomfort
○ Very severe discomfort



Q7

7. Has your stomach felt BLOATED during the past week? (Feeling bloated refers to swelling often associated with a sensation of gas or air in the stomach.)

- ○ No discomfort at all
- ○ Minor discomfort
- ○ Mild discomfort
- ○ Moderate discomfort
- ○ Moderately severe discomfort
- ○ Severe discomfort
- ○ Very severe discomfort

Q8

8. Have you been bothered by BURPING during the past week? (Burping refers to bringing up air or gas from the stomach via the mouth, often associated with easing a bloated feeling.)

- ○ No discomfort at all
- ○ Minor discomfort
- ○ Mild discomfort
- ○ Moderate discomfort
- ○ Moderately severe discomfort
- ○ Severe discomfort
- ○ Very severe discomfort

Q9

9. Have you been bothered by PASSING GAS OR FLATUS during the past week? (Passing gas or flatus refers to the need to release air or gas from the bowel, often associated with easing a bloated feeling.)

- ○ No discomfort at all
- ○ Minor discomfort
- ○ Mild discomfort
- ○ Moderate discomfort
- ○ Moderately severe discomfort
- ○ Severe discomfort
- ○ Very severe discomfort

Q10

10. Have you been bothered by CONSTIPATION during the past week? (Constipation refers to a reduced ability to empty the bowels.)

- ○ No discomfort at all
- ○ Minor discomfort
- ○ Mild discomfort
- ○ Moderate discomfort
- ○ Moderately severe discomfort
- ○ Severe discomfort
- ○ Very severe discomfort

Q11

11. Have you been bothered by DIARRHOEA during the past week? (Diarrhoea refers to a too frequent emptying of the bowels.)

- ○ No discomfort at all
- ○ Minor discomfort
- ○ Mild discomfort
- ○ Moderate discomfort
- ○ Moderately severe discomfort
- ○ Severe discomfort
- ○ Very severe discomfort

Q12

12. Have you been bothered by LOOSE STOOLS during the past week? (If your stools (motions) have been alternately hard and loose, this question only refers to the extent you have been bothered by the stools being loose.)

- ○ No discomfort at all
- ○ Minor discomfort
- ○ Mild discomfort
- ○ Moderate discomfort
- ○ Moderately severe discomfort
- ○ Severe discomfort
- ○ Very severe discomfort

Q13

13. Have you been bothered by HARD STOOLS during the past week? (If your stools (motions) have been alternately hard and loose, this question only refers to the extent you have been bothered by the stools being hard.)

- ○ No discomfort at all
- ○ Minor discomfort
- ○ Mild discomfort
- ○ Moderate discomfort
- ○ Moderately severe discomfort
- ○ Severe discomfort
- ○ Very severe discomfort

Q14

14. Have you been bothered by an URGENT NEED TO HAVE A BOWEL MOVEMENT during the past week? (This urgent need to go to the toilet is often associated with a feeling that you are not in full control.)

- ○ No discomfort at all
- ○ Minor discomfort
- ○ Mild discomfort
- ○ Moderate discomfort
- ○ Moderately severe discomfort
- ○ Severe discomfort
- ○ Very severe discomfort

Appendix E

State-Trait Anxiety Inventory for Adults^{TM}

Instrument and Scoring Key

License to Administer

Developed by Charles D. Spielberger
in collaboration with R.L. Gorsuch, R. Lushene, P.R. Vagg, & G.A. Jacobs

