Dietary Interventions Targeting the Gut Microbiota to Support Gastrointestinal and Respiratory Health of Athletes

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LIST OF ABBREVIATIONS

AMPS	Antimicrobial Peptides
ANOVA	Analysis of Variance
B-GOS	Bimuno Galactoologisaccharide
BMI	Body Mass Index
CLR	C-Type Lectin Receptors
CO_2	Carbon Dioxide
CRP	C reative Protein
DC	dendritic cell
ELISA	Enzyme Linked Immunosorbant Assay
FIO_2	Fraction of Inspired Oxygen
FOS	Fructooligosaccharide
FoxP3	Forkhead P3 Transcription Factor
GALT	Gut Associated Lymphoid Tissue
GI	Gastrointestinal
GIS	Gastrointestinal Symptoms
GOS	Galactooligosaccahride
GPR	G-protein Coupled Receptor
HDAC	Histone Deacetylase
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
I-FABP	Intestinal Fatty Acid Binding protein
IFN-γ	Interferon Gamma
IgA	Immunoglobulin A
IL-10	Interleukin 10
IL-1RA	Interluekin 1 receptor antagonist
IL-1β	Interleukin 1 beta
IL-22	interluekin 22
IL-6	Interleukin 6
IL-C3	Type 3 innate lymphoid cells
LBP	Lipopolysaccharide Binding Protein

1	NF-kβ	Nuclear Factor Kapa-light-chain-enhancer of activated B cells
2	NLR	Nucleotide Binding Oligomerization Like Receptors
3	O_2	Oxygen
4	PRR	Pattern Recognition Receptors
5	RCP	Respiratory Compensation Point
6	RCT	Randomised Control Trial
7	RPE	Rating of Perceived Exertion
8	SCFA	Short Chain Fatty Acids
9	SD	Standard Deviation
10	sIgA	Salivary immunoglobulin A
11	Th 1	T helper 1 cell
12	Th 17	T helper 17 cell
13	Th 2	T helper 2 cell
14	TLR	Toll Like Recptors
15	TNF-α	Tumor Necrosis Factor Alpha
16	URS	Upper Respiratory Symptoms
17	URTI	Upper respiratory Tract Infection
18	$\dot{V}_E$	Minute Ventilation
19	$\dot{V}O_{2max}$	Maximal Oxygen Uptake
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PUBLISHED MATERIALS

Unless otherwise indicated by reference to published resources, the work presented in this thesis is that of the author and has not been previously submitted for another degree to this or any other University. Some of the work in this thesis has been published in peer-reviewed journals and/or presented at conferences.

Peer reviewed oral communications

Parker, C.J., Hunter, K.A., Johnson M.A., Sharpe G.R., Gibson G.R., Walton G.E., Poveda C., Cousins B., Williams N.C**.** (2022). **Oral Presentation** - Effects of 24-week prebiotic intervention on self-reported upper respiratory symptoms, gastrointestinal symptoms, and markers of immunity in elite rugby union players. European College Sport Science Conference, Seville, Spain.

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Parker, C.J., Hunter, K.A., Johnson M.A., Sharpe G.R., Abbott, S., Williams N.C**.** (2024). **Oral Presentation** - Effects of a 6-week prebiotic intervention on indirect markers of gastrointestinal damage, gastrointestinal symptoms and self-reported upper respiratory symptoms following a simulated soccer match in the heat. European College Sport Science Conference, Glasgow, Scotland.

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Peer reviewed written publications

Parker C.J., Hunter, K.A., Johnson M.A., Sharpe G.R., Gibson G.R., Walton G.E., Poveda C., Cousins B., Williams N.C**.** (*2023)*. Effects of 24-week prebiotic intervention on self-reported upper respiratory symptoms, gastrointestinal symptoms, and markers of immunity in elite rugby union players. Eur J Sport Sci. 2023 Jun 18:1-8.

Clayton, D. J., Burbeary, R., **Parker, C**., James, R. M., Saward, C., Procter, E. L., ... & Varley, I. (2024). Combined Turmeric, Vitamin C, and Vitamin D Ready-to-Drink Supplements Reduce Upper Respiratory Illness Symptoms and Gastrointestinal Discomfort in Elite Male Football Players. *Nutrients*, *16*(2), 243.

ABSTRACT

Introduction: Acute illnesses that are associated with gastrointestinal (GI) discomfort and upper respiratory symptoms (URS) are one of the major causes for medical treatment and absence in athletes. GI discomfort may occur during exercise due to increased exercise-induced GI damage and permeability, with 96% of athletes from both individual and team-based sports experiencing at least one symptom. Athletes face numerous stressors that can increase the risk of upper respiratory illness. Heavy exercise, poor sleep, poor nutrition, life stress and travel can all temporarily impair immunity and potentially increase the likelihood of infection. Large squads and the sharing of facilities can then increase the risk of transmission amongst athletes. Performing exercise in extreme environments can exacerbate these issues, which is a concern as athletes are more likely to be exposed to such climates. There is limited evidence to date on the combined influence of environmental stressors and team sport exercise on URS, GIS and gut barrier function.

It is well established that the gut microbiome has a significant influence on GI health and immunity. Specific nutritional interventions may positively alter the gut microbiome and influence gut barrier function. Indeed, probiotic interventions have been shown to alleviate both GI discomfort and URS in athletic populations. However, it is unclear whether other dietary strategies that target the gut could be effective.

Thesis aims: Accordingly this thesis investigated: (i) the impact of exercising in hypoxia on blood biomarkers of GI damage, permeability, endotoxemia and discomfort, (ii) GI damage in response to a competitive soccer match and the impact of a turmeric, vitamin D and vitamin C combined dietary intervention on GI discomfort, damage and respiratory illness in professional footballers, (iii) the effects of a 24-week prebiotic trans-galactooligosaccharide (B-GOS) supplementation on GI discomfort, URS and markers of immunity in professional rugby players across a competitive season, (iv) the effects of a 6-week prebiotic B-GOS supplementation on markers of GI damage, GI discomfort, URS and markers of immunity in response to a simulated soccer match in the heat.

Experimental Results

Chapter 4: It was found that 60-min of treadmill running at 85% respiratory compensation point (RCP) caused greater GI damage when performed in normobaric hypoxia (F_IO_2=14.0%) than normoxia (F_IO_2=20.9%). Specifically, pre to post exercise Δ plasma I-FABP was almost 2-fold greater in hypoxia than normoxia (780 ± 350 vs. 388 ± 317pg/mL) (P = 0.008). Running in hypoxia also caused greater increase in surrogate markers of GI permeability and endotoxemia as shown by higher pre to post exercise Δ plasma claudin-3 (0.88 ± 1.66 vs. -0.65 ± 2.04ng/mL) (P = 0.005) and LBP (2659 ± 2568 vs. 607 ± 1730ng/mL) (P = 0.031) respectively. These findings were accompanied by higher GI discomfort during the hypoxia run than normoxia (10.9 ± 8.6 vs. 6.1 ± 4.4) (P=0.016). This is the first study to show that when exercise intensity is matched to environment hypoxia induces greater GI damage and discomfort. Thus, this provides preliminary evidence of a possible hypoxic and exercise mechanistic pathway. It also highlights the precautions athletes should take when exercising in hypoxic or high-altitude environments.

Chapter 5: This thesis shows for the first time that a competitive 90-minute soccer match causes GI damage as shown by elevated plasma I-FABP. It then demonstrated that the daily supplementation of a combined supplement containing 17.5 g of raw turmeric root (estimated to contain 700 mg curcumin), 1000 mg of Vitamin C, 3000 IU of Vitamin D_3, and 200 mg of black pepper (estimated to contain 10 mg of piperine) for 113 days was able to reduce exercise induced GI damage following a competitive match. The combined ingredient nutritional supplement also lowered URS incidence and the severity of GI discomfort. These data highlight that the combined administration of turmeric, vitamin D_3 and Vitamin C can be a potentially beneficial treatment for elite team-sport athletes, reducing the burden of acute illness and exercise-induced GI damage.

Chapter 6: Data in chapter 6 showed for first time that a 24-week prebiotic intervention was able to reduce the burden of upper respiratory illness and GI discomfort in male elite rugby union players over the course of a competitive season. Individuals assigned the daily consumption of a prebiotics

galactooligosaccharide (B-GOS) (2.9/day) experienced shorter URS episodes than those assigned a placebo (7.42 ± 2.83 days vs 9.82 ± 4.05 days) (P = 0.045). They also reported a lower incidence and less severe GI discomfort (P < 0.001, P = 0.041). Individuals in the prebiotic groups also had a higher salivary IgA secretion rate than the placebo group at 24-weeks (129.23 ± 38.15 vs 90.06 ± 33.45) (P = 0.004). This study indicates that regular ingestion with a prebiotic galactooligosaccharide can be beneficial for alleviating the impact of URS and GI discomfort in elite team sport athletes.

Chapter 7: Another highly novel finding of this thesis was that a 42-day intervention of B-GOS alleviated exercise -induced GI damage following a treadmill based simulated football match in the heat. This was accompanied by a reduction in GI discomfort severity with a 17% drop in the GI severity score (P=0.021). Like the previous chapter, the B-GOS group experienced shorter URS episodes (3.4 ± 5.1 days vs 9.0 ± 5.9 days) (P = 0.025) . However, this was in conjunction with less severe URS (12 ± 16.5 vs 34.5 ± 22.5) (P = 0.029). There was also an increase in salivary IgA secretion rate in the B-GOS group but not placebo immediately post exercise at day 42 (74 ± 204 vs -106 ± 124) (P = 0.016). This shows for the first time that a prebiotic galactooligosaccharide can influence GI barrier integrity during team-based exercise in the heat, possibly alleviate exercise-induced immunosuppression and confirms that it can reduce the burden of URS athletic populations.

1. Chapter 1 – General Introduction

1.1. Introduction and Rationale

Gastrointestinal (GI) discomfort and acute respiratory illness are prevalent in exercising individuals of all levels. Following physical injury, GI and upper respiratory disturbances are the second most common reason for an athlete to require medical attention (Engebretsen et al., 2013; Soligard et al., 2018). Common GI symptoms can include nausea, bloating, intestinal pain, flatulence and diarrhoea (Drew et al., 2017). Upper respiratory symptoms commonly relate to acute illnesses such as the common cold or influenza and are characterised by the presence of headaches, malaise, coughing, sore throat and nose congestion. Eighty-six percent of athletes have disclosed that they experience GI discomfort during exercise (Pugh et al., 2017) with 34% of females and 14% of males claiming it negatively impacts their performance in team-based sports (Wilson et al., 2023). During the summer and winter Olympic games, there is a reported 7-45% incidence rate of respiratory infection (Engebretsen et al., 2010; Soligard et al., 2015; Soligard et al., 2019; Valtonen et al., 2018). In team-based sports like rugby union and football athletes commonly report 4 URTI episodes per competitive season (Cunniffe et al., 2009; Dvorak et al., 2011). The impact that such common illnesses and symptoms can have on performance are profound. A study in Norwegian cross-country skiers observed that winning athletes with the highest performance level reported fewer illness days than less successful athletes (Swendsen et al., 2016). Likewise, football teams with fewer illnesses achieve greater points and league standings (Hägglund et al., 2013).

The aetiology of GI discomfort during exercise is likely multifactorial but the redistribution of blood flow is considered a key contributor. During intense or prolonged exercise, splanchnic blood flow is reduced to prioritise skeletal muscle perfusion. Indeed, portal vein blood flow can be reduced by 80% following 60-min of moderate intensity exercise (Reher et al., 2001). Resultant intestinal ischemia disrupts the epithelial and tight-junction complexes within the intestinal wall. Such disruption elevates intestinal permeability and the likelihood of gram-negative bacteria crossing into systemic circulation. The systemic appearance of gram-negative bacteria can initiate local and

systemic inflammation possibly leading to the onset of symptoms. Elevations in circulatory markers of intestinal damage, permeability and endotoxemia have all been observed following running and cycling (van Wjick et al., 2011; Edwards et al., 2021; Pugh et al., 2020; Pugh et al., 2017; Morrison et al, 2014; Kahru et al., 2017; Lis et al, 2015). Evidence is lacking on the impact of intermittent and team-based invasion sports on GI damage, permeability and endotoxemia. Chantler et al. (2022) reported elevations in plasma I-FABP following a bout of rugby union training, indicating greater GI damage. Pugh et al. (2017) also found that intermitted running, similar to that performed during team-based invasion sports increased plasma I-FABP and GI discomfort. These studies provide an insight into the impact of team-sports on GI structure, but further research is needed to assess permeability, endotoxemia and how whether this also occurs during competitive performances.

Although athletes may not experience more upper respiratory tract infections than the general population, they do face numerous stressors that may increase the risk. Heavy bouts of exercise have been shown to temporarily reduce immunity and increase the risk of illness. For example, 13% and 19% of runners reported symptoms of respiratory illness 1-3 weeks after the Los Angeles and Stockholm Marathons (Nieman et al., 1990; Ekblom et al., 2006). In addition to heavy exercise, sleep disruption, poor nutrition, lifestyle stress and travel are very common for elite athletes and can all contribute to immunosuppression (Fitzgerald 1988; Wentz et al., 2018; Drew et al., 2017; Svendsen et al., 2016). There is a debate whether any of these decrements actually lead to illness but it is clear that athletes face numerous stressors that likely contribute to the risk of them becoming ill during key competition and training periods.

Evidence shows that performing exercise in extreme environments can exacerbate the GI and immune response. Running in the heat caused greater elevations in intestinal permeability than when performed in cooler environments conditions (Yeh et al., 2013). A similar heightened response was observed when cycling and running in hypoxia with higher GI damage, permeability and endotoxemia when compared to normoxia (Hill et al., 2020; McKenna et al., 2023). However, it

should be noted that exercise intensity was not matched to the environment in either of these studies. This means that participants may have been exercising at a harder relative intensity in the hypoxia trials than normoxia, which could have contributed to the heightened GI response. Therefore, studies establishing whether these exacerbations still occur when intensity is match are warranted.

It is well established that the gut microbiome interacts with the immune system and is key for normal GI function (Roberfroid et al., 2010). Evidence also shows that individuals with GI conditions and respiratory diseases have an altered gut microbiome compared to healthy controls (Madan et al., 2012; Sagar et al., 2014). Bacterial strains such as bifidobacterium and lactobacilli are considered commensal bacteria, providing numerous health benefits to the host. Probiotics are food products that contain live bacteria including bifidobacterium and lactobacilli. Studies in athletes have shown improvements in GI discomfort and respiratory illness (Gleeson et al., 2011; Haywood et al., 2013; Pugh et al., 2019; Schreiber et al., 2022). Yet, no improvements have been shown in GI damage and permeability (Pugh et al., 2019) and some studies have shown little impact on illness (West et al., 2011; West et al., 2014). One issue with probiotics is that it is unclear which strains are most beneficial, what is the ideal colony forming unit and whether they transit to the gut.

Prebiotics are dietary fibres that have been proven to reach the large intestine and help the proliferation of resident gut microbes (Gibson et al., 2017). In elderly, overweight, asthmatics and IBS sufferers, a prebiotic galactooligosacchride mixture was shown to alter the gut microbiome, reduce inflammation and alleviate the burden of asthma (Vulevic et al., 2008; Vulevic et al., 2013; Williams et al., 2016; Vulevic et al., 2018). Yet, no known investigations have explored the impact of prebiotics in athletes. Evidence also suggests that both turmeric and vitamin D supplementation can influence the gut microbiome and function (Peterson et al., 2018). One study observed a reduction in exercise-induced GI damage following a 3-day turmeric intervention (Szymanski et al., 2017). Furthermore, prebiotics, turmeric and vitamin D supplementation may be an alternative to

probiotics. Research is needed to clarify the impact of these nutritional interventions on illness, GI discomfort, GI damage and permeability during exercise and possibly in the extreme environments when it is exacerbated.

1.2. Research Aims

The purpose of the series of studies was to investigate the following research questions

I. What are the effects of exercising in normobaric hypoxia on markers of GI damage, permeability and discomfort when intensity is at the same, environment-specific relative to the environment?

The research aims were to:

- Determine whether treadmill running in normobaric hypoxia still causes greater GI damage and discomfort when absolute speed is lower to compensate for the additional stress of hypoxia.

This will give us a greater insight into whether the exacerbated GI damage and discomfort seen in other studies is due to the environment or the fact that they were working at a greater relative intensity.

II. To investigate both the impact of a 90-min professional football match on GI damage and the effects of a combined supplement on acute GI and respiratory illness across the course of a professional season.

The research aims were to:

- Assess the effect of a 90-min competitive football match on a biomarker of gut damage (plasma I-FABP) in male professional footballers.
- Establish whether the daily consumption of a ready-to-drink supplement including turmeric, vitamin-D and vitamin C could alleviate exercise induced GI damage, GI discomfort and upper respiratory symptoms throughout the course of a competitive season in male professional footballers.

III. To assess whether a daily dose of prebiotic trans-galactooligosacchride (B-GOS) for 164 days can reduce GI discomfort, upper respiratory symptoms and markers of inflammation and immunity in elite rugby union players.

The research aims were to:

- Assess if the daily consumption of prebiotic B-GOS could reduce the burden of acute respiratory symptoms and GI discomfort during a competitive season in elite male rugby union players.
- Evaluate if prebiotic B-GOS can favourably alter levels of systemic inflammation and immunity through the assessment of pro-inflammatory cytokines and salivary IgA.

IV. Following on from the findings of study II & III, it was important to evaluate whether nutritional interventions that target the gut microbiome can alleviate GI damage and discomfort during exercise in extreme environments. Subsequently, a double-blind randomised control trial was conducted to investigate the effect of prebiotic B-GOS on markers of exercise-induced GI damage following a football-based treadmill protocol in the heat.

The research aims were to:

- Investigate whether a 6-week prebiotic B-GOS intervention could alleviate exercise-induced GI damage, permeability and discomfort during a simulated football match in 33^{o}C heat.
- To assess whether prebiotic B-GOS could support immunity assessed through salivary IgA concentrations.
- To confirm the findings of study III, assess if prebiotic B-GOS was able to alleviate the burden of URS and GI discomfort.

Chapter 2 – Review of Literature

2.1.1 – Gastrointestinal perturbations during exercise

The gastrointestinal (GI) tract is a complex network of organs working in tandem with all physiological systems (figure 1). At rest the main functions of the GI tract are to digest, absorb and excrete nutrients from our diet while also playing an important role in fluid and electrolyte balance, detoxification, and immune function. To ensure full function of the GI tract, parasympathetic activity and splanchnic blood flow are high, while neural and hormonal stimulation are well controlled (Schwellness & Wright, 2008). Exercise can reduce parasympathetic activity, splanchnic blood flow and disrupt neural and hormonal stimuli, which can reduce oxygen content, increase oxidative stress and inflammation, placing great strain on the GI tract (Schwellnus & Wright, 2008, Brouns & Beckers, 1993, Reher et al., 2001). The response of the GI tract to exercise varies due to the differing nature of sport and exercise (e.g. intensity, duration, posture, direction of movement). Indeed, both moderate and strenuous exercise are widely recognised for its beneficial effects in musculoskeletal, cardiovascular and gastrointestinal health, with decreased risk of colon cancer and gallbladder disease (Oruc & Kaplan, 2019). But both moderate and strenuous can cause (depending on duration, environment and age) can induce acute GI distress (Ter Steege, Van der Palen and Kolkman, 2008; Peters et al., 2001) (figure 2.1).Typical GI symptoms experienced during exercise are bloating, belching, flatulence, vomiting and the sudden urge to defecate, all of which can interfere with performance and negatively impact health (Peters & Bateman, 1983; Drew et al., 2017; Hellard et al., 2015; Svendsen et al., 2016; Wentz et al., 2018).

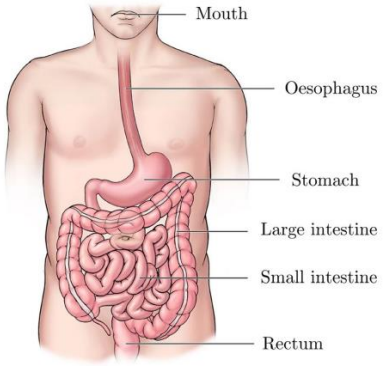

Figure 2.1. Organs of the human gastrointestinal tract. Figure adopted from Durcan et al., (2024).

A recent review identified a potential threshold of exercise intensity that may help individuals avoid alterations in the GI tract (Riberio et al., 2021). The authors concluded that exercising at an intensity equal or greater than 70% of maximal capacity for a duration of 60 minutes or more will increase the risk of GI distress. Furthermore, it would be plausible to advise exercising below this threshold to avoid any unwanted GI distress. However, this concept would be hugely oversimplified and impractical for most individuals as exercising above this proposed threshold is necessary in both training and competition (Seiler & Tønnessen, 2009). Within this consideration, it is extremely likely that GI symptoms are prevalent in competitive sport. Investigating the prevalence and what exercise modes and environments cause the most strain on the GI tract is imperative for athlete health, while identifying methods to reduce symptoms is important for performance.

2.1.2 - Prevalence of GI symptoms in Sport

GI symptoms are very common among athletes from a range of sports, with 86% of elite athletes reporting discomfort (Pugh et al., 2017). Concerningly, GI disturbances were one of the major reasons for an athlete to require medical attention at the summer and winter Olympic games (Soligard et al., 2015; Soligard et al. 2013; Engebretsen et al 2010; Engebretsen et al. 2013; Soligard et al., 2023), and specifically at the most recent Winter Olympics, GI related issues contributed to

13% of all illness reported (Soligard et al., 2023). GI discomfort during exercise has mainly been assessed in endurance athletes. During endurance events, the prevalence varies with 30-90% of athletes expressing GI discomfort (de Oliveira et al., 2014). As previously stated, the duration, intensity and environment can all affect GI discomfort. This is reflected by the prevalence of GI discomfort as an internet based observational study reported 45% of 1,281 runners experience at least on GI symptom (Ter Steege et al., 2008). Whereas, 93% of athletes reported GI discomfort during a long-distance triathlon in extreme environments, which include both high temperatures (peak: 32.1^{o}C) and altitude (peak: 3600m) (Jeukendrup et al., 2000) and severe symptoms such as vomiting, diarrhoea and faecal blood loss occurred in 37-89% of athletes during ultra-events endurance events (Baska et al., 1990; Hoffman & Foggard 2011; Rehrer et al., 1992; Stuempfle et al., 2013).

Like the Summer and Winter Olympic games, GI related illness are frequently reported at elite team sport events (Divorak et al., 2010; Schwellnus et al., 2012; Bjorneboe et al., 2016; Schwellnus et al., 2020). At major football and rugby union tournaments diarrhoea and vomiting contributed to 26% and 21% of illness (Divorak et al., 2010; Schwellnus et al., 2020). Despite these observations, there is a lack of quality research that specifically assesses GI discomfort during team-based activity. For example, two studies showed that GI symptoms increased during football specific exercise, but this was not the main scope of the studies (Harper et al., 2017; Stevenson et al., 2017). Another study reported one case of nausea following a 90-minute football match but again this study focussed on hydration and did not clarify how discomfort was measured (Guttierres et al., 2011). Again, nausea was elevated during a simulated basketball match, but the exact incidence was not reported (Gentle et al., 2014). Clearly, further investigations with the use of more specific assessment tools are needed to explore the impact of team-based exercise on the GI tract.

The variation in GI discomfort between studies is likely multifactorial. During an ironman, only 4% of athletes reported severe GI distress when running, yet this increased to 32% when cycling (Pfeiffer

et al., 2012, suggesting exercise mode has an impact. Hotter climates can also have an impact. Snipe et al. (2018) reported greater GI discomfort when running in 35°C compared to 22°C. Higher core temperature promotes the redistribution of blood flow from the GI tract to the periphery to support heat dissipation (Roswell, 1974), which can induce intestinal ischemia and damage the intestinal barrier, possibly leading to symptoms. Anecdotally, GI discomfort is common when ascending to high altitude. Jeukendrup et al. (2000) observed a 93% incidence rate in GI discomfort, 45% of which being rated as severe in triathletes during an event at an altitude of 3600m and peak temperatures of 32°C. Although the impact of the environment was beyond the scope of this study, the high incidence of GI discomfort does provide some preliminary evidence of exacerbation in such environmental conditions. For a more comprehensive review of the impact of exercise and the environment on the intestinal barrier please refer to section 2.1.5 and 2.1.9.

Various quantitative scales ranging between 4 and 10 points have been used to define the severity of GI discomfort (Riddoch & Trinick, 1988; Pfeiffer et al., 2009; Svedlund et al., 1988b; Nieman et al., 2006; Snipe et al., 2018). This has likely contributed to the variation and possible overestimation of GI discomfort in some studies. For example, one study in an ultramarathon cohort concluded that 96% of participants experienced at least one GI symptom. However, on a 5-point likert scale, many of these were classified as 1 ('mild') and were symptoms which would not affect exercise performance. Despite this, it is very clear that GI symptoms are prevalent in various sports and the impact that different exercise modes, duration and environments can have on GI health and symptoms needs further investigation. Potential interventions that may attenuate the onset and severity of symptoms are warranted.

2.1.3 - Potential causes of GI symptoms

Although the onset of GI discomfort is still not fully understood it is likely due to a series of interactive pathways. Research has even linked some pathways to the occurrence of specific symptoms (Peters et al., 1995, Bischoff, 2011, van Wijck et al., 2012a, Schwellnus & Wright, 2008).

For example, the gut microbiota could contribute to symptoms in multiple ways including effects of GI immune system activation and inflammation, membrane activity, intestinal motility, gut-brain communication, and the gas production from the fermentation and break down of foods (Stern & Brenner, 2018) (Please refer to section 2.4 for a more comprehensive review of the gut microbiota). It is also possible that the mechanical load and repetitive movement of the GI tract can lead to symptoms. This may explain why runners typically report more GI symptoms than cyclists (Pfeiffer et al., 2012, Peters et al., 2000). Psychological traits may also directly influence GI discomfort as individuals diagnosed with high stress and anxiety have higher incidences of GI discomfort (Worobetz & Gerrard, 1985). Similar findings have been reported in sport as triathletes with higher stress presented greater GI discomfort (Sullivan, 1987).

Exercise can also impact the functionality of the GI system, which may contribute to discomfort. Exercise mode, duration and intensity can affect motility, digestion and absorption (Rao et al., 2004, van Nieuwenhoven et al., 2004). Exercise performed at ≥70% peak power output or for ≥90 mins appear to be detrimental for gastric motility with further disturbances occurring in a dose-response relationship (Costa et al., 2017; Horner et al., 2015). A similar response has been observed for gastic emptying as this was slower following high intensity intermittent exercise than steady state exercise (Leiper et al., 2017). Impaired gastric emptying can lead to pronounced intra-gastric pressure, reduced orocaecal transit time (OCTT) and possibly malabsorption which may lead to discomfort (Horner et al., 2015; Costa et al., 2017).

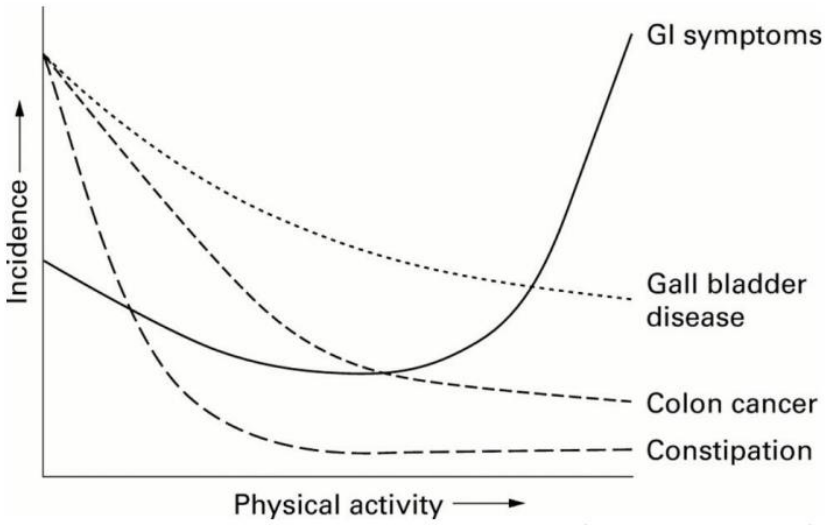

Figure 2.2 Schematic showing the relationship physical activity and the presence of GI discomfort and disease (peters et al., 2001).

The time of day can also influence the onset of GI discomfort. Changes in human metabolism, circulatory, neuroendocrine, and immunological status due to daily circadian rhythm may negatively affect GI integrity (Costa et al., 2008; Costa et al., 2010; Oliver et al., 2009; Oliver et al., 2015). It is also well documented that plasma cortisol and circulatory immune cells (leukocyte, granulocyte and lymphocyte counts) can differ between diurnal and nocturnal times (Costa et al., 2008; Costa et al., 2010; Born et al., 1997; Dinges et al., 1994). Together this can initiate systemic endotoxemia and feeding intolerance (Oliver et al., 2009) which may be amplified during prolonged endurance exercise causing GI discomfort (Costa et al., 2019; Costa et al., 2017). Despite this, only one study has explored this notion. Gaskell et al. (2020) observed higher concentrations of plasma cortisol and delayed orocecal transit time when running at night (21:00-0:00) compared to the day (09:00-12:00). This attributed to higher incidences in gastrointestinal symptoms. However, no additional perturbations were reported in intestinal damage or systemic endotoxemia at night compared to day. Therefore, it seems nocturnal exercise may exacerbate GI symptoms through alterations in the neuroendocrine–gastrointestinal pathway rather than barrier integrity. This finding is a concern as participation in 24-hour endurance events is becoming increasingly common which includes nocturnal segments (Knoth et al., 2012).

Exercise can also impair nutrient absorption and concurrently cause GI discomfort. Nutrient absorption can be determined by assessing urinary excretion of non-metabolizable glucose analogues, such as D-xylose which is passively absorbed, and 3-O-methyl-D-glucose which is actively absorbed (Lang et al., 2006). Running at 70% $\dot{V}O_{2max}$ decreased the absorption of carbohydrates when compared to running at 50%, 30% $\dot{V}O_{2max}$ and rest (Lang et al., 2006). Similarly, carbohydrate absorption measured by breath hydrogen (H_2) excretion was reduced in 68% of 25 healthy runners during the recovery period after 3 hours of running (Costa et al., 2017). Interestingly, H_2 correlated with GI symptom incidence and severity implying that impaired absorption leads to greater discomfort. In addition to carbohydrate, protein absorption may also be impaired after exercise. Protein absorption was lower following a bout of resistance exercise (van Wjick et al., 2013). This was accompanied by a 35% increase in plasma I-FABP suggesting it may be due to heightened GI damage.

2.1.4 - The Intestinal Barrier

The intestinal barrier provides a barricade between the body's internal and external environments, aiding the body's surveillance against harmful invading entities (Konig et al., 2016). The intestinal barrier has two primary functions, one of which is to prevent harmful entities such as foreign antigens, bacterial pathogens, toxins and proinflammatory factors from leaving the lumen and entering the internal environment (Podolsky 1999; Blikslager et al. 2007). The second function is to selectively filter essential dietary nutrients, electrolytes, and water from the intestinal lumen into circulation (Blikslager et al., 2007; Kunzelmann & Mall, 2002, Broer, 2008; Ferraris & Diamond, 1997). Structurally, the barrier is formed of epithelial cells and enterocytes (figure 2.3), which are connected by tight junctions consisting of specialized proteins such as occluding, zona-occludens and claudins (Podolsky, 1999; Kuennen et al., 2011).

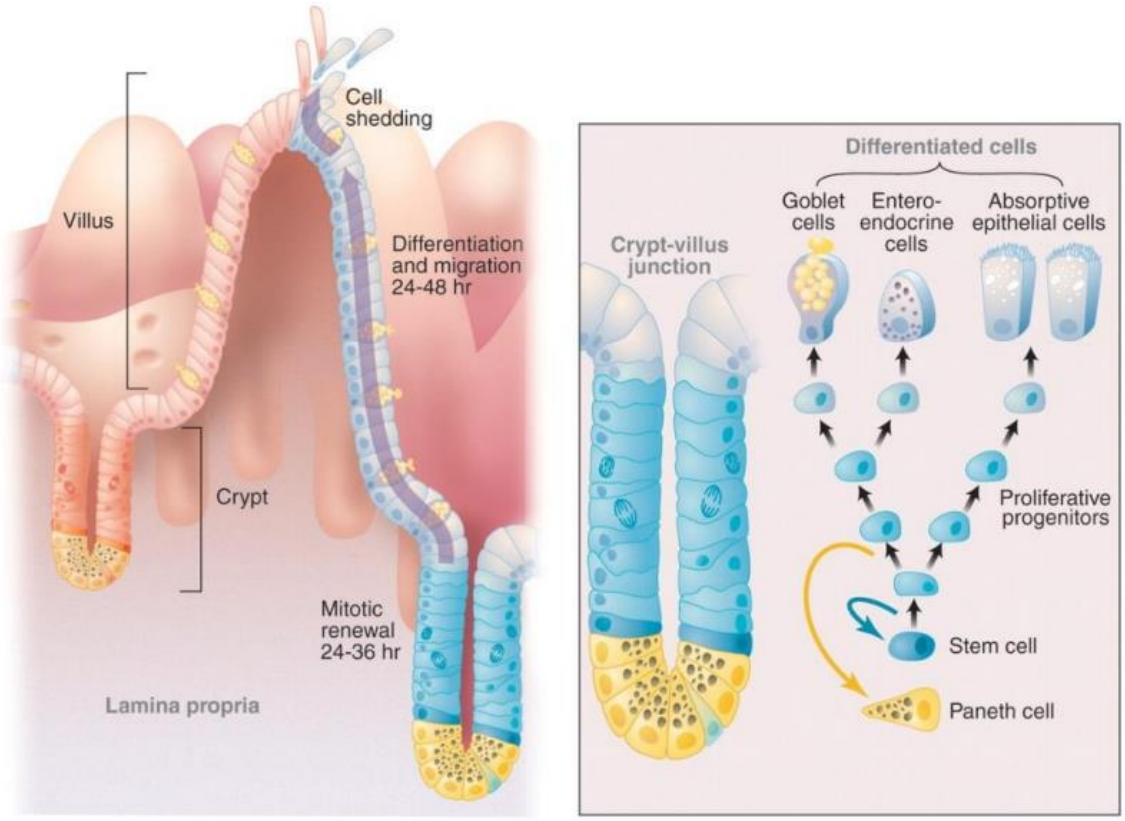

Figure 2.3 The above figure shows the structure of the epithelia within the small intestine. The left figure details the shape, height and depth of the intestinal villi and crypts that form the epithelium (Radkte & Clevers, 2005).

Tight junctions connect adjacent cells on the apical and lateral membranes forming the extracellular border around the cell (Farquhar & Palade, 1963) (Figure 2.4). They help marshal the paracellular movement of ions, water and other nutrients while preventing luminal toxins from entering circulation (Farquhar, 1963; Groschwitz & Hogan, 2009). Occludin and claudins (claudin-1, claudin-2 and claudin-3) are tetraspanning membrane proteins with two extracellular loops, and cytoplasmic N-terminal and C-terminal domains (Schneeberger & Lynch, 1992; Furuse et al., 1993; Hartsock & Nelson, 2008). The C-terminal domain is the main site for interaction with the zona-occludens and PDZ proteins and is required for the assembly at the tight junction (Rao, 2009; McCarthy et al., 1996). Occludins were considered the primary foundation of tight junction complexes as overexpression was associated with greater resistance (Furuse et al., 1996; McCarthry et al., 1996). More recent evidence suggests otherwise as occludin knock-out mice showed normal tight junction

resistance, and barrier function (Saitou et al. 2000). Nevertheless, occludins may play an important role in the Zonulin-1 and actomyosin cytoskeleton interaction, which through signalling molecules maintains the tight junction complex and barrier function (Groschwitz, 2009; Suzuki et al., 2009; Van Itallie et al., 2010). Furthermore, the claudin proteins (claudin-1, claudin-2 & claudin-3) are now considered to be the primary seal forming protein for the tight junction complex (Mitic, Van Itallie & Anderson, 2000; Furuse et al., 1998; Furuse et al., 2002). Indeed, overexpression claudin results in greater tight junction resistance, while claudin knock-out mice died within 1 day of birth (Furuse et al., 2002).

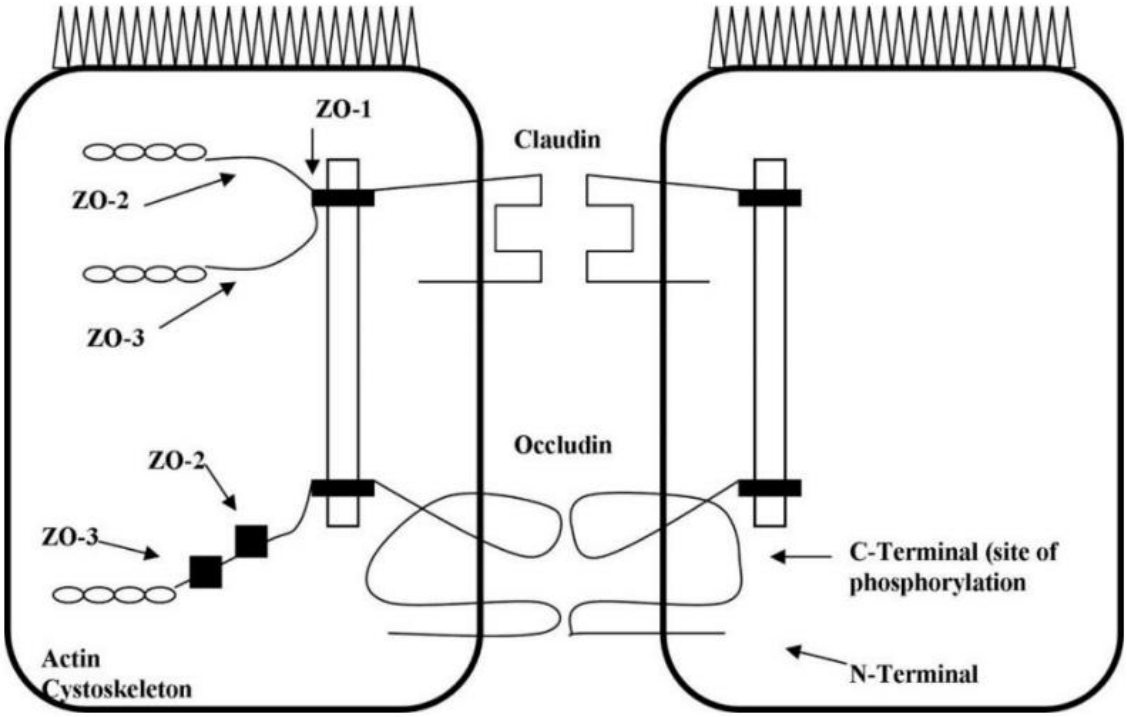

Figure 2.4 schematic of the claudins, occuldin and regulaltory zonulin-1, 2 and 3 proetins which form the tight junction complex (zuhl et al., 2014).

Both occludin and claudin formation at the tight junction are regulated through phosphorylation of several proteins, including different isoenzyme forms of protein kinase C (PKC) (Andreeva et al. 2006), protein kinase A (PKA) (D'Souza, Agarwal & Morin,2005), tyrosine kinase (Elias et al., 2009),

MAPK and several more (Dorfel & Huber, 2012). Occludin phosphorylation by conventional PKC and tyrosine kinases has been shown to decrease tight junction assembly (Andreeva et al., 2006, Elias et al., 2009) while phosphorylation by novel PKC (nPKC) improves tight junction resistance (Andreeva et al., 2006). Claudin phosphorylation by nPKC promotes fibril formation and tight junction assembly (Suzuki et al., 2009), while PKA has opposing effects (Banan et al., 2005). Changes or disruption of the tight junction structure or phosphorylation can increase intestinal permeability (DeMeo et al., 2002). Increased intestinal permeability can be defined as the non-mediated diffusion of large, normally prohibited molecules (>0.15 kDa molecular mass), form the intestinal lumen to the blood (Lambert, 2009; Travis & Menzies, 1992). Lifestyle events including diet, medication, exercise and stress can disrupt the intestinal structure and exacerbate intestinal permeability. Reports following exercise in temperate and hot environments indicate increased intestinal permeability (Jeukendrup et al., 2000; Pals et al., 1997; Marchbank et al., 2011), while similar responses are present after taking certain non-steroidal ant-inflammatory drugs (NSAIDS) and excessive alcohol (Playford et al., 2001; Bode, 2003). Interestingly, its alleged that psychological stress and mental illness can negatively affect intestinal permeability (Mayer, 2011). As intestinal permeability has been associated with numerous chronic intestinal diseases, it is worrying to find increased susceptibility during and following exercise. Although, the current impact of acute increased permeability following exercise in healthy adults in poorly understood.

2.1.5 – Impact of acute exercise on the gastrointestinal tract

As previously discussed, an important function of the GI tract is to provide a semi-permeable barrier between the internal and external environments. The GI tract contains large quantities of lipopolysaccharides (LPS) embedded in the walls of gram-negative bacteria. In healthy individuals at rest, LPS usually stay confined to the intestinal lumen and does not cross the GI barrier at a rate greater than the ability of the liver to remove it from circulation, meaning it causes little harm to health. However, during exercise performance particularly that of long duration or high intensity,

splanchnic blood flow is reduced to increase oxygen availability to skeletal muscle and vital organs. At the onset of steady state exercise, portal vein blood flow was reduced by 20% within 10 minutes and by 80% at 60 minutes during running at 70% $\dot{V}O_{2max}$ (Reher et al., 2001). A similar response was also reported during steady state cycling, as gastric arterialised PCO_2 increased by 1.7 kPa, though levels returned to baseline 1 hour after exercise (van Wjick et al., 2011). The resultant intestinal ischemia induces epithelial damage and disruption to the apical sites and epithelial cell types (i.e. enterocyte, goblet, Paneth and enteroendocrine cells) which are essential to GI barrier function (Grootjans et al., 2016). Substantial damage will break the multi-protein tight junction complexes, increasing intestinal permeability. Indeed, 60 mins of running at 70% $\dot{V}O_{2max}$ disrupts tight junctions and elevates intestinal permeability (Doklandy et al., 2016).

Injury within the epithelial cells stimulates NF-kB gene expressions (Irving et al., 2014; Kaparakis-Liaskos et al., 2015), which initiate a local inflammatory cascade by signalling release of pro-inflammatory cytokines such as interleukin 1B (IL-1B), tumor necoriss factor (TNF-a) and interferon gamma (IFN-γ). Such inflammation can also promote dysregulation of tight junctions and increase intestinal permeability (Capaldo et al., 2009). Substantial disruption to the intestinal barrier can heighten the translocation of bacterial endotoxins. Increases in circulatory lipopolysaccharide (LPS) and lipopolysaccharide binding protein (LBP) concentrations have been reported following endurance exercise (Jeukendrup et al., 2000; Camus et al., 1997; Gill et al., 2015; McKenna et al., 2022). Gram-negative bacteria such as LPS stimulate NF-kB gene expression through NOD receptors within the epithelium, which also contributes towards local and systemic inflammation (Irving et al., 2014; Kaparakis-Liaskos et al., 2015) and possibly the onset of GI symptoms such as nausea and regurgitation (Jeakuendrup et al., 2000; Stuempfle et al., 2015).

2.1.6 - Exercise-induced gastrointestinal damage

Gastrointestinal damage is commonly assessed through changes in circulatory concentrations of intestinal fatty-acid binding protein (I-FABP). I-FABP is a small (14-kD) cytosolic water-soluble protein

found only in mature enterocytes within the small and large intestine. Once damaged, enterocytes rapidly release I-FABP into circulation making it an early and sensitive marker of exercise-induced GI damage (Pelsers et al., 2003; Derikx et al., 2008; Grootjans et al., 2016). Circulatory I-FABP correlates with splanchnic hypoperfusion and intestinal ischemia making it a useful surrogate marker for reduced splanchnic blood flow (van Wijck et al., 2012; van Wijck et al., 2011).

I-FABP has been extensively researched in response to numerous exercise modes, intensities and duration. Prolonged running and cycling (> 1 hour) have been shown to consistently elevate I-FABP (van Wijck et al, 2011; Morrison et al., 2014; Kahru et al., 2017; Lis et al., 2015). The greatest mean concentrations of I-FABP have been observed after long duration (>3 hours) running, with values above 1000 pg/ml reported (Pugh et al., 2019; Costa et al., 2019), suggesting exercise duration may be key to the magnitude of intestinal damage. It is likely that the prolonged dehydration and splanchnic hypoperfusion during such events contributes to the elevated I-FABP (Gisolfi et al., 2000). Short term, high intensity exercise can still induce significant damage. Pugh et al. (2017) observed similar damage to that seen during after repetitive interval running (18 x 800m) at 120% $\dot{V}O_{2max}$. Edwards et al. (2021) investigated the impact of exercise mode, comparing intestinal damage between cycling and running when performed at the same intensity and duration. The authors found plasma I-FABP to be significantly greater post cycling than running, citing the pooling of blood flow towards the lower body as the main contributor.

Despite the variety of exercise protocols used to induce intestinal damage, limited research has investigated the impact of intermittent team sports on GI responses. Only one known study has assessed I-FABP concentrations following team-sport activity (Chantler et al., 2022). In this study, 21 professional academy rugby union players performed a 45-min training bout which had an emphasis on evasion skills, collision and within contact exertion. The mean total distance covered during the training protocol was 555m with a mean heart rate of 170 bpm. Interestingly, the training protocol caused a 70% increase in I-FABP and a 211% increase in intestinal permeability as shown by urinary

lactulose:rhamnose concentrations (for more information on intestinal permeability, please refer to section 2.1.7), indicating disruption to the intestinal barrier.

Like other team-based sports, football is an intermittent, invasive field-based sport involving repeated bouts of high intensity running and periods of low intensity movements and static recovery (Bangsbo et al., 2006; Mohr et al., 2003). Depending on playing positions players can cover 10-14km on average with most at lower intensities (Bradley & Noakes, 2013). However, a significant proportion is performed at high intensities with an average of >1400m spent during accelerating and decelerating movements and >500 changes in direction (Bradley et al., 2010; Carling et al., 2012; Di Mascio & Bradley, 2013; Di Salvo, et al., 2012). During match play, players compete at an average heart rate of 85% age predicted maximum which can translate to an 70% of their maximal oxygen uptake (Bangsbo et al., 2006; Krustrup et al., 2006). As previously explained, both HIIT exercise and rugby-based exercise increases plasma I-FABP concentrations (Pugh et al., 2017, Wallet et al., 2022; Chantler et al., 2022). Therefore, it is plausible to assume the prolonged aerobic involvement mixed with high-intensity intermittent movements of football would impose enough thermal load, blood redistribution and mechanical load to induce similar levels of intestinal damage. This is supported by the fact that GI disturbances impacted 26% of athletes at a major football tournament (Divorak et al., 2010), 21% at a rugby union tournament (Schwellnus et al., 2020) and 86% from other disciplines (Pugh et al., 2017). Yet, there is no current knowledge of whether GI damage and discomfort occurs during such performances.

Table 2.1 list of primary research studies reporting pre and post exercise I-FABP concentrations and correlations with GI discomfort. Data presented as mean ± SD unless stated otherwise.