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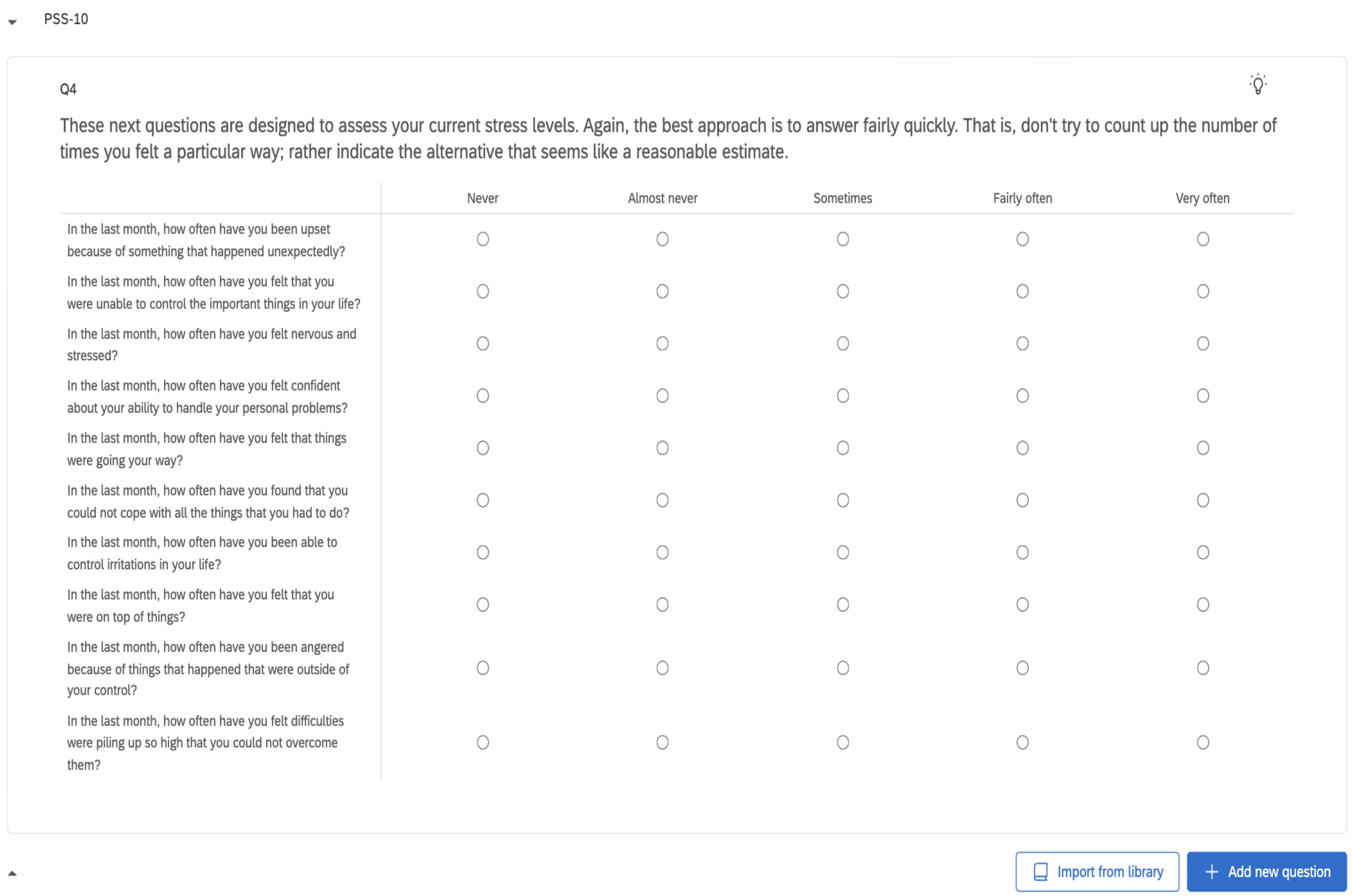
Appendix F



Q4

These next questions are designed to assess your current stress levels. Again, the best approach is to answer fairly quickly. That is, don't try to count up the number of times you felt a particular way; rather indicate the alternative that seems like a reasonable estimate.

	Never	Almost never	Sometimes	Fairly often	Very often
In the last month, how often have you been upset because of something that happened unexpectedly?	○	○	○	○	○
In the last month, how often have you felt that you were unable to control the important things in your life?	○	○	○	○	○
In the last month, how often have you felt nervous and stressed?	○	○	○	○	○
In the last month, how often have you felt confident about your ability to handle your personal problems?	○	○	○	○	○
In the last month, how often have you felt that things were going your way?	○	○	○	○	○
In the last month, how often have you found that you could not cope with all the things that you had to do?	○	○	○	○	○
In the last month, how often have you been able to control irritations in your life?	○	○	○	○	○
In the last month, how often have you felt that you were on top of things?	○	○	○	○	○
In the last month, how often have you been angered because of things that happened that were outside of your control?	○	○	○	○	○
In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	○	○	○	○	○