Study	Participants	Exercise Stressor	∆I-FABP Concentrations (pg.mL)	GI Discomfort
Van Wjick et al. (2012)	n= 9 male cyclists and triathletes	60 min cycling at 70% W_{max}	Pre-exercise: 295 ± 46 Post-exercise: 474 ± 74 *	No assessment
Morrison et al. (2014)	n= 7 trained male participants	15 min cycling at 50% HRR, 30 min running at 80% HRR, 30 min running time trial + 15 min cycling at 50% HRR in 30°C	Pre-exercise: 143 ± 59 Post-exercise: 949 ± 423 *	Assessed but no comparison to I-FABP
Barberio et al. (2015)	n= 8 endurance trained male participants	Running at 78% $\dot{V}O_{2max}$ (4mMol.L^{-1} blood lactate) until core temperature increases by 2°C or volitional exhaustion (~ 24 min) in 40°C	Pre-exercise: 640 ± 125 Post-exercise: 937 ± 149 *	No assessment
Van Wjick et al. (2013)	n= 12 recreationally trained male participants	30 min resistance exercise	Pre-exercise: 254 ± 31 Post-exercise: 344 ± 53 *	No assessment
Lis et al. (2015)	n= 13 male and female competitive cyclists	45 min steady state cycling at 70% W_{max} & 15 min time trial	Pre-exercise: 94 ± 83 Post-steady state exercise: 233 ± 188 * Post-time trial: 304 ± 191*	Assessed but no comparison to I-FABP
Sessions et al. (2016)	n= 7 endurance trained male and female participants	60 min running at 70% $\dot{V}O_{2max}$ in 30°C	Pre-exercise: 261 ± 160 20 min Post-exercise: 337 ± 207 *	No assessment
Karhu et al. (2017)	n= 17 trained runners 9 asymptomatic	90 min run at 80% 10km PB	Pre-exercise (symptomatic): 389 ± 327	Assessed but no comparison to I-FABP

	8 symptomatic		Post-exercise (symptomatic): 961 ± 949 * Pre-exercise (asymptomatic): 314 ± 152 Post-exercise (asymptomatic): 804 ± 599*	
Pugh et al. (2017)	11 male runners	18 x 400m runs at 120% $\dot{V}O_{2max}$	Pre-exercise: 481 ± 334 Post-exercise: 829 ± 448 *	Measured with no relationship to I-FABP
Pugh et al. (2019)	24 recreational runners (20 male; 4 female)	42.2 km on synthetic 400m running track, 16-17°C	Pre-exercise (probiotic group): 455 ± 190 Post-exercise (probiotic group): 1814 ± 1708 * Pre-exercise (placebo group): 460 ± 221 Post-exercise (placebo group): 1392 ± 867 *	Assessed but no significant relationship to I-FABP
Snipe et al. (2018)	10 endurance runners	120 min running at 60% $\dot{V}O_{2max}$ in 35°C	Pre-exercise: 285 (137-432) Post-exercise: 1515 (965-2065) *	Assessed but no relationship to I-FABP
Snipe et al. (2018)	24 endurance runners (13 male; 11 female)	120 min running at 60% $\dot{V}O_{2max}$ in 35°C	Pre- to post exercise Δ (males): 1389 Pre- to post exercise Δ (females): 1445	Assessed but no comparison to I-FABP

Lee et al. (2017)	21 recreationally active males	40 min cycle at 50% $\dot{V}O_{2peak}$ in ambient (18°C), hypoxia (F_IO_2 = 14%) or hot conditions (40°C)	Pre-exercise (ambient): 352 ± 249 Post-exercise (ambient): 380 ± 240 Pre-exercise (hypoxia): 449 ± 234 Post-exercise (hypoxia): 643 ± 232* Pre-exercise (hot): 370 ± 392 Post-exercise (hot): 652 ± 100*	No assessment
Hill et al. (2018)	10 recreationally active males	60 min running at 65% normoxic $\dot{V}O_{2max}$ in normoxia (F_IO_2 = 20.9%) and hypoxia (F_IO_2 = 14%)	Pre-exercise (normoxia): 600 Post-exercise (normoxia): 900 Pre-exercise (hypoxia): 620 Post-exercise (hypoxia): 1300*#	No assessment

McKenna et al. (2022)	9 recreationally active males	60 min cycling at 65% normoxic $\dot{V}O_{2max}$	Pre-exercise (normoxia): 759 ± 224 Post-exercise (normoxia): 828 ± 288 Pre-exercise (hypoxia): 708 ± 191 Post-exercise (hypoxia): 1215 ± 518*#	Assessed and significantly correlated to Δ I-FABP
Chantler et al. (2022)	19 elite male academy rugby players	45 min collision based rugby training	Pre-exercise: 2140 (1260-2730) Post-exercise: 3245 (1985-5143) *	Assessed but not compared to I-FABP

*** indicates significant difference to pre exercise value. # indicates significant difference to other trial.**

2.1.7 - Exercise-induced intestinal permeability

A consequence of significant damage and disruption to the intestinal epithelia and tight-junction complexes during exercise is increased intestinal permeability. Intestinal permeability is an index of mucosal integrity, specifically describing the control of material passing form inside the GI tract to the rest of body via the gut lining. Intestinal permeability can be assessed non-invasively through urinary excretion of an orally administered, non-metabolised, non-toxic water-soluble probe and measuring its recovery in urine. Traditionally, urinary concentrations of a larger (typically lactulose) and a smaller molecule (typically rhamnose or mannitol) are compared over a 5-hour period (Camilleri et al., 2010). The premise of this test is based off the notion that each sugar probe follows a different pathway once consumed (Bjanason et al., 1995). The smaller molecule can pass through the intestinal barrier paracellularly and transcellularly through aqueous pores of the enterocyte cell membrane or the lipid soluble brush border whereas, the larger molecule can only pass paracellularly through the tight junctions (Travis & Menzies, 1992). This means that higher concentrations of the larger molecule are due to structural disruption within the tight junctions, implying greater permeability (Camilleri et al., 2010; Travis & Mezies, 1992).

Assessing the urinary lactulose/rhamnose ratio was originally used in clinical populations (Keating et al., 1995; Sanderson et al., 1997, but this has been extended into exercise to investigate intestinal permeability during exercise stress and interventions (Marchbank et al., 2011; Mahmood et al., 2007). In exercise trials, the sugar-probe dose and the timing of ingestion has differed with some administering prior to exercise (van Nieuwenhoven et al., 1999, van Nieuwenhoven et al., 2004), 20 minutes into an exercise bout (Zuhl et al., 2014), 30 minutes into an exercise bout (Pals et al., 1997, Van Wijck et al., 2011, van Wijck et al., 2013b), or post-exercise (Smetanka et al., 1999). Such discrepancies make it difficult to interpret and compare between trials. Replicating the traditional method of 5-hours is also a challenge for investigators and participants. Thus, alternative assessments of intestinal permeability are warranted. Pugh et al. (2017) successfully assessed the

lactulose-rhamnose ratio in serum following a high intensity interval challenge. This procedure was based off previous literature (Flemming et al., 1996) and was found to be more sensitive when compared to urine (Pugh et al., 2017). Peak concentrations were also detected as early as 2-hours after ingestion making it more practical and time efficient.

Despite the innovative finding from Pugh et al. (2017), the assessment of dual-sugar probes requires specialised equipment and is costly. One alternative is the assessment of circulatory claudin-3. claudin-3 is a tight junction protein found in the duodenum and colon. The heightened appearance of claudin-3 in circulation indicates disruption of the paracellular barrier within the gut-lining and thus, increased intestinal permeability (Tsukita et al., 2001; Morin 2005). Elevated concentrations of circulatory claudin-3 have been reported following 60 min cycling in hypoxia (McKenna et al., 2022). Another marker of interest is circulatory zonulin. Zonulin is 47-kDa protein which modulates tight junctions in the small intestine and intestinal innate immunity (Fasano & Shea-Donohue, 2005; Wang et al, 2000; Smecuol et al, 2005). Initially, serum zonulin was strongly correlated with the lactulose/mannitol ratio (Sapone et al, 2006) but its reliability has come under scrutiny. A recent study concluded that a commercial zonulin enzyme linked immunosorbent assays (ELISA) do not reflect true zonulin concentrations and detect unknown proteins (Scheffler et al., 2018; Ajamian et al., 2019). This does not dispute the use of zonulin as a marker of intestinal permeability but rather advises researchers to interpret published zonulin data cautiously.

The amount of intestinal permeability appears to be highly influenced by the magnitude of exercise with higher body temperature and greater intensity causing more permeability (Costa et al., 2017 Pals et al., 2000; Pals et al., 1997; Zuhl et al., 2014). This was well characterised by Pals et al. (2000) who assessed small intestinal permeability following 60 minutes of running at three separate intensities in thermoneutral conditions. Intestinal permeability increased in a dose response following running at 40%, 60% and 80% $\dot{V}O_{2max}$, while rectal temperature also followed a similar response. This may explain why one of the highest rates reported of elevated intestinal permeability was following a high-intensity interval protocol (Pugh et al., 2017).

Even though markers of intestinal damage and permeability can give us an insight into the effects of exercise on the GI tract, the incidence of discomfort should be equally important. The onset of GI symptoms can be detrimental to exercise performance, causing the individual to decrease intensity or stop the activity. Despite this, a limited number of studies have assessed the link between GI damage, permeability, and symptoms. Studies have consistently shown increases in I-FABP during exercise but have failed to compare values to GI symptoms or have failed to measure symptoms altogether (Table 1). Similarly, increases in intestinal permeability have been reported following a range of exercise modalities and durations, but there has been a failure to report symptoms (table 2). Additionally, those few studies that have also reported GI discomfort have only showed mild or no cases. For example, GI permeability has been shown to increase following both a half and full marathon, but there was no correlation to GI discomfort (Smetanka et al., 1999; Oktedalen et al., 1992) implying no causal relationship, and that there may be another impacting factor. Interestingly, Costa et al. (2017) reported an inverse correlation between GI permeability and discomfort, where individuals with the highest permeability actually reported the lowest discomfort. To better understand the complexity of GI discomfort future studies should report both GI permeability and GI discomfort, but also highlight other confounding factors that may contribute to discomfort (e.g. dietary intake during field-based studies, environment, travel, anxiety).

Table 2.2 list of primary research studies reporting pre and post exercise intestinal permeability and correlations with GI discomfort. Data presented as mean ± SD unless stated otherwise.

Study	Participants	Exercise Stressor	Assessment of Permeability	GI Discomfort
Van Wjick et al. (2012)	n= 9 male cyclists and triathletes	60 min cycling at 70% W_{max}	Dual sugar probe (L 5g, R 2g) ingested 30 min after exercise started. Pre-exercise concentration : 0.01 ± 0.01 Post-exercise concentration: 0.03 ± 0.02	No assessment
Buchman et al. (1999)	n= 15 male and female marathon runners	Road marathon competition	Dual sugar probe (L 5g, M 2g) taken post-race Pre-exercise concentration: 0.03 ± 0.02 Post-exercise concentration: 0.07 ± 0.10	Assessed but no comparison to L:M ratio
Lambert et al. (2008)	n= 20 trained runners	60 min running at 70% $\dot{V}O_{2max}$	Dual sugar probe (L 5g, R 5g) taken immediately before running. Pre-exercise concentration: 0.035 (0.01-0.10) Post-exercise concentration: 0.063 (0.02-0.17)*	Assessed but no comparison to L:R ratio
March et al. (2017)	n= 18 healthy male participants	20 min running at 80% $\dot{V}O_{2max}$	Dual sugar probe (L 5g, R 1g) taken immediately after running.	No assessment

			Pre-exercise concentration: 0.35 ± 0.06 Post-exercise concentration: 0.95 ± 0.12	
Pals et al. (1997)	n= 6 active male and female participants	60 min running at 40%, 60% and 80% $\dot{V}O_{2peak}$	Dual sugar probe (L 5g, R 2g) taken 30 min after exercise started. Pre-exercise concentration: 0.048 ± 0.01 Post-exercise (40% $\dot{V}O_{2peak}$) concentration : 0.056 ± 0.01 Post-exercise (60% $\dot{V}O_{2peak}$) : 0.064 ± 0.01 Post-exercise (80% $\dot{V}O_{2peak}$) : 0.107 ± 0.02*	Assessed but not comparison to L:R ratio
Smetanka et al. (1999)	n= 7 marathon runners n = 6 resting controls	Road marathon competition	Dual sugar probe (L 5g, R 2g) taken 30 min after race completion. Runners post race concentration : 0.019 ± 0.01 Controls concentration : 0.022 ± 0.01	Assessed but not compared to L:R ratio

Van Nieuwenhoven et al. (1999)	n= 10 asymptomatic runners	90 min cycle at 70% W_{max}	Pre-exercise concentration: 0.02 (0.01-0.27) Post-exercise concentration: 0.01 (0.00-0.01)	No assessment
Pugh et al. (2017)	11 male runners	18 x 400m runs at 120% $\dot{V}O_{2max}$ and rest (control)	Dual sugar probe (L 5g, R 2g, S 1g, D 0.5g) measured in serum Runners post exercise concentration: 0.051 ± 0.016 Controls concentration: 0.137 ± 0.148 *	Assessed but no significant correlation to L:R ratio
Pugh et al. (2019)	24 recreational runners (20 male; 4 female)	42.2 km on synthetic 400m running track, 16-17^{o}C	Dual sugar probe (L 5g, R 2g) measured in serum Pre-exercise (probiotic group) concentration: 0.057 ± 0.022 Post-exercise (probiotic group) concentration: 0.099 ± 0.062 * Pre-exercise (placebo group) concentration: 0.061 ± 0.042 Post-exercise (placebo group) concentration: 0.081 ± 0.036 *	Assessed but no significant correlation to L:R ratio

Van Nieuwenhoven et al. (2004)	n= 10 asymptomatic runners	90 min run at 70% $\dot{V}O_{2max}$ 90 min cycle at 70% W_{max}	Dual sugar probe (L 5g, R 0.5g) taken immediately after exercise Res concentrationt: 0.02 (0.01-0.04) Post-cycling concentration: 0.03 (0.01-0.04) Post running concentration: 0.04 (0.02-0.05)*	No assessment
Van Wjick et al. (2011)	n= 6 healthy male participants	60 min cycling at 70% W_{max}	Dual sugar probe (L 1g, R 0.5g) taken 30 min after exercise strated Pre-exercise concentration (urinary): 0.022 ± 0.005 Post-exercise concentration (urinary): 0.042 ± 0.04 Pre-exercise concentrtaion (plasma): 0.002 ± 0.001 Post-exercise concentration (plasma): 0.006 ± 0.004*	No assessment
McKenna et al. (2022)	n=9 recreationally active males	60 min cycling at 65% normoxic $\dot{V}O_{2max}$	Plasma claudin-3 concentrations (ng.mL)	Assessed and Significantly correlated to Δ claudin-3

			Pre-exercise (normoxia): 13.71 ± 1.77 Post-exercise (normoxia): 14.25 ± 1.61 Pre-exercise (hypoxia): 13.82 ± 0.94 Post-exercise (hypoxia): 15.27 ± 1.22*	
Zuhl et al. (2015)	n= 8 endurance trained male and female participants	60 min running in the heat at 70% $\dot{V}O_{2max}$	Dual sugar probe (L 5g, R 2g) taken 30 min after exercise started Pre-exercise concentration: 0.022 ± 0.008 Post-exercise concentration: 0.06 ± 0.047*	No assessment

*** indicates significant difference to pre exercise value. # indicates significant difference to other trial.**

2.1.8 - Exercise-induced endotoxemia

Endotoxemia is a possible consequence of significant exercise-induced intestinal damage and permeability. Endotoxemia is a term used to describe the presence of circulating bacterial lipopolysaccharides (LPS). Once in circulation, LPS bind to LPS binding protein (LBP) and initiate systemic inflammation (Triantafilou et al, 2002; Diks et al, 2001). Clinically, an increase of $\geq$5 pg/mL from baseline in plasma or serum LPS with a reduction in anti-endotoxin antibody concentrations (e.g., IgG and IgM) is indicative of exercise-induced endotoxemia (Brock-Unte et al., 1988; Camus et al., 1998). Studies have assessed endotoxemia using a limulus amoebocyte lysate chromogenic assay with the greatest elevations found following exhaustive endurance exercise (Brock-Utne et al., 1988; Jeukendrup et al., 2000). An early study from Brock-Utne et al. (1988) found that 89% of exhausted athletes had endotoxin concentrations above the upper limit of 100 pg/mL following the comrade's marathon in South Africa (89.4km). Similar outcomes are supported in more recent studies, with a 37 and 71% increase in plasma LPS following a 24h ultra-marathon and Olympic course triathlon (Gill et al., 2015; Ashton et al., 2009).

Despite being used consistently as a measurement of exercise-induced endotoxemia, plasma LPS has several limitations. Firstly, the limulus amoebocyte lysate assay used to assess LPS is suggested to lack sensitivity (Novisky et al., 1998). Secondly, LPS is easily contaminated, has a short half-life and needs to be collected under LPS-free conditions. And thirdly, LPS fluctuates throughout the day making it difficult to measure in large cohorts or when numerous samples are collected (Novisky et al., 1998, Munford 2005). Alternatively, more recent studies have adopted the use of LBP and CD14 as surrogate markers of endotoxemia. LBP is an acute phase protein which attaches and transfers LPS to CD14 once released into circulation from the intestinal tract (Schimann & Latz, 2000). The activation of the LPS-LBP-CD14 complex leads to the secretion of pro-inflammatory markers, inducing systemic inflammation (Stoll et al., 2004). LBP is synthesised and released into the circulation in the presence of LPS and is considered as a surrogate biomarker for the activation of

LPS-induced innate immune response regarding its relatively long half-life (24-48 h) (Lepper et al., 2007). Both LBP and soluble CD14 (sCD14) are now recognised as clinical markers of endotoxin exposure (Sun et al., 2010) and have been shown be markers of Crohn's disease (Lakatos et al., 2011). Furthermore, more recent studies have used LBP or sCD14 as indirect markers of exercise-induced endotoxemia (Stuempfle et al., 2016, Pugh et al., 2019, Gaskell et al., 2021, McKenna et al., 2022).

The current literature implies that exercise of ≥2 h duration, as well as shorter higher intensity exercise (≥70% $\dot{V}O_{2max}$) in hot ambient temperatures are more likely to induce exercise-induced endotoxemia (Costa et al., 2017). This was confirmed in a recent field study, where a 40 pg/ml increase of circulatory endotoxin was reported after the first 37km stage of a 230km multistage ultra-marathon event in the heat (31-40°C), but then continued to rise to 80 pg/ml by the end of the event (Gill et al., 2015). Another study showed the impact of exercising at a high intensity in hot ambient temperatures as they reported greater elevations of LBP and sCD14 following repeated sprints in the heat (40°C) compared to cool (20°C) conditions (Wallet et al., 2022). To date, all studies reporting increases in circulatory LPS, LBP or sCD14 following exercise have included a protocol greater than 2 h in duration, performed at a high intensity (≥80% $\dot{V}O_{2max}$) or has been performed in an extreme environment (Brock Unte et al., 1988; Ashton et al., 2009; Gill et al., 2015; barbeiro et al., 2015; Stuempfle et al., 2016; Pugh et al., 2019, Gaskell et al., 2020; McKenna et al., 2022; Wallet et al., 2022) (Table 3).

The recovery time course of exercise-induced endotoxemia is unclear, though it is suggested to be dependent on the magnitude of response (Costa et al., 2020). Studies performed in a controlled environment mostly report a peak response immediately post- to 1 h post-exercise (Gill et al., 2016 Snipe et al., 2018; Snipe et al., 2018; Gaskell et al., 2020), returning to baseline within 4 hours. In comparison, exercise of a longer duration has been shown to cause a longer recovery time for LPS. Following a mountain based ultra-triathlon, LPS concentrations did not return to baseline until 24

hrs later (Jeukendrup et al., 2000). One consequence of elevated endotoxemia is the associated a systemic inflammatory response. Generally, strenuous exercise will increase inflammation and pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α). However, the inflammatory profile found following significant exercise-induced endotoxemia is reported to be like that of clinical sepsis (Costa et al., 2020).

Table 2.3. Primary research Studies reporting pre and post exercise Δ endotoxemia and correlations with GI discomfort. Data presented as mean ± SD unless stated otherwise.

Study	Participants	Exercise Stressor	Marker of Endotoxemia (Plasma LPS unless stated otherwise)	GI Discomfort
Yeh et al. (2013)	n= 15 recreationally active male and female participants	60 min running at 70% $\dot{V}O_{2max}$ in hot and cool environments	Cool environment : -1.1 pg.mL Hot environment : 5.0 pg.mL	No assessment
Nieman et al. (2006)	n= 25 male and female ultra-marathon runners	160 km ultramarathon race	0.1 pg.Ml	No assessment
Sessions et al. (2016)	n= 7 endurance trained male and female participants	60 min running at 70% $\dot{V}O_{2max}$ in 30°C	0.6 pg.mL	No assessment
Shing et al. (2014)	n= 8 male endurance runners	Running to fatigue at 80% ventilatory threshold in 35°C	4.0 pg.mL	No assessment
Jeukendrup et al. (2000)	n= 29 male and female triathletes	Mountain Ironman triathlon race	4.0 pg.mL	No assessment
Ashton et al. (2003)	n= 10 male physical education students	Incremental cycling test to exhaustion	10 pg.mL	No assessment
Pugh et al. (2019)	24 recreational runners (20 male; 4 female)	42.2 km on synthetic 400m running track, 16-17°C	Plasma CD14 Probiotic group : 5.9 µg.mL Placebo group: 5.4 µg.mL	Assessed but no relationship with CD14
Gill et al.,	n= 8 endurance trained male participants	120 min running at 60% $\dot{V}O_{2max}$ in 34°C	10.0 pg.mL	No assessment

Lim et al.,	n= 18 male endurance runners	Running at 70% $\dot{v}O_{2max}$ until core temp reaches 39.5°C or volitional exhaustion in 35°C	10.0 pg.mL	
McKenna et al. (2022)	n=9 recreationally active males	60 min cycling at 65% normoxic $\dot{V}O_{2max}$	Plasma LBP concentrations Normoxia : 0.46 µg.mL Hypoxia : 3.12 µg.mL	Significantly correlated to Δ LBP
Camus et al. (1998)	n= 9 male endurance runners	Marathon competition	16.0 pg.mL	No assessment
Barberio et al. (2015)	n= 8 endurance trained male participants	Running at 78% $\dot{V}O_{2max}$ (4mMol.L^{-1} blood lactate) until core temperature increases by 2°C or volitional exhaustion (~ 24 min) in 40°C	28.0 pg.mL	No assessment
Gill et al. (2015)	n= 19 male and female ultra-marathon runners	Multi-stage ultramarathon competition	40.0 pg.mL	No assessment
Gill et al. (2015)	n= 17 male and female ultra-marathon runners	24 hours continuous ultramarathon competition	122 pg.mL	No assessment
Stuempfle et al. (2016)	n= 20 male and female ultra-marathon runners	161 km ultramarathon competition	Plasma CD14 0.6 µg.mL	No assessment
Camus et al. (1998)	n= 12 male triathletes	Olympic course triathlon race	12.0 pg.mL	No assessment

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2.1.9 – The role of environmental conditions on GI damage, permeability and symptoms

It appears that GI perturbations occur during exercise in a dose-response relationship with either exercise duration or intensity. However, one other element that seems to have a significant influence is the environment, specifically ambient temperature. Like exercise intensity and duration, exercise-induced intestinal damage increases with the rise of environmental temperature (Snipe et al., 2017; Snipe et al., 2018). Increased intestinal damage, permeability endotoxemia was observed following continuous and sprint-based exercise in the heat (33-40^{o}C) but not in cooler environments (20-22^{o}C) (Yeh et al., 2013; Wallet et al., 2022). Exercising in the heat may also exacerbate GI symptoms as increases in core temperature were found to positively correlate with nausea and urge to regurgitate (Snipe et al., 2018). Furthermore, current evidence suggests that additional thermoregulatory strain induced by heat exposure exacerbates splanchnic hypoperfusion, intestinal ischemia and injury to the intestinal epithelium. Nevertheless, the contribution of thermoregulatory strain during exercise was challenged in a recent a study (Sheahen et al., 2018). Sheahen et al. (2018), attempted to isolate the impact of the heat by instructing participants to exercise at the same relative intensity in both hot (30^{o}C) and cool (20^{o}C) environments. They observed similar magnitudes in I-FABP between trials with no differences in core temperature. It is possible that no differences were found as the chosen temperature was not strong enough to elicit any additional changes. Regardless of whether it is the higher exertional or thermoregulatory stress that amplifies the GI response when exercising in the heat, it is imperative to find solutions to attenuate this response for benefiting performance and health.

Similar to exercising in the heat, ascending up to high attitude is associated with GI distress (Anand et al., 2006). When in hypoxic environments, sympathetic blood flow is increased (Fletcher, 2000), which may redirect blood flow away from splanchnic organs (Loshbaugh et al., 2006). Hypoxia will also induce hypoxemia, which will reduce oxygen saturation within the tissues and possibly the GI

tract. Together, this may disrupt the integrity of the GI tract and increase the risk of permeability, endotoxemia and GI discomfort. Exercising in hypoxia is becoming more common in athletic populations and military personnel (Czuba et al., 2017). Hypoxic exercise requires greater skeletal muscle demands and reduces arterial oxygen saturation (Joyner & Casey, 2014; Rowell et al., 1986; Roach et al. 2000). This may induce further splanchnic hypoperfusion and strain to the GI tract than exercising in normoxic conditions (Derikx et al., 2008). Indeed, moderate running (50-65% $\dot{V}O_{2max}$) in normobaric hypoxia induced greater amounts of GI damage and endotoxemia than a normoxic equivalent (Machado et al., 2017; Hill et al., 2020). This was supported by a more recent and extensive study by McKenna et al. (2022). McKenna et al. (2022) found greater concentrations of I-FABP, LBP and Claudin-3 after a 60-min cycling bout in hypobaric hypoxia than normoxia, indicating more GI damage, permeability and endotoxemia. Another niche element to this study was the inclusion of GI symptoms. Overall GI discomfort had a higher incidence and severity in hypoxia compared to normoxia. Additionally, overall GI discomfort and nausea significantly correlated with Δ I-FABP, Δ LBP and Δ Claudin-3 (McKenna et al., 2022), providing novel evidence that these markers are associated with GI symptoms when cycling.

It should be noted that all studies assessing the impact of hypoxia have performed exercise at an intensity based off a maximal oxygen uptake test performed in normoxia. Lower $\dot{V}O_{2max}$ have been reported at high altitudes (Calbet et al., 2003), meaning participants likely performed exercise at a higher % of $\dot{V}O_{2max}$ in the hypoxia trials. As exercise intensity has previously been shown to influence GI integrity (Pals et al., 1997), it is possible that the greater GI damage, permeability and endotoxemia is due to greater exertional strains than hypoxia. Furthermore, an investigation assesses whether a similar degree of GI damage, permeability and endotoxemia occur when exercise is matched to the environment is warranted. Despite this, the current literature regarding exercising in hypoxia should not be disregarded as it is very rare that an athlete would voluntarily reduce their intensity to match the environment. Therefore, identifying methods to attenuate GI disruption and symptoms is imperative for exercising individuals in hypoxia.

2.2 – Acute upper respiratory illness in athletes

To most people, the main role of a professional coach is to ensure an athlete is physically, technically, and tactically prepared for competition. However, a secondary role is to ensure the protection of their athlete's health and welfare, which will allow an athlete to train and perform effectively to be as successful as possible. After injury, acute illness is consistently shown to be the most common reason for an athlete to miss training or competition, with Upper Respiratory Tract Infections (URTI) being the largest contributor. The impact of URTIs on athlete availability and success was highlighted previously as URTI incidence was negatively correlated with annual training volume, implying that the less sick you are the more you can train (Martensson, Nordebo & Malm, 2014). Furthermore, this heightens the interest in developing methods to monitor acute illness, identify risk factors and to find possible avenues to reduce to onset of illness, particularly during important training periods and competition.

2.2.1 – The immune system

The immune system is a complex but highly organised system that is comprised of a network of cells, proteins, tissues, and organs that provide a potent, multi-layered defence against invading pathogens, viruses, bacteria, and other foreign elements. The various cellular and soluble elements that frame the immune systems defence against infection can be divided into the innate and adaptive arms of immunity. The innate immune response occurs immediately, acting as the first line of defence once a pathogen has been detected. The innate immune system comprises of physical and chemical barriers (e.g. the skin and mucosal membranes) and phagocytes (e.g. neutrophils, monocytes) which ingest microorganisms and other non-specific killer cells to remove them from the host.

A key response of innate immunity is the recruitment and activation of neutrophils at the site of infection (Witko-Sarsat et al., 2000). This response is initiated by the release of cytokines by

macrophages. Specifically, granulocyte and granulocyte-macrophage colony stimulating factors stimulate division of myeloid precursors on the bone, releasing millions of cells into circulation and resulting in neutrophil leucocytosis. There are two mechanisms at which neutrophils kill the cause of infection, one being oxygen dependent and one being oxygen independent. The oxygen dependent response involves the reduction of oxygen by an NADPH oxidase and influx of toxic oxygen metabolites, including hydrogen peroxide, hydroxyl radicals, and singlet oxygen. The oxygen independent route uses highly toxic cationic proteins and enzymes contained within the neutrophil cytoplasmic granules (Garred et al., 1995).

In contrast, the adaptive immune system provides the second barrier of defence. This system is highly specialised but takes longer to develop and deploy against the targeted pathogen. Unlike the innate response, the adaptive immune system works at a cellular level, mainly comprising of T and B lymphocytes. T and B lymphocytes proliferate and perform various duties in response to a pathogen, including antibody production, cytotoxic T cell killing and the development of T memory cells so that the necessary immune response can be performed when exposed to the same pathogen. Over time the adaptive immune system creates a catalogue of diverse lymphocytes, capable of binding to a unique pathogen associated molecule. The production of anti-bodies to a particular pathogen can take days or even weeks to develop, though this increases immunity to that pathogen. The most documented case is that of chicken pox as once an individual becomes infected, they are rarely reinfected in their lifetime.

The initial stage of adaptive immunity is when antigen-presenting cells, including dendritic cells, macrophages, or B cells detect, capture and process antigens. Specifically, dendritic cells upregulate major histocompatibility and costimulatory molecules and migrate to draining lymph nodes to interact with naïve T cells that are specific to that antigen to initiate an immune response (Iwasaki et al., 2004; Blum et al., 2013). The upregulated immune response and release of cytokines helps guide T cell differentiation into effector subsets (e.g. t helper cells, 1, 2 and 17 or regulatory T cells) (Jain &

Pasare, 2017). The resultant activation of CD8+ cytotoxic cells kill infected cells, while CD4+ helper t cells assist other immune cells, including B cells (Rastogi et al., 2022). Activation of B cells occurs via the detection of antigens through their B cell receptors. Once activated, B cells differentiate into plasma cells to allow the secretion of antibodies and memory B cells that streamline future responses if reinfection occurs (Sindhava et al., 2012; Rastogi et al., 2022).

2.2.2 – Prevalence of acute respiratory illness in athletes

To the concern of elite athletes and coaches, ~50% of medical consultations at the Olympic and paralympic games (Engebretsen et al., 2013; Engebretsen et al., 2010; Soligard et al., 2015; Soligard et al., 2014; Soligard et al., 2018), international football tournaments (Dvorak et al., 2011; Theron et al., 2013) and rugby union world cup (Schwellnus et al., 2012) were due to acute illness, with respiratory related illness being the most common (Dvorak et al., 2011; Mountjoy et al., 2010; Schwellnus et al., 2012; Engebretsen et al., 2012; Soligard et al., 2015; Derman et al., 2019; Derman et al., 2014)**.** In Olympic athletes ~70% of these episodes resulted in missed training or competition (Palmer-Green et al., 2013). Regarding the incidence of URTI in elite athletes, a recent meta-analysis conducted by the International Olympic Committee (IOC) reported an incidence of 5.9 episodes per 1000 days, equating to an average of 1.8 infections per year (Derman et al., 2022).

Team based sports, particularly those that include full contact offer unique challenges in regard to illness risk. Throughout training and a competition, players are continuously in contact with each other through high-intensity collisions and set-play scenarios (Weaving et al., 2019). During such physical contact, skin-on-skin abrasions and the transfer of bodily fluids may increase the likelihood of virus transmission and illness (Stacey & Atkins, 2000). Indeed, throughout a regular playing season the approximate URTI incidence per player was 4.1 in rugby union (Cunniffe et al., 2009), 2.3 in American football (Fahlman et al., 2005) and 1.5 in Australian rules football (Fitzgerald et al., 2019). In addition to viruses, contact sport athletes have been found to become colonised with bacterial infections faster and more frequently than no-contact sport athletes (Jimenez-Truque et al., 2017).

In addition to physical contact, high incidences of illness are likely due to regular international travel, frequent competitive schedules and the sharing of facilities and equipment. Concerningly, it has been reported that 25% of the surfaces within the changing facilities of a collegiate sports club tested positive with influenza (LaBelle et al., 2020), while other commonly shared items such as soaps, towel and water bottles pose a significant threat for the transmission of methicillin-resistant staphylococcus aureus (MRSA) infection (Nguyen & Mascola, 2005).

2.2.3 – Risk factors of acute respiratory illness in athletes

In the context of sport and exercise science, exercise immunology can be seen as a relatively new discipline having come to age in the latter stages of the twentieth century. Indeed, since 1990, over 5000 peer reviewed papers have been published, covering various themes and populations within sport and exercise (Simpson et al., 2020). It is well established that physical activity has numerous health benefits for human health, especially in regard to musculoskeletal and cardiovascular health (World Health Organisation, 2010). Likewise, regular exercise at a moderate intensity has also been shown improve immune responses to vaccination, increase anti-inflammatory properties and improve immune markers associated with disease states (Duggal et al., 2019; Gleeson et al., 2011; Hojman et al., 2018; Suzuki, 2019). Despite this, intense bouts of exercise and periods of greater training loadmay cause acute, temporary decrements in mucosal and cellular immunity, possibly increasing the risk of upper respiratory symptoms and impaired immune responses to vaccination (Peake et al., 2017). Together, this body of research has created the notion at which regular exercise ($\leq$ 45 mins) at a moderate intensity are 'immunoenhancing', whereas repeated bouts of long-lasting (> 120 mins), intense exercise can be 'immunosuppressive' (Simpson et al., 2020). This is best highlighted by the early concepts titled the 'J-shaped' and 'open window' hypothesises. The J – shaped hypothesis was developed by Nieman in the 1990s, whereby moderate intensity exercise can enhance immune function, but as training loadincreases the risk of upper respiratory tract infection also increases (Neiman, 1994). Despite this concept and the reported perturbations of immune

function following intense exercise, it is unclear whether this short period of immunosuppression is sufficient enough to compromise the ability of the host to resist infection (Martensson, Nordebo & Malm, 2014; Ekblom, Ekblom & Malm, 2006; Walsh et al., 2011; Campbell et al., 2018). One of the first challenges to this theory, was an epidemiological study from Ekblom and co-authors in 2006. Ekblom et al. (2006) failed to show any relationship between training volume in the 6-months preceding and upper respiratory infection in the 3-weeks following the Stockholm Marathon. This went against previous reports of heightened incidences following marathon and ultramarathon events (Peters & Bateman, 1983; Nieman et al., 1990). From then on, researchers have focused on other risk factors in addition to heavy exercise for illness and infection within athletic cohorts.

Upon the challenges against the 'open window hypothesis, the contribution of other stressors in the development of acute respiratory illness has had great consideration. Furthermore, risk factors primarily associated with the general population have also been suggested to impact immunity in athletic and military personnel. These include seasonal changes (winter & influenza) (Hellard et al., 2015; Svendsen et al., 2016), high levels of psychological stress, anxiety and depression (Drew et al, 2017) poor sleep (< 6 hours per night) (Wentz et al., 2018) and long-haul travel (Svendsen et al, 2016). Like physical exertion, heightened psychological stress and poor sleep influence immunity via the activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous-system. In the general population, dose-response relationships between both psychological stress and the common cold and sleep quantity,quality and the common cold are reported following intra-nasal inoculation with rhinovirus (Cohen et al., 1991; Cohen et al., 2009). Whereas increased training load only resulted in small increases in elite swimmers (Hellard et al., 2015) and no changes in elite cross-country skiers (Svendsen et al., 2016). Elite athletes in most sports will likely undergo long-haul domestic or international travel. Long-haul flying has been associated with increased incidence of URTI and gastrointestinal disturbances in elite athletes (Svendsen et al., 2016; Schwellness et al., 2012). Furthermore, it is conceivable that aspects of psychological wellbeing, sleep and travel contribute at least in part to the onset of acute illness during heavy periods of training.

For our immune system to function correctly and defend against invading viruses, pathogens, and bacteria there must be an adequate supply of energy provided from glucose, amino acids and fatty acids. Specifically, amino acids are necessary to produce important proteins such as immunoglobulins, cytokines, and acute phase proteins (Calder 2013). Furthermore, malnutrition of these proteins and nutrients is detrimental to our immunity, as shown by higher incidences of infection-related mortalities in developing countries (Woodward, 1998; Pelletier et al., 1995). Similarly, severe energy restriction can also influence immunity via the hypothalamic-pituitary-adrenal axis (Dhaber, 2014). Micronutrients may also play an important role in our immune defence, as iron, zinc and magnesium may influence nucleotide and nucleic acid synthesis, whereas vitamins C and E may enhance antioxidant defences (Calder, 2013). More specifically, vitamin D may also directly influence immunity by regulating gene expression (Calder, 2013). Aside from macro and micro-nutrients, fibres, probiotic, and prebiotics may affect our immune system by modifying the gut microbiota (Colbey et al., 2018) and zinc in oral lozenges may directly inhibit viral activity in the oropharyngeal region, both reducing the onset of upper respiratory illness (Hemilä, 2017).

2.3 – The human gut microbiota

2.3.1 – The gut microbiome in humans

Within the human GI tract lies the largest composition of microorganisms, collectively known as the 'microbiome'. In total, around 10^{14} bacteria reside in the tract, containing at least 100 times as many genes as the human genome (Gill et al., 2006; Vaishampayan et al., 2010). This microbial ecosystem harbours over 100 individual microbial species each with different functions that affect the health and behaviour of the human host (Eckburg et al., 2005). Due to peristaltic motility in the proximal small intestine and the anti-microbial effects of the luminal fluid (consisting bile, pancreatic secretions and acid in the stomach) the upper gastrointestinal tract contains relatively small numbers of bacteria (less than 10^6 organisms per ml in the upper gut), with gram-positive aerobes or facultative anaerobes such as lactobacilli and enterococci being the main inhabitants (Bures et al.,

2010; Quigley 2010). Conversely, the distal gut (colon) contains the greatest number of microbes, partly due to the slow transit time, favourable pH and substrate availability found in this section of the gastrointestinal tract (Penning et al., 2000). Together, these bacteria have a symbiotic relationship with the host conferring numerous physiological benefits, including the synthesis of vitamins and the fermentation of indigestible elements of host's diet such as plant polysaccharides (Backhed et al., 2005, Neish, 2009).

The composition of the gut microbiota largely influences overall gut health and subsequently, general human health. The gut microbiota confers numerous health benefits to the host, including protection against intestinal epithelium injury, nutrient absorption and regulation of host fat (Palmer et al., 2006). The bacterial profile can be altered by many factors including surgery, anti-biotics, ageing, diet and sedentary behaviour (Mitsuoka 1990, Backhed et al., 2005). The disruption of commensal bacteria has been associated with chronic diseases such as colonic cancer and inflammatory bowel disease (Gorbach et al., 1990, Rakoff-Nahoum et al., 2004).

2.3.2 - Bacterial Groups

There are four predominant bacteria phyla in the human GI tract, Bacteroidetes (Gram-negative), Firmicutes (Gram-positive), Proteobacteria (Gram-negative) and Actinobacteria (Gram-positive) and these have been shown to be variable in terms of species composition and distribution between individuals (Durban et al., 2010, Vrieze et al., 2010). Bacteroidetes typically occupy between 20 and 53% of total bacteria in different individuals, and the *clostridium* XIVa cluster (that falls under the Firmicutes phyla) is seen as the most dominant cluster, making up around 24-47% of total bacteria in the gut (Suau et al., 1999, Wang et al., 2003). The colon is dominated by obligate anaerobic bacteria whereas the facultative anaerobes (e.g. *Escherichia, Enterobacter and Enterococcus)* are less predominant, contributing less than 0.1% of the total bacteria dues to the strict anaerobic

environment of the distal gut (Backhed et al., 2004, Eckburg et al., 2005). These microorganisms possess a range of enzymes that can convert xogenous and endogenous compounds into a variety of different metabolites (Gorbach et al., 1990). Another function is the processing of components of the diet and the deposition of extracted energy into host adipose tissue (Backhed et al., 2005).

Resident intestinal bacteria play an important role in host resistance to pathogens, 'colonisation resistance' is among one of the ways of describing the process leading to elimination of orally introduced organisms that have not been limited or eradicated by the harsh environment of the upper gastrointestinal tract (van der Waaij et al., 1971). In a study on mono-associated gnotobiotic mice infected with *E. coli* versus conventional mice undergoing the same treatment, it was observed that there was a significant difference in the excretion rate of *E. coli*, it being more than 8 times higher in the gnotobiotic mice (Freter et al., 1983). This demonstrated that the primary mechanism behind the control of bacterial populations in the colon could be due to limitation of growth from competitive metabolic populations.

Adherence to the intestinal epithelial wall (competition for adhesion sites with the indigenous bacteria) and bacteria-bacteria interactions (competition for carbon and energy sources) are vital for a pathogento to successfully invade the gut in the presence of the indigenous microbes (Berg 1996, Sansonetti 2002). Substrates for bacterial transformation can reach the colonic microbiota through direct oral ingestion, bile secretion into the upper bowel, or secretion across the mucosa (Gorbach et al., 1990). The inability of a pathogen to invade and colonise the large intestine is aided by the lowering of the gut pH by organic acids (Bohnhoff et al., 1964, Cummings 1981). Short chain or volatile fatty acids (principally acetic, propionic and butyric acid) are greatest in the proximal colon formed from carbohydrates, proteins and peptides which are anaerobically fermented by colonic bacteria (Cummings et al. 1991, Macfarlane et al., 2003).

2.3.3 The Gut microbiome in athletes

Despite its complexity and large microbial composition, various elements of our lifestyle can alter the ecosystem within our gastrointestinal tract. The development of our gut microbiota begins during infancy and is highly influenced by the mode of delivery at birth, method of infant feeding and use of antibiotics (Li et al., 2018). Disruption in the development of the gut microbiota during infancy can be detrimental, increasing the risk of autoimmune, allergic and metabolic diseases later in life (Tanaka & Nakayama, 2017; Francino, 2014). After approximately three years, the gut microbiota stabilises, becoming more resilient and resembling that of an adult (Uhr et al., 2019). However, throughout adult life there are elements that can still influence the gut microbiota both positively and negatively.

It is well established that there are differences in microbiome composition and diversity between athletes and sedentary individuals. Observations show that athletes exhibit higher levels of alpha diversity and bacteria which have health-associated effects (Mohr et al., 2020), with differences being both predicted and attributed by athletic performance. Clarke et al. (2014) were one of the first to assess the differences between athletes and sedentary controls. Through 16s rRNA amplicon sequencing, a group of elite rugby union players were observed to have a higher diversity of gut microorganisms with 22 phyla detected compared to just 11 and 9 found in low and high BMI controls. Specifically, the elite athletes had a significantly lower number of Bacteroidetes and higher proportions of *Akkermansiaceae* (family) and *Akkermansia* (genus) than high BMI controls. Whereas, in comparison to low BMI controls, elite athletes had higher proportions of *Lactobacillaceae*, *Bacteroides* and *Lactobacillus* (Clarke et al., 2014). The authors also found that differences in gut diversity were positively correlated with protein intake and creatine kinase levels, implying that diet and exercise are drivers in microbiome diversity.

In addition to microbial diversity, there is also links between physical fitness and the functionality of the gut microbiota and its metabolites. Metabolic phenotyping and functional metagenomic analysis

of the gut microbiome in elite rugby players showed that athletes have a greater abundance of metabolic pathways than non-athlete controls, specifically those associated with amino acid and antibiotic biosynthesis and carbohydrate metabolism (Barton et al., 2017). This finding was accompanied by higher amounts of faecal metabolites, with greater concentrations of acetate, butyrate, propionate, isobutyric acid, isovaleric acid and valeric acid. These microbial produced SCFAs are associated with enhanced muscle turnover and numerous health benefits (Koh et al., 2016) (please see more information in section 2.4.4). Like their previous work, Barton et al. (2017) found that acetic, butyric and propionate acid all correlated with fibre and protein intake, whereas isobutyric, isovaleric and valeric acid were correlated with microbial diversity. This again shows that exercise and diet can influence the metagenomic and metabolic pathways in addition to the gut microbiome.

It is worth noting that the two aforementioned studies used elite rugby union players as their chosen athletes. Not all exercise is equal, sports can be categorised according to their static or dynamic requirements. For example, static sports are those that primarily involve intramuscular forces, maximal strength and can be measured by maximal voluntary contractions (e.g. weightlifting). Dynamic sports involve changes muscle length and joint movement, measured using maximal oxygen uptake and are associated with cardiovascular fitness (e.g. endurance running). Then there are some sports which combine both elements (e.g. football, rugby, field hockey). To gain a greater understanding of the impact of various sports on the human gut microbiome, faecal and urine samples were collected from 37 elite Irish athletes across 16 sports (O'Donovan et al., 2020). The authors found that Streptococcus suis, Clostridium bolteae, Lactobacillus phage LfeInf and Anaerostipes hadrus were found to be associated with those groups with a moderate dynamic component, which includes sports such as fencing. Sports that include high dynamic and low static components like field hockey were associated with greater counts of Bifidobacterium animalis, Lactobacillus acidophilus, Prevotella intermedia and F. prausnitzii, while Bacteroides caccae was

found to be associated with sports that include high dynamic and static components, such as rowing (O'Donovan et al., 2020).

2.3.4 - Benefits to the host

Aside from digestion, the intestinal epithelium provides a resilient barrier against resident microbes and invading pathogens (Sansonetti 2002). Commensal gut bacteria contribute significantly to mucosal immunity through the supply of anti-inflammatory signals to the mucosa and associated cells in the body (Barbosa et al., 2010). Toll like receptors (TLR) are an evolutionarily conserved family of receptors that function in innate immunity via recognition of conserved patterns in bacterial molecules. One main function of TLR is to identify microbial infections, which is critical for the initiation of inflammatory and immune defence systems (Gewirtz et al., 2001; Rakoff-Nahoum et al., 2004). The production of pro and anti-inflammatory cytokines assists the immune response to invading pathogens. Epithelial inflammatory chemokine secretion may be activated by both commensal and invading pathogens; therefore, it is only activated when bacterial flagellins (a protein contained in bacterial flagella) have translocated from the luminal domain (containing the indigenous population) to the basolateral membrane domain (Gerwirtz et al. 2001).

The intestinal mucosa has the function of specific immunological protection, largely mediated by secretory immunoglobulin A (Sansonetti 2002). Interaction of commensal bacterial products with host microbial pattern recognition receptors play a critical role in resistance to epithelial injury and presumably in other aspects of epithelial homeostasis, hence recognition of commensal bacterial products by the receptors used for defence against pathogens represents a critical component of symbiosis between host and indigenous microbes (Rakoff-Nahoum et al., 2004).

2.3.5 – Interactions between the gut microbiota and the immune system

Alongside extracting energy and the production of metabolites, the microbial residents within our gastrointestinal tract play an important role in the defence against local and systemic infections. If a pathogen were to infiltrate the GI tract, it must first overcome three barriers of defence, including the intestinal microbiota, the intestinal epithelial layer, and the mucosal system (Iacob, Iacob and Luminos, 2018). The main mechanism at which the microbiota defends against invading pathogens is referred to as 'colonisation resistance', whereby commensal and invading microbes compete for space and resources, resulting in the elimination of the invading microbes (Libertucci & Young, 2019; Lawley & Walker., 2013; Chiu et al., 2018). Commensal bacteria use signalling molecules through the mechanism called quorum sensing to continuously monitor the environment for invading microbes and to maintain gut homeostasis (Lazar et al., 2018). However, in some cases pathogens can also use quorum sensing to avoid these immune responses and increase pathogenicity (Iacob et al., 2018). The influence of the microbiota in preventing pathogen colonisation has previously been demonstrated in the mice (Freter et al., 1983), whereby gnotobiotic mice had 8 times the amount of *E.coli* than a conventional group of mice following exposure. Furthermore, alterations to the microbiota community may disrupt host immunity and result in low-grade inflammation, reduced colonisation resistance and an increased susceptibility to infection (Forgie, Fouhse & Willing, 2019).

Similarly to the gut microbiota, the intestinal epithelial barrier protects the host against pathogens and infection (Okumura & Takeda, 2017). As previously discussed, the intestinal barrier is a single-layer dynamic structure of tight-junction proteins, functioned to separate commensal bacteria in the gut and the external environment. Certain pathogenic bacteria can disrupt the intestinal barrier through the release of toxins (Libertucci & Young, 2019). In defence, the epithelial barrier is reinforced with a mucus lining that is directly exposed to the luminal microbial contents. Considered the initial innate response within the gut, this lining denies direct contact between pathogens and epithelial cells. The effectiveness of the epithelial barrier is coordinated through host and microbe interactions which can recognise microorganisms via pattern-recognition receptors (PRRs). PRRs consist of a large consort of extracellular and intracellular receptors that recognise specific microbe-

associated patterns (MAMPs) (Wiertsema et al., 2021). Typical PRRs can include toll like receptors (TLR), C-type lectin receptors (CLRs), nucleotide binding oligomerization (NOD)-like receptors (NLRs), and cytosolic sensors of DNA and RNA (Wiertsema et al., 2021). Once a pathogen has been identified the activation of PRRs results in the release of chemokines and cytokines which initiate a protective immune response (Fukata & Arditi, 2013). MyD88 is a key component of PRR signalling, linking PRR activation to the activation of NF-kB, which is a master regulator of inflammation (Wiertsema et al., 2021). In addition, intestinal epithelial cells produce numerous antimicrobial molecules and anti-microbial peptides (AMPs), including secretory IgA and defensins. These provide bactericidal, anti-inflammatory and anti-endotoxic properties (Vandamme et al., 2012), limiting the interaction between pathogens and the epithelium.

In addition to colonisation resistance, the gut microbiota can enhance the local immune response via the production of metabolites (Chen & Stappenback, 2019). Upon the absorbance of undigested dietary components, commensal bacteria produce metabolites such as short-chain fatty acids (SCFA), tryptophan metabolites and bile acid derivatives, which have all been shown to promote immunity and suppress inflammatory responses within the intestine. SCFAs can enhance immunity through several mechanisms, including histone deacetylase (HDAC) inhibition, G-Protein-coupled receptor (GPR) signalling, acetyl-CoA production, and metabolic integration. Additionally, SCFAs increase the production of AMPs and mucus by the epithelial cells, stimulate colonic T-cells and aid the proliferation of IL-C3 and IL-22 which help dampen inflammation within the intestinal barrier and gut microbiota (Schnupf, Gaboriau-Routhiau & Cerf-Bensussan, 2018; Chun et al., 2019).

It is becoming more apparent that in addition to its influence on local and mucosal immunity, the gut microbiota can orchestrate systemic immune responses. As previously discussed, the immune system comprises of two parts, the innate and adaptive immune system. The gut microbiota can influence both systems through a variety of mechanisms (Blander et al., 2017). One mechanism is through is its ability to influence T cell coordination. T cells are a major component of the adaptive

immune system with the key role of identifying antigens. The gut microbiota can affect the differentiation of T cell populations into T-helper (th) Th1, Th2 and Th17 cells or into T cells with a regulatory phenotype (Francino, 2014; Owaga et al., 2015). The regulation of T cells is predominantly driven by SCFA, with butyrate being the most influential (Arpaia et al., 2013). SCFA can also reprogram the metabolic activity of B cells and inhibit the generation of Th17 cells which can suppress the inflammatory response (Arpaia et al., 2013; Grainger, Daw & Wemyss, 2018).

In addition to T cell and adaptive configuration, the gut microbiota can also modulate the innate immune system. The gut microbiota can release signals which stimulate the lymphoid cells of the spleen, modulate neutrophil migration, activate macrophages and aid the maturation of natural killer (NK) cells (Schnupf et al., 2018; Francino, 2014; Owaga et al., 2015; Khosravi et al., 2014; Gorjifard & Goldszmid, 2016). Moreover, recent evidence also suggests that some bacterial species can regulate inflammatory responses via the decrease of plasma corticosterone, an anti-inflammatory steroid that dampens inflammation to mucosal damage (Menezes-Garcia et al., 2020).

In short, the gut microbiota has a heavy influence on both local and systemic immunity. Therefore, disruption to the gut microbiota may increase the risk of medical complications associated with local and or systemic inflammation. One relationship that has been extensively researched is the gut-lung axis. Individuals with asthma or other respiratory diseases are reported to have negative alterations within the gut microbiota (Russell et al., 2013; Russell et al., 2013; Korpela et al., 2016; Noverr et al., 2005; Arrieta et al., 2015). Due to this relationship and the impact that the gut microbiota has on innate and adaptive immunity, it is plausible that it is key in the development of upper respiratory tract infections. Probiotics and prebiotics have previously been shown to reduce the incidence and severity of URTI and gastrointestinal disturbances in the general population (Zhao, Dong & Hao, 2022; Hughes et al., 2011). Furthermore, targeting the gut microbiome through this method may be beneficial to athletes during training and competition.

2.4 – Impact of dietary interventions that target the gut and its resident microbes

Despite its complex structure, various elements can alter our gastrointestinal tract and enhance gastrointestinal functioning. Due to the influence of the gut microbiome on gastrointestinal health, dietary interventions that target this have received great attention. Interestingly, our dietary habits account for 20% of the gut microbiome with changes observed after just 48hrs of short and long-term dietary interventions (Sonnerburg & Bäckhed, 2016). Most foods are thought to impact the gut microbiome in some way, but fruit and vegetables rich in fructans and galatcoologiosaccharides (GOS) are found to be the most effective at increasing commensal bacteria (So et al., 2018).

2.4.2 – Probiotics and mechanisms

In addition to mass changes in diet, the gut microbiome can be altered using dietary supplements. The two which have received most attention are probiotics and prebiotics. Probiotics are defined as "live organisms which, when administered in adequate amounts, confer a health benefit on the host" (WHO, 2001). Probiotics are commercially available in various forms, including capsules, powder, or dairy and fermented products. The amount and type of bacterial strains within probiotics can vary between products, with some contain single strain or multi strain doses. However, the most widely used probiotics usually contain *Bifidobacterium, Enterococcus, Lactobacillus, Saccharomyces bouladii, and Escherichia coli* Nissle 1917 bacteria (Plaza-Diaz et al., 2019). The variations in probiotic strains have led to some reservations when evaluating the benefits of probiotics. Due to the complexity of the gut microbiome and the influence specific strains have, it is suggested that some benefits may be strain specific (Allen et al., 2017). Despite this, a recent Cochrane review revealed that probiotics can reduce the amount of people with at least one URTI by 24%, the amount of people to suffer with at least three URTI by 41%, the overall incidence of URTI by 18% and the duration of episodes by 1.22 days (Zhao, Dong & Hao, 2022). This has led to a clinical and

commercial surge in the use of probiotics, with probiotics being prescribed alongside antibiotics in some countries (Goshal et al., 2021).

One of the proposed mechanisms at which probiotics act is via the release of immunomodulatory and anti-inflammatory molecules which stimulate immune cells and help regulate inflammatory pathways (Pyne et al., 2015). Specifically, probiotic bacteria interact with epithelial cells, DC's, monocytes/macrophages and lymphocytes (D'Amelio & Sassi, 2018), potentially enhancing the immune response. Additionally, probiotics may increase IFN-Y production which may elevate IgA production through enhanced T lymphocyte activation (Cox et al., 2010). Although probiotics target the gut, the stimulation of the immune cells can travel to other mucosal sites including the respiratory tract (Glück & Gebbers, 2003). This may explain why probiotics may help reduce the onset of URS. Regarding gut health, probiotic bacteria can enhance tight junction protein production through the activation of TLR-2 (Lamprecht & Frauwallner, 2012). Once probiotics are consumed, TLRs within the gut cell surface initiate an inflammatory cascade which stimulates nuclear kB (NF-Kβ) transcription which can help preserve epithelial integrity. This is necessary for the maintenance of the intestinal barrier and to reduce the risk of increased intestinal permeability and endotoxemia (Roberts et al., 2016). Another mechanism is that probiotics may increase the competition for bacteria to attach to receptor cites, favouring commensal bacteria. Probiotics may do this through the reduction in luminal pH, increasing the competition of nutritional sources and the production of bacteriocin or bacteriocin like substances (Collado et al., 2010). Lastly, probiotics are reported to increase volatile acids, as shown by elevated levels of SCFAs in faecal samples. In a randomised control trial with healthy adults, a probiotic containing strains of L. gasseri CECT5714 and L. coryniformis CECT5711 increased the production of butyrate, propionate and acetate compared to a control group who consumed a yoghurt (Olivares et al., 2006). Similar findings have been reported in infant and elderly diagnosed with gastrointestinal illness (Wang et al., 2014, Scneider et l., 2005, Nagata et al., 2011, Nagata et al., 2016, Maldonado et al., 2010). Both in vitro and in vivo studies, have observed that the production of SCFAs is associated with numerous physiological, biochemical,

and molecular effects in the intestine, brain, muscle and other key organs (Canfora et al., 2015). For example, in the intestine, bifidobacteria produce acetate, which can enhance epithelial integrity and protect against infection (Plaza-Diaz et al., 2023).

2.4.3 – Probiotic in athletic populations

As previously stated, respiratory and gastrointestinal illness are the second most common reason for an elite athlete to require medical attention (Soligard et al., 2015; Soligard et al., 2013; Engebretsen et al., 2010; Engebretsen et al., 2013; Soligard et al., 2023). Numerous studies have evaluated the effects of a single-strain probiotic on URS. Cox et al. (2010) conducted a cross-over trial on healthy elite male distance runners for 28 days. Daily supplementation of a probiotic containing Lactobacillus fermentum VRI-003 resulted in a reduction in the number of days where URS were present (Cox et al., 2010). However, West et al. (2011) failed to replicate these findings following an 11-week intervention with the same probiotic in competitive cyclists. The authors observed that the load (duration x severity) of URS was reduced in males taking the probiotic but was increased in females taking the probiotic, leading the authors to state that there was too much uncertainty to establish a decisive outcome (West et al., 2011). Positive effects were found when active individuals administered a commercially available probiotic containing *Lactobacillus casei Shirota* for 16 weeks during the winter period (Gleeson et al., 2011). The probiotic group experienced fewer URS episodes which was accompanied by an increase in sIgA at 8 and 16 weeks, suggesting some modulation of the immune system. Interestingly, the same group failed to replicate these results in two further studies when using a *Lactobacillus salivarius* and the same *Lactobacillus casei Shirota* probiotic (Gleeson et al., 2012; Gleeson et al., 2016). The contradicting results from the authors may be due to the different strain used, the time of year and lower URS across the cohort. Despite this, in the latter study the authors did observe a reduction in plasma CMV and EBV antibody titers, which may benefit immune status (Gleeson et al., 2016). Elsewhere, a probiotic intervention of just 13 days lowered the cumulative days of URS when compared to a placebo (Komano et al., 2018). Implying

that starting the administration of probiotics in the weeks leading to a competition may be beneficial for an athlete.

Improvements in URS have also been reported following the administration of multi-strain probiotics. Strasser et al. (2016) observed a 2-fold reduction in URS episodes during a 3-month probiotic intervention containing *Bifidobacterium bifidum W23, Bifidobacterium lactis W51, Enterococcus faecium W54, L. acidophilus W22, L. brevis W63, and Lactococcus lactis W58*. A more recent study explored the effects of a 30-day probiotic supplementation on URS during the 7-days post marathon (Tavares-Silva et al., 2021). Despite a low number of participants (7-probiotic, 7-placebo), a probiotic containing *L. acidophilus* LB-G80, *L. paracasei* LPc-G110, *L. subp. Lactis* LLL-G25, *B. animalis subp*. *Lactis BL*-G101, and *B. bifidum* BB-G90 caused a 29% reduction in URS incidence (Tavares-Silva et al., 2021). To date, only two known studies have investigated the impact of probiotics in team sport settings. In a group of elite rugby union players, 14 out of 30 did not experience a URS or GI episode during the probiotic intervention, compared to only 6 on the placebo (Haywood et al., 2013). Similar improvements were not replicated in another study with elite rugby union players, possibly due to a low incidence in URS episodes (Pumpa et al., 2019). However, supplementation with a probiotic containing *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Bifiodbacterium lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* did maintain salivary alpha-amylase levels during an international competition period (Pumpa et al., 2019), suggesting a maintenance of immune function.

There have also been mixed results for GI discomfort when administering both single and multi-strain probiotics. During a 3-month intervention of lactobacillus rhamnosus probiotic, the duration of GI symptoms was reduced in the 2-weeks after a marathon race but not during the intervention period (Kekkonen et al., 2007). In another study using a single-strain probiotic, a 11-week intervention of lactobacillus fermentum failed to reduce GI symptoms during training in cyclists and

triathletes (West et al., 2011). A similar result occurred when active individuals underwent a 150-day intervention with a multi-strain probiotic (West et al., 2014). The small or insignificant effects of probiotics may be due to the ineffectiveness of the probiotic strains used, the criteria used to diagnose GI symptoms and generally low incidence of discomfort. In more recent research, results have been more encouraging. Pugh et al. (2019) observed a reduction in the incidence of moderate GI symptoms during the 3rd and 4th weeks of a 28-day multi-species probiotic intervention in marathon runners. GI symptom severity was also reduced in the final third of the marathon which enabled a maintenance in running speed (Pugh et al., 2019). Pumpa et al. (2019) found a reduction in the onset of GI infections in elite rugby union players and schreiber et al. (2021) noted an improvement in GI symptoms during cycling-based exercise following a 90-day supplementation with a multi-species probiotic.

Even though the intestinal barrier has been heavily linked to gastrointestinal disturbances, few studies have investigated the impact of probiotics on intestinal integrity following exercise. Following a crossover design, daily supplementation of *Lactobacillus salivarius* for 4-weeks reduced exercise-induced permeability as shown by lower sucrose concentrations (Axelrod et al., 2019). Interestingly, this was accompanied by alterations in the gut microbiome but no changes in inflammation or faecal zonulin concentrations. In another study, a 4-week supplementation of an Escherichia coli containing probiotic reduced I-FABP concentrations after a 60 min run at various intensities (60-80% $\dot{V}O_{2max}$) (Mooren et al., 2020). However, it should be noted that this study did not include a control or placebo trial and there was only a small difference in the ΔI-FABP (56 pg/mL) between the pre and post intervention visits. Pugh et al. (2019) failed to show any between group differences in I-FABP concentrations or intestinal permeability following a marathon. A similar finding was observed after a 120 min submaximal cycle and 100KJ time trial (Pugh et al., 2020). Unlike previous studies, both investigations used a 4-week supplementation of a multi-species probiotic. It is also possible that the intensity was not sufficient at increasing enough intestinal

damage to see a benefit. Participants were also fed maltodextrin throughout the 120-min cycle which may have dampened the effects of the probiotic.

It is apparent that there is some evidence supporting the use of probiotics in athletes, particularly for improving URS and GI symptoms. However, there are concerns as it is unclear which strain is most effective and whether the supplementation of probiotics can help reduce the onset of damage and permeability during exercise.

2.4.4 – Prebiotics and mechanisms

Likewise, to the supplementation of probiotics, the small addition of prebiotics to one's diet can substantially influence the human gut microbiome. The term "prebiotic" was originally defined in 1995 as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health" (Gibson & Roberfroid, 1995). Since then, the definition has evolved numerous times. More recently, the International Scientific Association for Prebiotics and Probiotics (ISAPP) expanded the definition to include the functionality of prebiotics, defining prebiotics as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al., 2017).

The key characteristics of a prebiotic are as follows: 1) non-digestible by endogenous enzymes in the human gut; 2) selectively fermented by specific genera/species of resident gut microbiota; and 3) that this results in a targeted increase in specific bacteria that confers health benefits to the host (Roberfroid et al., 2010). In theory, any food ingredient that avoids digestion in the upper GI tract and transits to the lower GI tact has the potential to be a prebiotic. Nevertheless, only a select few carbohydrates have repeatedly shown convincing evidence of prebiotic action. The three major carbohydrates used in the current literature are inulin, fructooligosccharides (FOS) and galactooligosaccahrides (GOS). Both FOS and GOS are insensitive to gastric acid and are immune to

hydrolytic enzymes in the upper GI tract; as such they can transit to the lower GI and stimulate resident bacteria. FOS and GOS are proven to follow the key prebiotic characteristics (Gibson 2004; Bouhnik et al., 2004), but GOS is reported to have a greater prebiotic effect than short chain FOS (Bouhnik et al., 2004). Although FOS and GOS are the most used, other non-digestible carbohydrates (glucooligosaccharides, isomaltooligosaccharides, lactosucrose, polydextrose, soybean oligosaccharides, and xylooligosaccharides) are receiving great attention, with promising evidence but not sufficient to class them as prebiotics (Bandyopadhya & Mandal, 2014).

The fermentation of prebiotics can alter the composition and improve function of the microorganisms within the gut microbiome through the provision of energy sources and the byproducts of the fermentation process (Flint et al., 2007). The most important by-products of prebiotic fermentation are SCFAs. Butyrate, propionate and acetate are the most abundant within the gut and have the most influence on gut composition. These SCFA's can act as substrates for other microorganisms, improving the growth and activity of other species (Belenguer et al., 2006). As most by-products are acids, they can also influence the environment within the gut. SCFAs can decrease the pH, with a one-unit alteration sufficient to change the composition of the gut microbiome (Walker et al., 2005; Duncan et al., 2009).

Elevations in SCFAs has been linked with various health benefits to the host. SCFA molecules are small enough to diffuse through the gut enterocytes and enter blood circulation, influencing the gastrointestinal tract and other systems in the body (Den Besten et al., 2013). One benefit of SCFAs is the modulation of immune cells. It is widely reported that butyrate can modulate macrophages (Liu et al., 2012; Maa et al., 2010; Chang et al., 2014), neutrophils (Kim et al., 2013), regulatory T cells, $CD4^+$ and $CD8^+$ T cells (Singh et al., 2014; Gurav et al., 2015) and DCs (Millard et al., 2002).

Another positive action of SCFAs is the protective effects on the gut paracellular permeability (Wang et al., 2012; Tong et al., 2016). Butyrate specifically, may be able to enhance and maintain epithelial integrity by increasing claudin-1 (Ma et al., 2012; Wang et al., 2014), and the redistribution of

occluding and zona occludens-1 in the cell membrane (Voltolini et al., 2012). Butyrate can also stimulate the goblet cells within the intestinal mucosa, this can improve the production and quality of mucus, which can protect against the infiltration of toxins, pathogens and allergens (Liang et al., 2022).

2.4.5 – Prebiotics and reducing URS and GIS

Similarly to probiotics, the indirect effects of SCFAs on immunity and intestinal barrier integrity has highlighted the potential for prebiotics to reduce the risk of acute URS and GI illness. Specifically, *Bifidobacterium* sp. appear to be very effective at fermenting starch, fructans (Belenguer et al., 2006) and producing SCFA's. Inulin, GOS and FOS have been shown to have bifidogenic effects at smaller doses (Costabile et al., 2010, Ramnani et al., 2010; Eli et al., 2008; Bouhnik et al. 2004) making them an ideal prebiotic. Arslanoglu et al. (2007) found that in healthy infants, a 6-month supplementation of formula with the addition of GOS and FOS (8g/L) reduced the incidence of URS compared to a group consuming standard infant formula. This finding was then replicated in a group of healthy infants with a parental history of atopy (Arslanoglu et al., 2008). More recently, a 4-month treatment of GOS (7.5g/day) was also able to reduce the incidence in healthy infants (Paganini et al., 2017). A lower dose of GOS (3.6g) and Beta-glucan (26 mg) was able to reduce the duration as well as the incidence of URS during a 28-week supplementation in healthy children aged 3-4 years (Li et al., 2014). These findings suggest that prebiotics are beneficial in younger ages when the gut microbiome is in the early stages of development.

In adult populations there has been an inconsistency in results. Langkamp-Henken et al. (2004) found that a relatively low daily dose of FOS (4.41g) for 6-months reduced the duration of URS but not the incidence. Improvements have also been reported in university students following an 8-week supplementation of GOS (Hughes et al., 2011) and partially hydrolysed guar gum in hospital patients (Takahashi and Kozawa, 2021). In contrast, a larger dose of xlyooligosaccharide (8g/day) had no effect on URS incidence in healthy adults (Childs et al., 2014). Unlike other studies, this study

adopted a cross-over design. Although this design can be viewed as more robust, in this case it is possible that a 4-week washout was not sufficient for the gut microbiome and immunological effects to have returned to pre-intervention state. Nevertheless, a randomised control trial demonstrated null effects of a rice bran exo-polymer based prebiotic and a tea beverage containing maltodextrin on URS (Choi et al., 2014). The inconsistency within the current literature implies that not all prebiotics may have the same impact on URS. GOS and FOS may be most effective as they have greater bifidogenic effects. The literature also suggests that in adult populations, prebiotics may reduce the duration of URS rather than the incidence. Which may explain why studies that only investigated the incidence did not find any positive results.

Regarding gastrointestinal symptoms, most studies have focused on individuals suffering from irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and other gastrointestinal disorders. A metanalysis concluded that overall, prebiotics do not affect response, GI symptoms or quality of life in patients with IBS and other functional bowel disorders (Wilson et al., 2019). However, the authors did note that the findings between studies varied as non-inulin type fructan prebiotics may be beneficial. For example, a 12-week supplementation of partially hydrolysed guar gum (6g/day) and a 43-day FOS intervention (5g/day) both improved GI symptoms and intensity in IBS patients and individuals who suffer from digestive disorders respectively (Niv et al., 2016; Paineau et al., 2008). Nevertheless, two other studies failed to find any improvements in IBS symptoms following a 4-week (6g/day) and 6-week (20g/day) supplementation of FOS (Hunter et al., 1999; Olesen et al., 2000). In addition to the type of prebiotic, the ideal dose of prebiotic is unclear and may partly explain the discrepancies between studies. Indeed, there is some evidence to suggest that higher doses may have a negative effect on symptoms (Benjamin et al., 2011; Joossens et., 2012).

Athletes and exercising individuals face numerous stressors that may put them at a greater risk of respiratory illness and GI perturbations. Based off the literature in infants and adults, prebiotics may

ameliorate some of these risks like that of probiotics. However, unlike probiotics, only one know study has investigated the effects of prebiotics on upper respiratory illness or GI perturbations in athletes. Specifically, this study compared the effects of a prebiotic mixture containing GOS, FOS, inulin, polydextrose, strawberry powder and maltitol on the incidence and severity of URS in footballers compared to a synbiotic containing the same prebiotic mixture and a multispecies probiotic (Zhang et al., 2023). After a 6-week supplementation, URS incidence and duration was lower in synbiotic group compared to the prebiotic group. Interestingly, sIgA was also increased in the synbiotic group but not the prebiotic group, suggesting an immunological effect of the synbiotic but not the prebiotic. However, in this study there is limited information provided on the assessment of URS, the prebiotic supplement contains low amounts of various prebiotic types, and the lack of a control group hides any potential benefits of the prebiotic group. Thus, stronger research is needed.

Beta glucans are nondigestible carbohydrates found within the cell membranes of yeast, fungi, and oats. Although they are not technically a prebiotic, Beta glucans can surpass digestion in the small intestine and reach the large intestine where they can have greater bifidogenic effects than inulin (Wang et al., 2020). Twenty-eight days of supplementation with yeast beta glucan caused a 37% reduction in the number of URS days within 7-days post marathon compared to placebo (Mcfarlin et al., 2013a). The same beta glucan was shown to increase sIgA by 32% following a 10-day intervention (Mcfarlin et al., 2013b), suggesting it may have immunomodulatory effects that help reduce the onset of URS following strenuous exercise. In another study, 12-weeks supplementation of beta glucan from mushroom *Pleurotus osteratus* similarly reduced URS in top level athletes from a range of sports (Zhang et al., 2021). Clearly further investigations are needed to assess the impact of different prebiotics in sport.

One type of prebiotic which has received great attention is galactooligosaccharides (GOS). GOS are very resistant to heat and acidity and have been shown to be more bifidogenic than the more

commonly used FOS (Bouhnik et al., 2004). GOS is formed by enzymatic treatment of lactose by β-galactosidase to produce several oligomers of different chain lengths (Prenosil, Stuker & Bourne, 1987). It can be produced from lactose in cows' milk, but the main raw material is obtained from whey-derived lactose. GOS is then produced by β-galactosidases that have transgalactosylation activities, which results in the formation of 4- or 6-galactosylactose, oligosaccharides, transgalactosylated disaccharides and non-reducing oligosaccharides (Ahngus, Smart & Short, 2005).

As GOS is manufactured from raw materials, the specificity of its actions can be manipulated. One way is by including β-GOS synthesised by bifidobacterial (Macfarlane, Steed & Macfarlane, 2008). The prebiotic used in the current thesis was produced using a novel strain of *Bifidobacterium bifidum* NCIMB 41171 isolated from a faecal sample from a healthy human volunteer with an ability to express β-galactosidase activity was used in synthesis reactions to produce GOS from lactose (Tzortzis, Goulas & Gibson, 2005). This product is commercially available and marketed as BiMUNO by Clasado and referred to as a trans-galactooligosaccharide (B-GOS). BiMUNO has been shown to increase the number of bifidobacteria in vitro and to a greater effect that inulin in vivo (Tzortzis et al., 2005).

The bifidogenic effects of BiMUNO were assessed in humans following a 7-day intervention of either 3.6g/day or 7g/day (Depeint et al., 2008). Bacteria numbers were determined using fluorescent in situ hybridization in stool samples collected before and after each intervention. Just 7 days treatment of BiMUNO was sufficient to increase bifidobacteria numbers, with the 7g/day treatment showing greater bifidogenic effects than a previous commercially available GOS supplement. Additionally, a dose response occurred between bifidobacteria number and the dose of B-GOS used. Furthermore, this study showed that B-GOS may provide greater bifidogenic effects than other commercially available GOS and that higher doses have a greater effect.

Regarding the effects of B-GOS on health outcomes, one study found that a 10-week supplementation with B-GOS increased the numbers of beneficial bacteria, particularly

bifidobacteria in a group of elderly volunteers (Vulevic et al., 2008). The alterations in the microbiome were associated with significant increases in NK cell activity and IL-10 production, and reductions in pro-inflammatory cytokines IL-6, IL-1β and TNF-α. In another study, a 3-week intervention with B-GOS improved exercise-induced bronchoconstriction in asthmatics (Williams et al., 2016). This was accompanied by a reduction in pro-inflammatory cytokines both in the asthmatic and control group. In a more recent study, a smaller dose of B-GOS (2.75g/day) for 2-weeks reduced bloating, flatulence and abdominal pain in general volunteers who presented GI symptoms during a short screening period (Vulevic et al., 2018).

Together, these studies suggest that B-GOS has the potential to alter the gut microbiome and provide numerous health benefits, including the reduction in respiratory and GI associated symptoms. This makes it an ideal supplement to target the perturbations faced by athletes. Another benefit is that in contrast to probiotics, rather than introduce live microbes to the gut, B-GOS targets probiotic bacteria that already inhabit the large intestine. Meaning it does not need to compete against inhabiting bacteria and has higher chance of transiting to the gut. Furthermore, it can be argued that prebiotics are more efficient and practical at influencing the gut microbiome than probiotics. However, this is would only be the case if microbiome contains commensal bacteria. If the microbiome is not present due to disease, age or antibiotic use the probiotics or synbiotics may be a more effective option.

2.4.6 – Turmeric and Curcumin

It has become apparent that other dietary supplements with a history of improving health may do so through modulation of the gut microbiome. Turmeric is a natural ingredient acquired from Curcuma Long.L, a tuberous herbaceous plant in the ginger family. As the plant grows in tropical climates, it has been a staple ingredient in various meals from Iran, India, China, Malaysia, and China (Gupta et al., 2013). In addition to its role as a food ingredient, turmeric has been widely used for medical treatments of various diseases for at least 2500 years in Asian countries (Gupta et al., 2013).

Specifically, it is the curcumin component of turmeric that is suggested to provide the various health benefits. Curcumin is a lipophilic polyphenol substance that constitutes 2-5% of the turmeric powder (Jurenka, 2009; Deogade & Ghate, 2015).

Curcumin was defined as “substance that gives the yellow colour” by Vogel and Pelletier approximately 200 years ago. In the mid 1900’s, curcumin was state to be a biologically active ingredient, providing antibacterial properties and thus, be effective at preventing Staphylococcus aureus, Salmonella paratyphi, Mycobacterium tuberculosis, and Trichophyton gypseum types. Curcumin is also proposed to have cholesterol-lowering, antidiabetic, anti-inflammatory and antioxidant properties. This has led to its use in preventing diseases such as cancer, autoimmune, neurological, metabolic, lung, liver, and cardiovascular diseases (Gupta et al., 2013; Prasad et al., 2014).

Recent evidence suggests that curcumin and turmeric may have effects on the gut microbiome. In rats fed a high fat diet, curcumin dietary supplementation altered the gut microbiome to that of a leaner phenotype and reduced the endotoxemia and intestinal inflammation associated with the high—fat diet (Feng et al., 2017). In another study, the supplementation of curcumin partially helped the restoration of the gut microbiome in ovariectomised rats (Zhang et al., 2017). Specifically, 12-weeks of curcumin supplementation was shown to reduce both *Anaerotruncus* and *Helicobacter pylori* which are associated with elevated stress and gastric cancer in murine models (Golubeva et al., 2015; Ohtani et al., 2007). Furthermore, it is possible that curcumin supplementation can positively influence the gut microbiome.

The impact of turmeric and curcumin on the human gut microbiome is less clear. Peterson et al. (2018) has conducted the most comprehensive study in humans to date. Fourteen adult participants underwent an 8-week supplementation with either turmeric (1000 mg turmeric root, 1.25 mg black pepper), curcumin (1000 mg curcumin C3 complex, 1.25 mg black pepper) or a placebo. At baseline, week 4 and week 8 a faecal sample was collected for the analysis of the gut microbiome via 16S

rDNA sequencing. The number of taxa detected in the study ranged from 172 to 325 bacterial species. At post-treatment, a 15% reduction in bacterial species was detected in the placebo whereas the turmeric and curcumin group had increased by 7 and 69% respectively (Peterson et al., 2018). Responsive subjects within the turmeric and curcumin groups had a defined signature which included uniform increases *Clostridium spp., Bacteroides spp., Cronobacter spp., Enterobacter spp., Enterococcus spp., Klebsiella spp., Parabacteroides spp.,* and *Pseudomonas spp*. Several of these species are linked to health benefits including anti-inflammatory effects, enhanced crosstalk with the immune system and the production of SCFAs (Guo et al., 2020; Hooper et al., 2002).

It is also possible that turmeric and curcumin supplementation could help protect the gastrointestinal barrier against ischemic induced injury. In vitro and in vivo studies have shown that curcumin can provide anti-inflammatory via the detoxification of bacteria derived LPS and subsequent attenuation of IL-1β signalling. As a result, this can inactivate p38 MAPK, inhibit NF-$_k$B and enhance the organisation tight junction proteins such as zonula occludens-1, claudin-1, and claudin-7 (Ghosh et al., 2018; Song et al., 2010; Tian et al., 2016; Wang et al., 2017). Despite this, only one known study has assessed whether turmeric and/or curcumin supplementation influences the intestinal barrier during exercise in humans. The authors found that there was less exercise-induced GI damage and inflammation following a 60-min treadmill run at 65% $\dot{V}O_{2max}$ after a 3-day curcumin supplementation (500mg/day) than placebo (Syzmanski et al., 2017). Specifically, plasma I-FABP increased more from pre to post (87%) and 1 hour-post (33%) during the placebo arm than curcumin (58% and 18%) respectively. IL-1RA also increased more from pre to 1 hour-post in placebo than curcumin (153% vs 77%) (Syzmanski et al., 2017). Both pre to post and 1hour-post TNF-α and IL-10 were only increased during the placebo arm, not curcumin (Syzmanski et al., 2017). This suggests that curcumin may ameliorate exercise GI damage through its anti-inflammatory properties. In rats, a 2-day administration of curcumin was able to ameliorate intestinal ischemia/reperfusion induced damage with the dampening of TNF-α and upregulation of zonulin-1 cited as the key mechanisms (Tian et al., 2016).

As discussed previously, curcumin supplementation can alter the gut microbiome. Sözen et al. (2015) showed similar anti-inflammatory effects of a smaller dose (20mg/kg/day) for 3-days in rats. Following a 60 min ischemic and 120 min reperfusion challenge, less circulatory concentrations of TNF-α, IL-6, IL-1β and CRP were observed in the rats under the curcumin condition (Sözen et al., 2015) suggesting an anti-inflammatory effect. Interestingly, the proposed mechanism for the anti-inflammatory response was the reduction in bacterial translocation across the membrane. Furthermore, it is plausible that alterations to the gut microbiome may have reduced pathogenic bacteria in favour of commensal bacteria. In summary, turmeric and specifically curcumin may provide anti-inflammatory, anti-oxidative and cause alterations to the gut microbiome which may all individually or collectively help maintain barrier integrity to ischemic stress. However, the lack of research studies in humans, particularly in response to exercise stressors means further research is needed to assess the full potential of turmeric and curcumin.

2.4.7 Vitamin D

As previously discussed, upper respiratory illness is a key issue in athletic populations. Vitamin D_3 supplementation has received wide attention in the athletic population as vitamin D sufficiency is a prevalent issue among athletes such as swimmers (Dubnov-Raz et al., 2015), footballers (Hamilton et al., 2014), wrestlers (Barcal et al., 2016), and taekwondo athletes (Jung et al., 2018). Concerningly, vitamin D deficiency has been negatively associated with URTI in British adults (Berry et al., 2011), while vitamin D deficient athletes are more likely to exhibit URS than athletes with optimal vitamin D concentrations (He et al., 2013). This is likely because vitamin D affects innate and adaptive immunity through its VDR action (Barker et al., 2014; Gombart et al., 2005; Wrzosek et al., 2013). Specifically, the activation of TLR has been linked with vitamin D-mediated AMP production (Krutzik et al., 2008). Calcitriol (1,25(OH)2D3) is a hormone and active form in vitamin D which is synthesised in the mucosal lining of the intestine, lung, bone tissue, skin epithelium and parathyroid glands. Once activated, 1,25(OH)2D3 interacts with VDR to induce the production of AMPs including sIgA

and plasma cathelicidin (Guo et al., 2014). Research has shown that both sIgA and plasma cathelicidin positively correlate with vitamin D status in athletes (Berry et al., 2011).

A meta-analysis based off 46 RCT studies revealed that a daily dose of 400-1000 IU of vitamin D has a small but protective effect against URTI in the general population (Jolliffe et al., 2021). Through another meta-analysis, Autier et al. (2017) also found a benefit of vitamin D on URTI, but majority of studies included children or medical patients. The impact of vitamin D in exercising individuals is less clear, however there are several promising studies. For example, in a group of a vitamin D-insufficient taekwondo athletes, a 5000 IU/day supplementation of vitamin D_3 for 4-weeks during winter training was able to reduce the burden of URTI with no improvements in the placebo group (Jung et al., 2018). A more comprehensive study in the military firstly found that 21% of military personnel were vitamin D insufficient during the winter months and that the vitamin sufficient personnel were 40% less likely to suffer from a URTI (Harrison et al., 2022). And secondly, that compared to the placebo group, the daily administration of vitamin D_3 for 8-weeks (1000 $IU \cdot d^{-1}$ for 4 wk and then 400 $IU \cdot d^{-1}$ for 8 wk) helped attain vitamin D sufficiency and reduce the severity and number of days that a URTI was present (Harrison et al., 2022). The authors did not find any alterations in either sIgA or cathelicidin, suggesting that the reduction in URTI burden may have been due to a reduction of inflammation during the infection rather than a direct effect on innate immunity (Walsh 2019; Ayres & Schneider, 2012).

There is growing evidence that vitamin D also influences the gut microbiome and gut health (Waterhouse et al., 2019). Indeed, four out of five studies have observed alterations in the gut microbiome during vitamin D supplementation (Bashir et al., 2016, Canteral et al., 2015, Kanhere et al., 2018; Shi et al., 2017), whereas vitamin D deficiency results in greater intestinal barrier disruption and inflammation (Assa et al., 2014). Vitamin deficiency has also been associated with an increased risk of inflammatory bowel disease (IBD) (Adrizzone et al., 2011), though its role in the development of intestinal inflammation is unclear. Specifically, it appears that the nuclear receptor

for vitamin D (VDR) are significant to its interactions with the gut microbiome and barrier. Colonic epithelial VDR expression is reduced in IBD patients (Liu et al., 2013), and VDR gene-knockout mice develop greater intestinal inflammation (Froicu et al., 2003). Through an extensive analysis of the genome-wide hots microbiota associations, Wang et al. (2016) observed that a variation of human VDR gene "generates" the gut microbiome, establishing the association between vitamin D, VDR and the human gut.

Vitamin D can contribute to gut homeostasis by maintaining intestinal barrier integrity and through healing of the intestinal epithelium (Nicholson et al., 2012). As previously mentioned, vitamin D deficiency results in greater intestinal damage and an increased risk of IBD. Assa et al. (2014) observed stark differences in vitamin sufficient and deficient mice after challenged with enterohemorrhagic Escherichia coli O157:H7. Mice that were fed a non-vitamin D diet experienced a decrease in intestinal barrier function and permeability with no alterations in the vitamin-D fed group. Vitamin D may protect the intestinal barrier through the expression of VDR-associated intracellular junction proteins (i.e. occludin, claudin, vinculin, and zonula occludens (ZO-1, ZO-2) which connect the epithelial cells within the intestinal barrier (Zhang et al., 2013). Furthermore, the influence that vitamin D and VDR has on innate immunity, the gut microbiome, intestinal barrier, and inflammatory response make it an intriguing treatment for reducing acute respiratory and GI illness, and exercise-induced GI damage. But, likewise to turmeric/curcumin, the lack of studies in exercising humans needs addressing in future research.

2.5 General Summary

Athletes face various stressors that can increase the risk of gastrointestinal and respiratory symptoms. Continuous and high-intensity intermittent exercise causes a redirection in blood flow which shunts blood away for the splanchnic region, resulting in splanchnic hypoperfusion and ischemia (van Wjick et al., 2011; Pugh et al., 2017). Such ischemic stress can damage the intestinal barrier, disrupting the epithelial and tight junction protein structures. Significant damage can lead to heighten permeability and the translocation of endotoxins across the intestinal lumen. Once in circulation, these endotoxins (LPS) initiate local and systemic inflammation which can impair GI function and the onset of Gastrointestinal symptoms. Gastrointestinal symptoms are reported by 86% of athletes from a range of sports (Pugh et al., 2017), with 14% of team-based athletes citing gastrointestinal symptoms as the cause for impaired performance (Wilson et al., 2023). Exercise in both the heat and hypoxia have been shown to exacerbate GI damage and the onset of symptoms compared to when performed in normal conditions.

Heavy exercise, sleep disturbances, poor diet and the sharing of facilities can all increase the risk of an athlete experiencing upper respiratory symptoms or illnesses. Evidence shows that intense exercise can acutely lower mucosal immunity as shown by reductions in sIgA concentrations (Walsh et al., 2002; Laing et al., 2005). Potentially making them more susceptible to infection. Like gastrointestinal symptoms, performing such exercise in the heat can exaggerate this response. Upper respiratory illness is the second most common reason for an athlete to require medical attention. Clearly suitable interventions that can ameliorate the prevalence and burden of upper respiratory and gastrointestinal symptoms are warranted in athletic populations.

The human gut microbiome is a dense ecosystem containing trillions of bacteria. Collectively these bacteria influence our gastrointestinal health, function, and immune system. This indicates that the gut microbiota may be a significant therapeutic target in the prevention of URS and GIS in athletic populations. Positive manipulation of the gut microbiota can be achieved through the feeding of pre

and probiotics, and possibly the administration of curcumin and vitamin D containing supplements. Probiotics have been shown to reduce URS in athletes, yet the same consistency has not been shown for reducing GIS. It is also unclear which type of probiotic bacteria is most beneficial for athletes. Furthermore, alternative supplements that target the gut microbiome warrants further investigation to establish if this manipulation can help maintain gut function and reduce the burden of acute URS and GIS in athletes.

Chapter 3 - General Methods

3.1 Location of testing and ethical approval

All exercise and biochemical analysis for study 1 and 4 was conducted in the physiology and biochemical laboratories of the Sport and Exercise Science Department, Nottingham Trent University. For study 2, data and samples were collected at the training ground of a professional football club from league one and the stadiums where competitive fixtures were played. All analysis was performed at Nottingham Trent University. Experimental visits for study 3 were carried out at the training ground of a professional rugby union club from the English Gallagher Premiership, with all analysis was performed at Nottingham Trent University.

3.2 Participants

Participants were recruited through adverts placed in Nottingham Trent University, local sports clubs, and leisure centres. Before recruitment started all studies were approved by the Nottingham Trent University Human Ethics Committee. All participants were given information on the nature of each experimental study both in writing and verbally before providing written informed consent. Participants completed a self-reporting medical questionnaire to confirm they were free from illness, had no history of gastrointestinal disorders and were not currently taking any pharmaceuticals. Prior to first experimental visit of study 1 and 4, participants completed a 24 h food diary which was then replicated for all subsequent experimental visits. Participants were also instructed to refrain from intense exercise (48 h prior), alcohol (24 h prior), caffeine (12 h prior) and arrive at the laboratory at least 2 h post-prandial. Specific participant characteristics (i.e. body mass, height, age) are described in each study chapter.

3.3 Assessment of cardio-respiratory measures

3.3.1 Assessment of respiratory compensation point (RCP) and $\dot{V}O_{2peak}$

Respiratory compensation point was assessed using an incremental exercise test on a motorised treadmill (HP Cosmos, Germany). Oxygen uptake and carbon dioxide production were measured continuously on a breath-by-breath basis using a metabolic cart (Vyntus CPX, Carefusion, Germany). The test commences at 8 $km \cdot h^{-1}$ at a 1% gradient, followed by an increase in speed of 1 $km \cdot h^{-1}$ every minute. After 1 minute at 18 $km \cdot h^{-1}$, treadmill speed was fixed, and the gradient was increased by 1% each minute until volitional exhaustion. Once breath by breath data was averaged over 10s, $\dot{V}_E$ and $\dot{V}CO_2$ were plotted to determine the RCP. $\dot{V}O_{2peak}$ was determined from the mean of the last 10s of the final minute stage.

3.3.2 Assessment of lactate Threshold

Lactate threshold was determined from an incremental running speed lactate test on a motorised treadmill (HP Cosmos, Germany). The test involved a series of 3-minute stages performed at a 1% incline. The first stage started at a speed of 9 $km \cdot h^{-1}$ and increased by 1 $km \cdot h^{-1}$ after each 3-min stage. At the end of each stage there was a 30-second break for the collection of a fingertip capillary blood sample for the assessment of lactate. The participant would continue until the lactate threshold was achieved, which was defined as the fastest speed with less than a 1 $mmol.L^{-1}$ increase in blood lactate concentration above the preceding levels (Astrand et al., 2003).

3.4. Anthropometric Measurements

Body mass was measured using a calibrated electronic scale (SECA 877 Scale, SECA, Birmingham, UK) to the nearest 0.1 kg). For study 2 and 3 participants wore lightweight clothing and were, for study 1 and 4 participants weighed their nude body mass privately. Height was measured using a portable stadiometer (SECA stadiometer, SECA, Birmingham, UK) with participants standing bare foot, with

heels together, their back up against the stadiometer, looking straight ahead. Participants were asked to take a deep breath until the headboard touched the top of their head.

3.5. Perceptual Scales

To assess Rating of perceived exertion (RPE) a scale ranging from 6-20 was used (Borg, 1962). Thermal sensation was measured using a 0 (Unbearably cold) to 8 (Unbearably hot) scale (Young, Sawka, Epstein, Decristofano, & Pandolf, 1987). Rating of fatigue was measured using a 0 (not fatigued at all) to 10 (total fatigue & exhaustion-nothing left) scale (Micklewright, St Clair Gibson, Gladwell & Al Salman, 2017). Global GI symptoms were measured on a 0 (no problem at all) to 9 (worst its ever been) scale relating to stomach upset, nausea, bloating, heartburn, flatulence, burping and other similar symptoms (Pugh et al. 2017).

3.6. Hydration

Urine osmolality was assessed before and after exercise in the study described in chapter 7 using a handheld osmometer (Osmometer-Osmocheck™; Vitech Scientific Ltd, West Sussex, UK) to ensure participants arrived in a hydrated state. Firstly, a sample of distilled water was pipetted onto the osmometer to recalibrate. Once the water had been removed, a sample of urine was pipetted onto the osmometer and then osmolality was assessed. All samples were assessed in duplicate. A value under 800 mosmol.kg^{-1} was considered hydrated (Perrier et al., 2015). If a sample was above 800 mosmol.kg^{-1}, the participant would be instructed to consume water and rest for 30 minutes and another sample was collected. To avoid participants arriving in a dehydrated state, they were instructed to consume a pint of water within 2 hours of each visit.

3.7. Environmental Chamber

During the studies describe in chapters 4 and 7, all experimental trials were performed within a walk-in environmental chamber (Model WIR52- 20HS; Weiss Technik, Gwent, UK). This was used to create hypoxic, hot and temperature conditions for the participants. In the study described in chapter 4, fraction of inspired oxygen (F_IO_2) in the chamber was set at 14% for normobaric hypoxia trials and 20.9% for the normoxia trials. For the study described in chapter 7 All experimental trials were performed in 33^oC and 50% relative humidity to create a hot environment.

3.8. Blood and Saliva Collection and Analysis

Venous blood samples during the study detailed in chapter 4, 6 and 7 were drawn from a vein in the antecubital foss region of the forearm, using a 21 gauge butterfly need (BD safety blood collection kit, BD, Plymouth, UK). For all venous blood samples, 6ml samples were collected with the participant in a seated position. Once collected, the needle was removed and a tissue with firm pressure was applied to avoid any superficial haematoma.

As described in chapter 5, capillary blood samples were collected from the fingertip using a 300µL trumpet shaped collection tube (Microvette®, Sarstedt, Nümbrecht, Germany). Following completion of each blood sample, medical tissue was firmly held on the puncture site to avoid any superficial haematoma.

3.8.1. Lactate

Lactate was assessed from whole blood samples in chapters 4 and 7. Whole blood was collected from the fingertip in a 7.20 µL sodium heparinised capillary tube and then shaken in an Eppendorf containing a glucose/lactate haemolysing solution. Once mixed, samples were analysed immediately using an automated analyser (Biosin, EKF diagnostic).

3.8.2. I-FABP

I-FABP was measured in plasma via an enzyme-linked immunosorbent assay (ELISA) (Hycult Biotechnology, Uden, Netherlands). This process was completed in accordance with the manufacturer's instructions. Whole blood in chapter 4 and 7 was collected into a 6ml heparin coated vacutainer (BD, Plymouth, UK) and immediately centrifuged at 1500xg for 15 minutes at 4°C. Whole blood in chapter 5 was collected from the fingertip using an EDTA coated collection tube (Microvette®, Sarstedt, Nümbrecht, Germany) and immediately centrifuged at 13000 rpm for 10 minutes. The resultant plasma samples were aliquoted into clean Eppendorfs and stored at -80 °C until further analysis. Once thawed, plasma samples were added to a sample dilution buffer and then transferred to pre-coated microtiter wells, coated with an antibody. Following a 60-min incubation, all wells were washed to remove any unbound substances and a biotinylated tracer antibody. A second incubation period and wash then removed any unbound antibody reagent and then a streptavidin-peroxidase conjugate was added to each well. A final wash was completed, before a TMB solution was added to help develop the colour of the wells in relation to the amount of I-FABP present. A stop solution was added to each well after 30-mins to stop the colour development and to allow absorbance to be measured via a microplate reader at 450nm.

3.8.3. LBP

A sandwich-based ELISA (Hycult Biotechnology, Uden, Netherlands) was performed in accordance with the manufacturer's instructions to analyse LBP concentrations in plasma samples. Whole blood in chapter 4 and 7 was collected into a 6ml EDTA coated vacutainer (BD, Plymouth, UK) and immediately centrifuged at 1500xg for 15 minutes at 4°C. Once centrifuged, plasma samples were aliquoted into clean eppendorfs and stored until further analysis in a -80 °C freezer. Upon thawing, samples were pre-diluted before being added to the dilution buffer solution, this ensured the samples reach the required 1000x dilution. Once diluted, samples were then then added to their designated well on a 96-well microtiter plate, coated with an antibody. After a 60-min incubation and wash, a biotinylated tracer antibody was added to each well. A second 60-minute incubation

and wash were performed, before the addition of a streptavidin-peroxidase conjugate to each well. TMB was then added after a final incubation and wash to encourage colour development in relation to the amount of LBP within each well. Stop solution was then added and plate absorbance was read using a microplate reader at 450nm.

3.8.4. Claudin-3

Plasma claudin-3 was analysed using a sandwich-based ELISA protocol (Elab Science, United States) performed in accordance with the manufacturer's instructions. Whole blood for claudin-3 was collected in chapter 4 into 6ml EDTA vacutainers (BD, Plymouth, UK) and then centrifuged within 30-mins at 1000xg for 10 mins at 4^{o}C. Aliquoted plasma samples were then stored in a -80 ^{o}C freezer until subsequent analysis. Prior to analysis, a standard working solution was added to each pre-coated well. Samples were then added to each well and the plate was incubated for 90-mins at 37 ^{o}C. The ELISA plate was then emptied, not washed and then a biotinylated detection ab working solution was added to each well. The plate was then incubated for another 60-mins before the first wash procedure. A HRP conjugate working solution was then added to each well before another 30-min incubation. Following another wash step, a substrate reagent was added to each well to encourage colour development. Stop solution was added to stop colour development and the plate was then read using a microplate reader at 450nm.

3.8.5 CRP

Plasma CRP concentrations were measured using an ELISA protocol (R & D systems, bio-techne, UK). Whole blood was collected in chapter 6 into a 6ml EDTA vacutainer and immediately centrifuged for 15-mins at 1000xg. The resultant plasma was then aliquoted into clean eppendorfs and frozen immediately in a -80 ^{o}C freezer. Prior to analysis, plasma samples required a 100-fold dilution, this was succeeded by adding 10µL of sample to 990µL of calibrator diluent. Assay diluent was added to each well on the microplate, then each diluted sample was added to its designated well. The entire

plate then underwent a 2-hour incubation at room temperature. Following a wash to remove any unbound entities, human CRP conjugate was added to each well and there was another 2-hour incubation period. Following another wash step, a substrate solution was added to each well and the microplate was protected from light to encourage colour development. Stop solution was then added to each well to stop colour development. The microplate was then assessed for absorbance via a microplate reader at 450nm with a wavelength correction of 570nm as per manufacturers instructions.

3.8.6 TNF-α

A sandwich-based ELISA was used to assess TNF-α in plasma samples (R & D systems, bio-techne, UK). Whole blood was collected for the assessment of TNF-α in chapter 6 via a 6ml EDTA vacutainer which was immediately centrifuged for 15-mins at 1000xg as per manufacturer's instructions. Plasma was then aliquoted into clean eppedorfs and frozen in a -80^{o}C freezer until later analysis. Assay diluent was added to each well on a precoated 96-well microplate. Sample was then added to each well and the entire plate underwent a 2-hour incubation period on an orbital microplate shaker. A wash procedure was performed to remove any unbound entities, and a high sensitivity conjugate was added to each well. Following a 60-min incubation another wash step was performed and streptavidin polymer-HRP to each well. After another incubation and wash period, substrate solution was added to each well and the plate was protected from light to ensure colour developed. Stop solution was then added and then the plate was read using a microplate reader at 450nm with a wavelength correction of 570nm.

3.8.7 Saliva Collection and Analysis

Unstimulated saliva was collected via the passive drool method for the analysis of salivary immunoglobulin A in chapters 6 and 7. Participants were instructed to consume and rinse their mouth with water and remain seated for 10-minutes. They then then tilted their head forward and

released saliva slowly into the collection tube for 2-minutes. The volume of saliva collected was then recorded. The saliva was then evenly aliquoted into two clean Eppendorf's and immediately stored at -20^{o}C and then moved to a -80^{o}C within 48 hours until further analysis. A sandwich-based ELISA was used to assess immunoglobulin A in saliva samples (Salimetrics, USA) Prior to analysis, all samples were diluted using a 1:5 ratio. A diluted antibody enzyme conjugate solution was then added to each sample and incubated for 90-mins. The incubated samples were then added to the 96-well microtiter plate and placed onto a plate rotator at 400 rpm for 90-mins at room temperature. A wash procedure was performed to remove any unbound entities and a TMB substrate solution was added to each well. The plate was then placed on a rotator for 5-mins at 500 rpm and then incubated in the dark for a further 40-mins. Following this incubation, stop solution was added to each well and the plate was placed on a rotator for another 3-mins at 500 rpm to stop colour development. The plate was then read using a microplate reader at 450 nm with a wavelength correction of 492 nm.

3.9 Gastrointestinal and upper Respiratory Symptom Scales

3.9.1 Rating of specific GI Symptoms

During and/or after exercise, participants completed a more detailed questionnaire (adapted from Gaskell et al., 2019) to assess any specific symptoms of gastrointestinal discomfort. Participants were asked to rate the severity of 14 different symptoms during the previous exercise period. All symptoms were rated on a 11-point Likert scale ranging from 0 to 10. The rating of symptom severity was explained to all participants prior as follows: a rating of 0 implies that there was no incidence of that symptom, a rating of 1-4 implies there was a mild presence (i.e., sensation of GIS, but not substantial enough to interfere with exercise workload), a rating of 5-9 implies there was a severe presence i.e., GIS substantial enough to interfere with exercise workload, and a rating of 10 implies there was a severe presence of that symptom warranting the cessation of exercise. Symptoms such as regurgitation and defecation are only rated as 0 (no presence) or 10 (extremely

severe presence) as these would always require the participant to stop exercising. The rating of all symptoms was summed to give a total gastrointestinal symptom score, helping characterise total gastrointestinal severity. Furthermore, the minimum total gastrointestinal severity was 0 and the maximal was 140. Symptom severity could also be divided into upper, lower, and other gastrointestinal symptoms, with 6 and 5 and 3 symptoms in each category respectively.

3.9.2 Gastrointestinal Symptom Rating Scale

To assess weekly gastrointestinal discomfort in chapter 7 the Gastrointestinal symptom rating scale (GSRS) (Svedlund et al., 1988) was used. Participants were asked to rate the severity of 15 different symptoms in relation to the previous seven days. Symptoms were rated on a seven-point Likert scale, ranging from no discomfort to very severe discomfort relating to, abdominal pain, hunger pains, nausea, heartburn, acid regurgitation, diarrhoea, loose stools, stomach rumbling, abdominal distension, belching, increased flatulence, constipation, hard stools, and the feeling of incomplete evacuation. This questionnaire was chosen as it assesses gastrointestinal symptoms over the course of seven days, reducing the burden on participants. It has also previously been used as an online resource in a similar cohort and study (Pugh et a., 2020) and has been validated with large groups (Spiegel et al., 2014).

3.9.3 Daily Upper Respiratory Symptom Questionnaire

To assess the occurrence of upper respiratory tract illness in chapters 5, 6 and 7, the Jackson cold symptom daily questionnaire (Jackson et al., 1958). Participants were required to state if they believe they are suffering from a common cold/acute respiratory illness. They would then rate the severity of 8 Jackson score symptoms (headache, chilliness, sneezing, sore throat, malaise, cough, nasal discharge, and nasal obstruction). Each symptom was rated on a 4-point likert scale, 0 equating to not present; 1, mild; 2, moderate; and 3, severe. For all of these chapters, an episode of URS was defined using similar criteria to Martineau, Hanifa and Witt (2015) and Davison et al. (2020). Which

included, a 3 or more-day period whereby (i) all 8 Jackson symptoms totalled ≥14 with a subjective impression of a cold (question), or (ii) a total Jackson score of ≥ 14 with nasal discharge for at least 3 days, or (iii) a total Jackson score <14 with a subjective impression of having a cold and at least 3 days of nasal congestion. Within the questionnaire, participants were required to state any medication they took during the illness episode, whether they visited their general practitioner and if this influenced their ability to perform exercise.

4. Chapter 4 – Investigating the effects of exercise at the same, environment-specific, relative intensity in normobaric hypoxia on gastrointestinal barrier permeability and gastrointestinal damage, and subjective feelings of gastrointestinal discomfort.

4.1 Abstract

Objectives Gastrointestinal (GI) discomfort is a common issue reported by many athletes, which can have serious implications for health and performance. Exercising in hypoxia may accelerate the onset of intestinal damage and GI symptoms due to heightened intestinal ischemia. Therefore, the current study aimed to investigate differences in enterocyte damage (intestinal fatty acid binding protein (I-FABP)), intestinal permeability (Claudin-3) endotoxemia (lipopolysaccharide binding protein (LBP) and GI symptoms between normobaric hypoxia and normoxia when running at the same relative intensity. **Methods** In a counterbalance design, 12 participants completed two experimental trials, one in normoxia (NORM) (F_IO_2=20.9%) and one in normobaric hypoxia (HYP) (F_IO_2=14.0%). Each experimental trial involved a 60-minute treadmill run at 85% of the respiratory compensation point (RCP) determined in both 14.0% F_IO_2 and 20.9% F_IO_2. Blood samples were obtained pre, immediately-post (post0) and 60-min post exercise (post60). The occurrence and severity of GI discomfort were assessed immediately post exercise using a questionnaire that rated the presence of 14 symptoms. **Results** The pre-post0 Δ was greater in HYP than NORM for I-FABP (780 ± 350 vs. 388 ± 317 pg/mL) (p = 0.008), LBP (2659 ± 2569 vs. 607 ± 1730 ng/mL) (p = 0.031) and Claudin-3 (0.88 ± 1.66 vs. -0.65 ± 2.04 ng/mL) (p = 0.005). GI discomfort severity during exercise was greater in HYP than NORM (10.9 ± 8.6 vs. 6.1 ± 4.4) (P = 0.016). **Conclusion** These data indicate that compared to running in normoxia, running in normobaric hypoxia is associated with greater enterocyte damage, increased intestinal permeability, and endotoxemia, along with more severe GI symptoms.

4.2 Introduction

Gastrointestinal (GI) discomfort is a common issue reported by many athletes, which can have serious implications for health and performance (Jeukendrup et al., 2000; Stuempfle & Hoffman 2015; Costa et al., 2016; Pugh et al., 2017). Although, the exact mechanisms of exercise-induced GI symptoms are not fully understood, splanchnic hypoperfusion and intestinal ischemia are considered key contributors (van Wjick et al., 2011). Indeed, splanchnic blood flow can fall by 80% during 60 mins of submaximal exercise (Rehrer et al., 2001; Nielsen et al., 2002), leading to dysregulation of the enterocyte and tight junction complexes which form the intestinal barrier (Zuhl et al., 2014a). Sufficient dysregulation to the intestinal barrier will increase permeability which may increase the translocation of lipopolysaccharide, initiating local and systemic inflammation (Triantafilou & Triantafilou 2002). Such disruptions may impair normal GI functioning and result in GI symptoms.

Various groups (e.g., mountaineers, athletes, military personnel, tourists, and emergency workers) may perform exercise in hypoxic environments for training and leisure purposes causing greater skeletal muscle stress and arterial oxygen desaturation (Dufour et al., 2006; Czuba et al., 2011; Joyner and Casey 2014; Rowell et al. 1986). This may exacerbate splanchnic hypoperfusion and place further strain on the GI tract (Derikx et al., 2008). Indeed, several studies have shown that markers of GI injury, permeability, and endotoxemia, along with GI symptoms are greater when exercising in hypoxia compared to normoxia (Machado et al., 2017; Hill et al., 2020; McKenna et al., 2022). However, it must be noted that for each of these studies, participants exercised at the same absolute intensity for both normoxia and hypoxia trials meaning that relative intensity may have been higher in hypoxia, exacerbating intestinal injury and symptoms.

Due to the difficulty of assessing splanchnic blood flow and GI ischemia, indirect markers of GI damage and permeability are commonly used, including blood concentrations of intestinal fatty acid-binding

protein (I-FABP), lipopolysaccharide binding protein (LBP) and claudin-3. I-FABP is derived exclusively from mature enterocytes of the small and large intestine making it a sensitive marker of acute GI damage (Peters et al., 2003; March et al., 2017). Plasma I-FABP correlates positively with splanchnic hypoperfusion (van Wjick et al., 2011), and is elevated in the blood following endurance exercise (van Wjick et al., 2011; van Wjick et al., 2013; Morrison et al. 2014; Barberio et al., 2015, Dokladny et al., 2016). Claudin-3 is a tight junction protein predominantly found in the epithelium of the small intestine. Furthermore, elevated plasma concentrations of claudin-3 are indicative of raised intestinal permeability (Tsukita et al., 2001). LBP is an acute phase protein primarily released from the liverwhich attaches to lipopolysaccharides (LPS), forming the LPS-LBP complex, which subsequently binds to CD-14 and to the myeloid differentiation factor 4/myeloid differentiation factor 2 complex (Schumann & Latz, 2000). This results in the activation of signal transduction pathways and the production of cytokines and pro-inflammatory mediators (Guha & Mackman, 2001), which can promote the breakdown of the tight junctions within the intestinal barrier and enhance intestinal permeability (Capaldo et al., 2009). As LPS is heavily produced within the gut, higher circulating concentrations of LPS or LBP are associated with disruptions within the intestinal barrier. This makes LBP an indirect marker of intestinal permeability and endotoxemia (Schumann et al., 1990). Both claudin-3 and LBP have been shown to be elevated following exercise (Yeh et al., 2013; Gaskell et al., 2020; McKenna et al., 2022).

Interestingly, hypoxic environments may acutely impact the GI tract by increasing sympathetic outflow (Fletcher, 2000), which may redirect blood flow away from splanchnic organs (Loshbaugh et al., 2006). Reduced partial pressure in oxygen will also induce hypoxemia in the blood. Together, these can induce intestinal ischemia and disrupt the integrity of the intestinal barrier. This may partly explain why GI discomfort is reported when ascending to high altitude (>2500m) (Anand et al., 2006).

Removing the confounding influence of exercise intensity, the aim of the current study was to investigate whether exercising in normobaric hypoxia causes greater GI injury, permeability,

endotoxemia and GI discomfort than when performed in normoxia. It was hypothesised that exercising in normobaric hypoxia at the same relative intensity as normobaric normoxia will increase the indirect markers of GI injury.

4.3 Methods

4.3.1 Study design and participants

Using a counterbalanced design, twelve healthy endurance trained males (age = 25.7 ± 7.4 years, height = 180 ± 10 cm, body mass = 77.4 ± 7.8 kg, $\dot{V}O_{2max}$ = 56.9 ± 5.5 $ml.kg^{-1}.min^{-1}$) completed four laboratory visits. The visits comprised of two preliminary visits, one in normoxia ((F_IO_2=20.9%) NORM) and one in normobaric hypoxia ((F_IO_2=14%) HYP) and two experimental trials one in NORM and one in HYP. All exercise was performed in a normobaric environmental chamber at 20°C and 45% relative humidity. Participants were instructed to arrive at least two hours post prandial and to have avoided exercise and alcohol during the previous 24 hours. All participants were not currently taking regular medication or nutritional supplements and had no history of GI-related medical issues. Participants were informed of the risks associated with the study and provided written informed consent. All procedures were performed in accordance with the declaration of Helsinki and were approved by the Nottingham Trent University Human Ethics Committee.

4.3.2 Preliminary visits

Participants completed two preliminary visits, one in NORM and one in HYP. In each visit, participants completed an incremental task to failure on a motorised treadmill (HP Cosmos, Germany) to obtain the respiratory compensation point (RCP) and maximal oxygen uptake ($\dot{V}O_{2max}$). Running started at 9 km⊙ h^{-1} and a 0% gradient, with running speed increasing 1 km · h^{-1} every minute until 18 km⊙ h^{-1} was reached. After one minute at 18 km⊙ h^{-1}, the treadmill inclined by 1% every minute until task failure. Ventilatory gas exchange variables were measured breath-by-breath throughout the maximal test using a metabolic cart (Jaeger®CPX, Vyntus®, Vyaire Medical Inc, United Kingdom), which was

calibrated with known gases (O_2 = 15.96%, CO_2 = 4.99%). Participants wore a facemask (Hans Rudolph 7450, Hans Rudolph Inc, USA) and expired air was sampled using a digital volume transducer, and O_2 and CO_2 sensors. Ventilatory and gas exchange variables were reduced to 10s averages, with $\dot{V}O_{2max}$ considered the highest $\dot{V}O_2$ value over any 10s. The respiratory compensation point (RCP) was determined by plotting minute ventilation ($\dot{V}E$) against carbon dioxide production ($\dot{V}CO_2$). The deflection point was then identified as the RCP.

4.3.3 Experimental visits

Participants arrived at the laboratory at the same time of day and were instructed to record their diet in the 24 hours preceding the first experimental trial via a food diary and to then replicate this for the following experimental trial. Following 10-min of seated rest, heart rate (HR) (Polar FT1 HRM, Polar Electro, Kempele, Finland), capillary blood lactate concentrations ($[La^-_B]$) was determined, and a venous blood sample was taken. At rest and throughout exercise, estimated arterial oxygen saturation was measured from the forehead (S_pO_2) (Nonin 8500 Pulse Oximeter, Nonin Medical Inc, Minnesota, USA). Participants then entered the environmental chamber (TISS series 201003-1, TIS services UK) and remained seated for 10 minutes. Each exercise trial consisted of 60 minutes of running at 85% of the RCP in either NORM or HYP. Ventilatory gas exchange variables were measured breath-by-breath and reduced to 15 min averages. HR, rating of perceived exertion (RPE) (Borg, 1982) and S_pO_2 were recorded every 15 min. $[La^-_B]$ was measured every 15 minutes. In both trials, to ensure the participants were naïve to the experimental environment, the exterior digital display was covered, and the compressor fans were constantly running during both trials.

Blood sampling and analysis

Capillary blood samples were obtained from the fingertip for the analysis of $[La^-_B]$. Blood was collected using a glass capillary tube and placed into a haemolysing solution. Once mixed, this was immediately analysed (Biosen C-Line, EFK Diagnostics, Germany). Six ml blood samples were obtained from the

antecubital vein using a 21G needle and vacutainers containing EDTA pre-, immediately post (post0) and 60 min post (post60) exercise. Once drawn, samples were immediately centrifuged at 1500xg for 15 min, and plasma was separated into clean Eppendorfs and frozen at -80^{o}C. Enterocyte damage, and intestinal injury were assessed by measuring plasma I-FABP (Hycult Biotechnology, Uden, Netherlands) and claudin-3 (Elab Science, United States). LBP was used as an indirect marker of LPS translocation and endotoxemia (Hycult Biotechnology, Uden, the Netherlands) respectively. ELISA kit procedures were performed according to the manufacturer's instructions. From the samples in the current study, it was determined that for I-FABP, the intra- and inter-assay variation was 3.1% and 5.6%. For CLDN-3, the intra- and inter-assay variation was 7.9% and 6.5%. For LBP, the intra- and inter-assay variation was 4.7% and 5.4%.

4.3.5 Assessment of GI discomfort

To assess GI discomfort during exercise, all participants completed a questionnaire immediately post exercise. Each participant rated the incidence and severity of 14 symptoms on a subjective analogue scale ranging from 0 to 10 (0 = absent, 1-4 = mild, 5-8 = severe, 9-10 = serious) (Gaskell et al. 2019). The scores from all 19 symptoms were totalled to provide a total GI symptom severity score. This was repeated for symptoms related to the upper and lower GI tract to give a separate severity score for both the upper and lower regions.

4.3.6 Statistical analysis

All statistical analyses were performed using the statistical package for social sciences (IBM SPSS version 28, New York, United States). All physiological data (HR, SPO_2, La^-_B, I-FABP, CLDN-3, LBP, and 15 min averages of breath-by-breath values) and RPE were confirmed to be normally distributed following a Shapiro-wilk test and analysed using a two-way (trial x time) repeated measures analysis of variance (ANOVA). Partial eta squared are reported as effect sizes for the two-way ANOVA, with 0.01, 0.06 and 0.14 considered as a small, medium, and large effects, respectively. Due to technical

difficulties, ventilatory gas exchange variables were not collected from 3 participants. [La^-_B], I-FABP, CLDN-3 and LBP were also analysed using a two-way repeated measures ANOVA. If a significant interaction or main effects were found, pairwise comparisons with Bonferroni corrections were used to determine differences between conditions. To assess the absolute change for I-FABP, claudin-3 and LBP, the post0 and post60 concentrations were subtracted from the pre concentration to give pre to post0 and post60 changes (Δ). Between trial differences were then assessed using a paired-sampled t test. GI symptoms from the post exercise questionnaire were totalled into categories (total GI, upper GI, and lower GI) and between trial differences were assessed using a Wilcoxon Signed-rank test. Spearman's rank correlations were performed to assess relationships between total GI symptom severity and pre-post0 Δ in I-FABP, LBP and claudin-3. All data are presented as mean ± standard deviation unless stated otherwise. Statistical significance was set at $P < 0.05$.

4.4 Results

4.4.1 Exercise Intensity

$\dot{V}O_{2max}$ was lower in hypoxia (51.4 ± 5.8 $ml.kg^{-1}.min^{-1}$) than normoxia (56.9 ± 5.5 $ml.kg.min^{-1}$) ($P < 0.001$). Similarly, treadmill speed at 85% of the RCP was lower in HYP (9.8 ± 0.6 $km.h^{-1}$) than Norm (11.2 ± 0.7 $km.h^{-1}$) ($P < 0.001$).

4.4.2 Plasma I-FABP

There was no main effect of trial ($F(1, 11) = 3.72$, $P = 0.080$, $\eta^2 = 0.25$), but there was for time ($F(2, 22) = 30.09$, $P < 0.001$, $\eta^2 = 0.73$) for plasma I-FABP. Plasma concentrations were higher at post0 than both pre and post60 in both trials ($P < 0.001$). There was a trial x time interaction effect ($F(2, 22) = 5.52$, $P = 0.011$, $\eta^2 = 0.33$) with higher concentrations at post0 in HYP than NORM ($P = 0.014$). There were no between trial differences at pre or post60 ($P = 0.486$) (figure 4.1a). Pre to post0 Δ I-FABP was greater in HYP (780 ± 350 pg/mL) (99%) than NORM (388 ± 317 pg/mL) (52%) ($P = 0.008$) (figure 4.1b). There were no differences in pre to post60 Δ I-FABP between trials ($P = 0.489$).

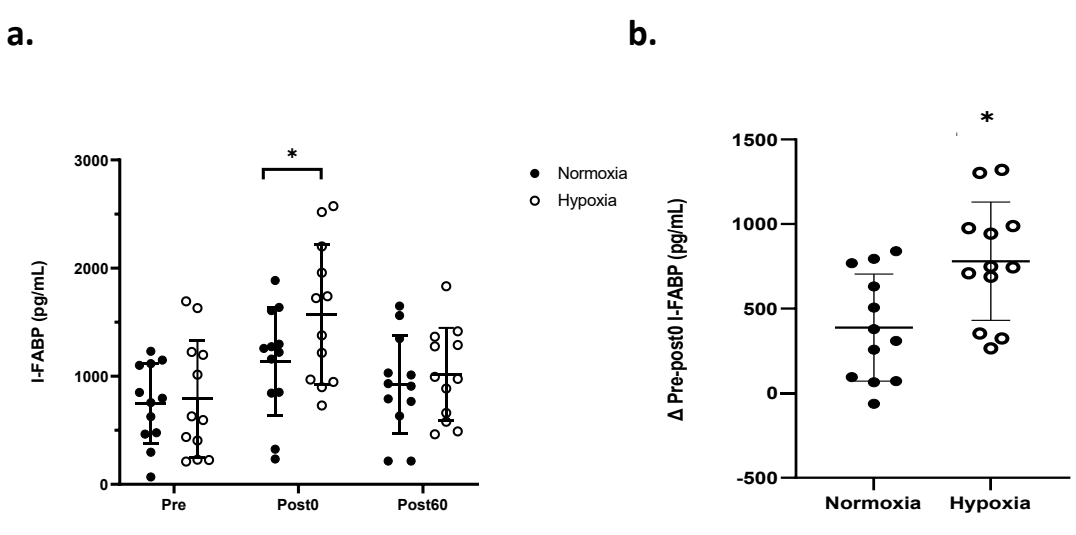

Figure 4.1 Panel 'a' shows plasma I-FABP before (Pre), immediately post (Post0) and 60 min post (Post60) a 60 min treadmill run in NORM and HYP. Panel 'b' shows pre-post0 Δ for plasma I-FABP after a 60 min treadmill run in NORM and HYP. Data is presented as mean ± SD with points representing individual participants. In panel 'a', * Indicates significant difference between conditions. In panel 'b' * indicates significant difference to normoxia.

4.4.3 Plasma Claudin-3

There was no main effect of trial ($F(1, 11) = 3.39$, $P = 0.572$, $\eta^2 = 0.03$) or time ($F(2, 22) = 1.54$, $P = 0.37$, $\eta^2 = 0.12$) for plasma claudin-3. There was no trial x time interaction effect ($F(2, 22) = 3.37$, $P = 0.053$, $\eta^2 = 0.24$) (figure 4.2a). Pre to post0 Δ claudin-3 was greater in HYP (0.88 ± 1.66 ng/mL) (9%) than NORM (-0.65 ± 2.04 ng/mL) (-1%) ($P = 0.005$) (figure 4.2b). There was no difference in pre to post60 Δ claudin-3 between trials ($P = 0.083$).

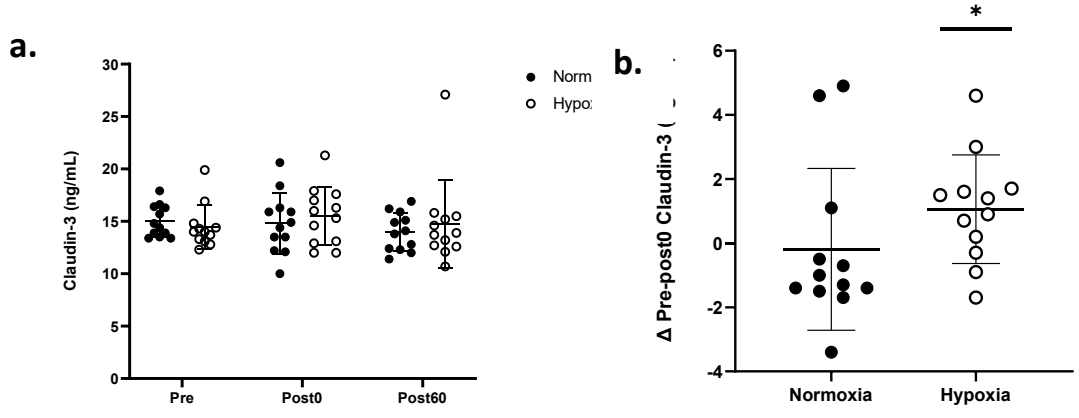

Figure 4.2 Panel 'a' shows plasma claudin-3 before (Pre), immediately post (Post0) and 60 min post (Post60) a 60 min treadmill run in NORM and HYP. Panel 'b' shows pre-post0 Δ for plasma claudin-3 after a 60 min treadmill run in NORM and HYP. Data is presented as mean ± SD with points representing individual participants. In panel 'a', * Indicates significant difference between conditions. In panel 'b' * indicates significant difference to normoxia.

4.4.4 Plasma LBP

There was a main effect of trial ($F(1, 11) = 11.63$, $P = 0.006$, $\eta^2 = 0.51$) and time ($F(2, 22) = 5.61$, $P < 0.011$, $\eta^2 = 0.34$) for plasma LBP. Plasma concentrations were higher at post0 than pre in both trials ($P = 0.017$). There was trial x time interaction effect ($F(2, 22) = 4.41$, $P = 0.025$, $\eta^2 = 0.29$) with higher concentrations in HYP at post0 ($P = 0.014$) and post60 ($P = 0.019$) compared to NORM (figure 4.3a). Pre to post0 Δ LBP was greater in HYP (2659 ± 2568 ng/mL) (37%) compared to NORM (607 ± 1730 ng/mL) (9%) ($P = 0.031$) (figure 4.3b). Pre to post60 Δ LBP was greater in HYP (2889 ± 2986 ng/mL) (46%) than NORM (100 ± 2195 ng/mL) (4%) ($P = 0.020$).

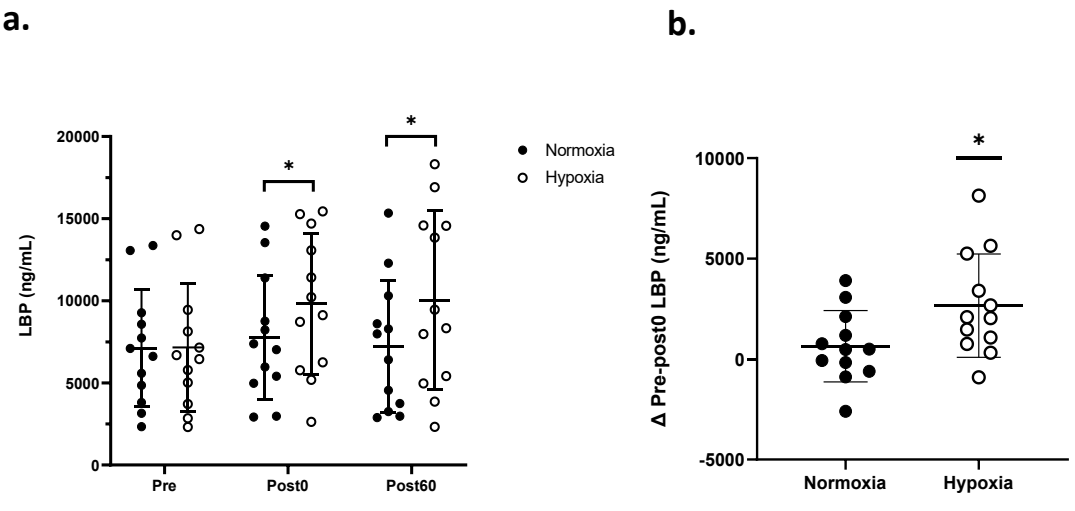

Figure 4.3 Panel 'a' shows plasma lipopolysaccharide binding protein (LBP) before (Pre), immediately post (Post0) and 60 min post (Post60) a 60 min treadmill run in NORM and HYP. Panel 'b' shows pre-post0 Δ for plasma LBP after a 60 min treadmill run in NORM and HYP. Data is presented as mean ± SD with points representing individual participants. In panel 'a', * Indicates significant difference between conditions. In panel 'b' * indicates significant difference to normoxia.

4.4.5 Gastrointestinal discomfort

Total GI symptom severity score was greater in HYP (10.92 ± 8.61) than NORM (6.08 ± 4.44) (P = 0.016). The lower GI symptom severity score was greater in HYP (4.08 ± 4.79) than NORM (2.08 ± 2.14) (P = 0.039). Specifically, flatulence was greater in HYP than NORM (P = 0.013). GI symptom incidence was greater in HYP (2.77 ± 2.71) than NORM (1.54 ± 1.20) (P = 0.026). There were no relationships between total GI symptom severity score and pre to post Δ I-FABP (r(10) = .044, P = 0.892), pre to post Δ LBP (r(10) = .129, P = 0.690) or pre to post Δ Claudin-3 concentrations (r(10) = .020, P = 0.950) in NORM. Similarly, there were no relationship between total GI symptom severity score and I-FABP (r(10) = .033, P = 0.920), LBP (r(10) = -.102, P = 0.753) or Claudin-3 concentrations (r(10) = -.001, P = 0.997) in HYP.

4.4.6 Ventilatory responses

There was a man effect of trial ($F(1, 8) = 11.12$, $P = 0.010$, $\eta^2 = 0.58$), time ($F(4, 32) = 14.54$, $P < 0.001$, $\eta^2 = 0.65$) and trial x time interaction ($F(2, 22) = 4.41$, $P = 0.025$, $\eta^2 = 0.29$) for end tidal CO_2, with lower values in HYP (33.8 ± 1.9) than NORM (38.1 ± 3.8) at all time points ($P < 0.05$). There was a time effect for RER ($F(4, 32) = 19.28$, $P < 0.001$, $\eta^2 = 0.54$), minute ventilation ($F(4, 32) = 264.56$, $P < 0.001$, $\eta^2 = 0.97$), $\dot{V}O_2$ ($F(4, 32) = 210.31$, $P < 0.001$, $\eta^2 = 0.96$) and VCO_2 ($F(4, 32) = 211.60$, $P < 0.001$, $\eta^2 = 0.96$), but there were no main effects for trial or trial x time interactions for any of these ventilatory variables ($P > 0.05$) (figure 4).

4.4.7 Oxygen saturation (S_pO_2)

There was a main effect of trial ($F(1, 11) = 12.88$, $P < 0.001$, $\eta^2 = 0.92$), time ($F(5, 55) = 81.55$, $P < 0.001$, $\eta^2 = 0.88$) and a trial x time interaction ($F(5, 55) = 83.68$, $P < 0.001$, $\eta^2 = 0.88$) for S_pO_2, with lower S_pO_2 at 0, 15, 30, 45 and 60 min in HYP ($P < 0.001$) (figure 4b).

4.4.8 Heart rate

There was a main effect of trial ($F(1, 11) = 5.77$, $P = 0.035$, $\eta^2 = 0.34$) and time ($F(5, 55) = 812.94$, $P < 0.001$, $\eta^2 = 0.99$), but no trial x time interaction ($F(5, 55) = 1.57$, $P < 0.236$, $\eta^2 = 0.13$) for HR (figure 4a).

4.4.9 Blood Lactate

There was a main effect of trial ($F(1, 11) = 14.64$, $P = 0.003$, $\eta^2 = 0.57$), time ($F(4, 44) = 19.58$, $P < 0.001$, $\eta^2 = 0.64$) and a trial x time interaction ($F(4, 44) = 3.93$, $P = 0.008$, $\eta^2 = 0.26$) for La^-_B (figure 4d). La^-_B was higher in HYP than NORM at 15 ($P < 0.001$), 45 ($P = 0.006$) and 60 min ($P = 0.037$) (figure 4d).

4.4.10 Rating of perceived exertion

There was a main effect of trial ($F(1, 11) = 10.91$, $P = 0.007$, $\eta^2 = 0.50$), time ($F(3, 33) = 29,98$, $P < 0.001$, $\eta^2 = 0.73$) and a trial x time interaction ($F(3, 33) = 3.51$, $P = 0.026$, $\eta^2 = 0.24$) for RPE. RPE was higher in HYP than NORM at 30 ($P = 0.003$), 45 ($P = 0.015$) and 60 min ($P = 0.012$) (figure 4c).

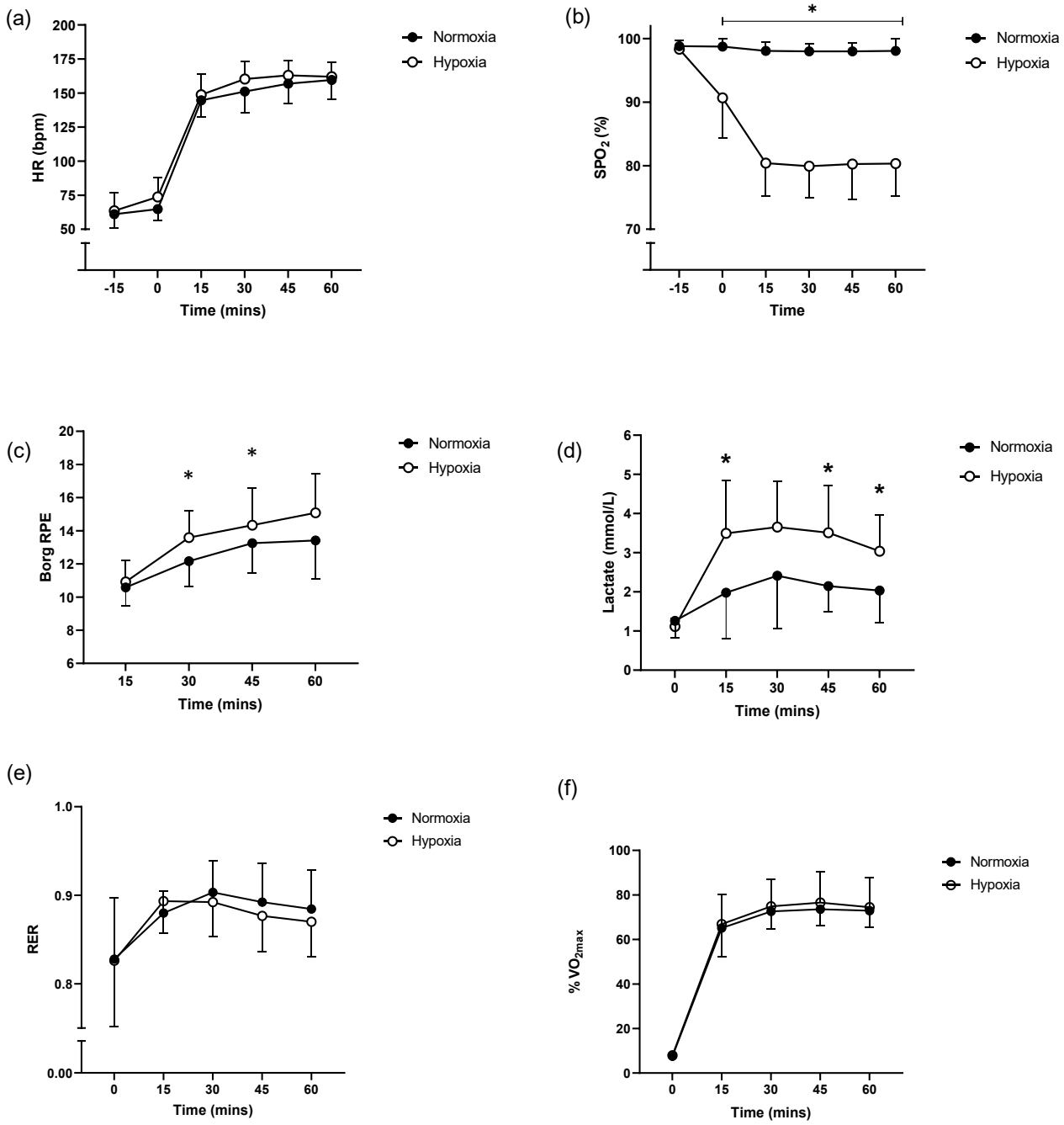

Figure 4.4 Physiological responses and perceived exertion during one hour of treadmill running in NORM and HYP. (a) heart rate; (b) oxygen saturation; (c) rating of perceived exertion; (d) Lactate; (e) RER; (f) % of $\dot{V}O_2$max. In panel a and b, time point -15 min is in normal ambient conditions, whereas time point 0 min is after 10 min rest in the environmental chamber in the respective conditions. * Indicates significant difference between conditions as confirmed by pairwise comparisons ($P < 0.05$).

4.4.11 Summary of Findings

- **60 min running in normobaric hypoxia increased I-FABP by 99% compared to 52% in normoxia.**
- **60 min of running in normobaric hypoxia caused a 9% increase in plasma claudin-3 with no increases in normoxia.**
- **Plasma LBP was increased by 37% and 46% at immediately post exercise following 60 min running normobaric hypoxia compared to 9% and 4% in normoxia.**
- **The severity and incidence of GI discomfort was greater when running in normobaric hypoxia than normoxia.**

4.5 Discussion

The aim of the current study was to investigate whether exercising in normobaric hypoxia when matched to the environment causes greater GI damage, permeability, endotoxemia and GI discomfort than when performed in normoxia. The key findings were that exercising in normobaric hypoxia (F_IO_2=14.0%) induced greater intestinal damage, endotoxemia and discomfort than when performed in normoxia (F_IO_2=20.9%) as shown by greater elevations in plasma I-FABP, LBP and claudin-3. This implies that the hypoxic environment is a contributing factor for the elevated intestinal damage and endotoxemia experienced following exercise in hypoxic environments.

4.5.1 biomarkers of GI damage and permeability in response to exercise in hypoxia.

It is well documented that an acute bout of moderate continuous exercise can disrupt GI function and structure, possibly leading to discomfort (van Wjick et al., 2011; Gaskell et al., 2019; Pugh et al., 2019. Pals et al., 1997, Pugh et al., 2017). Although the cause is likely multifactorial, the redistribution of blood flow and the resultant splanchnic hypoperfusion are highlighted as major contributors to the disruptions reported in GI function (Reher et al., 2001; van Wjick et al., 2011). I-FABP is an intracellular protein found within the mucosa of the small and large intestine (Pelsers et al., 2003). The leakage of I-FABP into systemic circulation can indicate structural damage to the intestinal barrier and has been associated with impaired GI function and intestinal disease (Adriaanse et al., 2013; Vreugdenhil et al., 2011; Thuijls et al., 2010). Recent studies suggest that performing exercise in hypoxic environments may amplify GI disruption and discomfort. Indeed, exercise in hypoxia has previously been shown to increase plasma I-FABP between 44-70%, compared to no increases when performed in normoxia (Lee et al., 2017; Hill et al., 2020; McKenna et al., 2022). However, it must be noted that these past studies were performed at the same absolute exercise intensity in both normobaric hypoxia and normaoxia. Furthermore, it is likely participants were exercising at a harder relative intensity in hypoxia than normoxia, which may explain the greater GI disruption. In the current study, despite participants

running at a lower speed in hypoxia compared to normoxia (9.8 km.h^{-1} vs 11.2 km.h^{-1}), there were greater I-FABP concentrations after exercise in HYP. Specifically, exercise in HYP caused a 99% increase in I-FABP compared to elevations of 52% in NORM. We are the first to show that these elevations in I-FABP still exist despite matching exercise intensity to the environment. This provides clearer evidence that the hypoxic environment may be a driving factor for greater damage to the GI barrier during exercise in hypoxia.

A consequence of significant damage to gastrointestinal barrier is the increased likelihood of endotoxins being translocated across the intestinal lumen. LPS reside within the cell walls of gram-negative bacteria that inhabit the GI tract. Upon bacterial death and increased intestinal permeability, lipopolysaccharides can translocate across the intestinal barrier and trigger an inflammatory response via the activation of monocytes, macrophages and toll-like receptor 4 signaling (Stoll et al., 2004). To initiate inflammation, lipopolysaccharides must attach to LBP and transfer to the CD14 receptor (Hailman et al., 1994). This has led to LBP being considered as an indirect marker of gastrointestinal permeability and endotoxemia (Schumann et al., 1990). In the present study LBP increased more following exercise in HYP than NORM at post0 (37% vs 9%) and at post60 exercise (46% vs 4%), indicating increased gastrointestinal permeability and mild endotoxemia. This is in agreement with Machado et al. (2017), who observed a 45% increase in circulating endotoxins following running in HYP (4200m) and no change in NORM. Heightened LBP in HYP may be a consequence of significant damage to the gastrointestinal structure (Sessions et al., 2016), which is plausible given the exacerbated gastrointestinal damage seen by elevated I-FABP following hypoxic exercise in the current study.

Like I-FABP and LBP, claudin-3 increased more during exercise in HYP than NORM. Claudin-3 is a tight junction protein that forms part of the intestinal barrier in the colon and duodenum. Disruption to claudin-3 and subsequent appearance in systemic circulation is indicative of increased intestinal permeability via the paracellular space (Tsukita et al., 2001; Morin 2005). The greater concentrations

observed in HYP are in line with McKenna et al. (2022) who reported that after 60 min of cycling at the same absolute work rate claudin-3 increased by 10% in a simulated altitude of 4300m but did not change in normoxia. However, the novelty of the present study is that it is the first to show that circulating claudin-3 is greater following exercise in HYP than NORM even when exercise intensity is matched to the environment. This indicates that hypoxia may be the key driver of the exacerbated exercise-induced GI barrier dysregulation seen during hypoxic exercise.

4.5.2 GI symptom response to exercise in hypoxia.

GI symptoms have commonly been reported following running-based exercise (Jeukendrup et al., 2000; Pugh et al., 2017, Costa et al., 2017; Pugh et al., 2019; Miall et al., 2018; Gaskell et al., 2021). We found a higher incidence and a greater severity for GI symptoms in HYP than NORM, agreeing with McKenna et al. (2022). The aetiology of GI symptoms during exercise is complex and likely multifactorial but intestinal injury and subsequent endotoxemia are frequently proposed as possible mechanisms. Nevertheless, we failed to show any correlation between symptoms and markers of intestinal damage or permeability (I-FABP, LBP & Claudin-3). This corroborates with previous reports that GI symptoms and intestinal injury do not correlate (Jeukendrup et al., 2000, Pugh et al., 2017). In contrast, the only other study to have explored the effects of hypoxia on GI function in exercise reported correlations between overall GI symptom scores and I-FABP, LBP and Claudin-3 (McKenna et al., 2022). Discrepancies between our findings and McKenna et al. (2022) could be due to the exercise mode (cycling vs running), the fact that exercise intensity was matched in the current study and the GI symptom tool used to assess GI discomfort. Indeed, higher incidences of GI symptoms were reported during running than cycling (Edwards et al., 2021), which may have influenced any possible correlations in the present study.

It is well established that continuous exercise causes splanchnic hypoperfusion and ischemic stress on the GI tract (Reher et al., 2001; van Wjick et al., 2011). Hypoxemia induces a greater sympathetic

output and vasoconstriction, which can exacerbate splanchnic hypoperfusion (Fletcher 2000) and thus, damage the intestinal barrier. Therefore, despite not measuring splanchnic blood flow and O_2 delivery it is still fair to speculate that exercising in hypoxia caused greater splanchnic hypoperfusion which may partly explain the exacerbated GI damage and permeability. It is also possible that this in conjunction with local tissue hypoxia, energy depletion and tissue acidosis induced further damage to the intestinal barrier (Sasaki & Takashi, 2007; Meng et al., 2018; Khanna et al., 2019).

In addition to changes in blood flow, hypoxic exposure may disrupt the symbiosis between the host and the gut microbiome which can damage the intestinal mucosa (Round & Maxmanian, 2009). In mice, both repetitive intermittent and continuous hypoxic exposure for 8 and 12h per 24h over the course of 28 and 72h increased the number of harmful bacteria (*Dorea*, *Oscillibacter*, *Enteractinococcus*, *Paenibacillus*, *Globicatella*, and *Flaviflexus* genera) within the gut (Li & Shi, 2024; Ma et al., 2023). This was accompanied with fractures within the crypt structure, the loss of epithelial cells and a reduction of goblet cells within the small intestine (Ma et al., 2023). Elsewhere, similar alterations in the microbiome were suggested to be the cause of elevated of interleukin-6, tumor necrosis factor-alpha and the upregulation of nuclear factor-kappa B (NF-kB), leading to local inflammation and apoptosis of the intestinal epithelial cells (Li et al., 2018). Although the exact timeline of how rapidly hypoxic exposure alters the gut microbiome and intestinal structure is not clear in the current literature. The fact that significant effects are seen as early as 28h could mean that the addition of hypoxic exposure during 60minutes of exercise may have had acute effects on the microbiome contributing to local inflammation and the GI damage seen by higher plasma concentrations or I-FABP, LBP and claudin-3 in HYP. However, whether these changes do occur as rapidly as 1h is not clear from the current literature. Therefore, further investigations are needed to confirm the rate at which hypoxic exposure can impact GI structure and the microbiome.

4.5.3 Strengths and limitations

To date, all studies investigating exercise-induced GI responses in hypoxia have asked participants to exercise at the same absolute intensity in hypoxia as normoxia, completing preliminary VO2max assessments in normoxia to determine exercise intensity in hypoxia rather than performing the test in a hypoxic environment. Due to lower $\dot{V}O_{2max}$ reported at high altitude, we decided to investigate whether GI responses persisted when intensity was matched to environment. A limitation of this is the relevance it may have on real life applications. It is very common for athletes and military personnel to exercise in hypoxia due to the additional physiological stress it induces when compared to normoxia (Czuba et al. 2011; Dufour et al., 2006). It may be difficult to ask an individual to purposely reduce their intensity when at hypoxia. The duration and nature of exercise used in the present study may not be completely relevant to all athletes. Many athletes perform intermittent bouts of work or time-trial based training in hypoxia. Future research should investigate whether these forms of exercise still cause GI damage, permeability, and symptoms at hypoxia. Another limitation was the type of GI symptom assessment tool used and the timing of when GI discomfort was recorded. This limits direct comparisons to previous literature and may be a reason for no correlations with markers of intestinal injury as previously reported (Mckenna et al., 2022). Lastly, we did not include a trial where participants were seated in hypoxia for 60 mins. This may have provided additional information on the sole effects of acute hypoxia on GI barrier damage and permeability.

4.5.4 Conclusion

In conclusion, we have shown for the first time that 60 min of moderate intensity running in acute hypoxia (F_IO_2=14%) significantly elevates markers of intestinal injury, permeability and symptoms compared to normoxia at the same relative intensity. This suggests that exacerbations of intestinal injury, permeability and symptoms are due to the concurrent physiological strain induced by exercise and hypoxia rather than exercise intensity alone. We did not show any correlations between markers of intestinal injury and symptoms, thus the underpinning mechanisms are unclear for the onset of symptoms. Future research should also investigate splanchnic blood flow when exercising in hypoxia

in humans. Our findings may be of interest to athletes and military personnel performing exercise in hypoxia and future work should investigate viable prevention strategies.

5. Chapter 5 – Effects of a combined turmeric, vitamin C and vitamin D ready-to-drink supplement on upper respiratory illness, gastrointestinal damage and gastrointestinal discomfort in male professional football players.

Note – the data presented here form part of larger data set that has been peer-reviewed and published (Clayton et al., 2024). All data here in Chapter 5 was collected and analysed by Connor Parker whilst supporting the larger study.

*Clayton, D. J., Burbeary, R., **Parker, C**., James, R. M., Saward, C., Procter, E. L., ... & Varley, I. (2024). Combined Turmeric, Vitamin C, and Vitamin D Ready-to-Drink Supplements Reduce Upper Respiratory Illness Symptoms and Gastrointestinal Discomfort in Elite Male Football Players. Nutrients, 16(2), 243.*

5.1 Abstract

Objectives Elite footballers are exposed to numerous risk factors which may increase their susceptibility to upper respiratory and gastrointestinal illness. Turmeric, vitamin C and vitamin D can influence immune and gastrointestinal function. The aim of the present study was to assess the effects of ready-to-drink supplement containing turmeric root, vitamin C and vitamin D_3 on upper respiratory symptoms (URS), gastrointestinal symptoms (GIS) and gastrointestinal damage in elite male footballers. **Methods** Twenty-three footballers completed 3 weeks of no intervention control (CON), followed by 16 weeks of consuming 60 mL.day of a commercially available supplement containing raw turmeric root (17.5 g, estimated to contain 700 mg of curcumin), vitamin C (1000 mg), and vitamin D_3 (3000 IU/75 mcg) (SUP). URS and GIS were measured daily. Immediately (0 h), 40, and 64 h after six competitive matches (two in CON, four in SUP), intestinal fatty acid binding protein (I-FABP) was assessed. **Results** URS incidence ($p < 0.001$), GIS ($p < 0.05$), and plasma I-FABP at 0 h ($p < 0.05$) were greater during CON versus SUP. **Conclusion** This study indicates that turmeric root, vitamin C, and vitamin D supplementation over 16 weeks can reduce URS, GIS, and post-match I-FABP in elite footballers.

5.2 Introduction

Injury and illness have been shown to disrupt athlete availability for training and competition in elite sport (Parry & Drust, 2006; Raysmith & Drew, 2016). An acute bout of strenuous exercise can compromise gastrointestinal (GI) barrier integrity and immune function which may contribute to the risk of GI discomfort and upper respiratory symptoms (URS) (Costa et al., 2020; Yamauchi et al., 2011). Concerningly, 86% of elite athletes report exercise associated GI symptoms (Pugh et al., 2017) and in team sports, significant relationships have been evidenced among increased training loads, upper respiratory illnesses, and decreases in a primary antibody in saliva, IgA (Mortatti et al., 2012; Moreira et al., 2011; Cunniffe et al., 2011). In professional football, it has been demonstrated that illness accounts for 6.3% and 8.5% of absences in training and match-play, respectively (Parry & Drust, 2006). Football teams with less player absences achieve a higher league ranking and a greater number of points per match (Hägglund et al., 2013), hence illnesses could have a direct effect on a team's success.

Although the exact mechanisms of exercise-induced gastrointestinal symptoms (GIS) are not fully understood, splanchnic hypoperfusion is considered a key contributor (van Wjick et al., 2011). Submaximal exercise for 60 min can reduce portal vein blood flow by 80% (Rehrer et al., 2001), leading to intestinal ischemia, disrupting GI barrier integrity, and increasing permeability (Zuhl et al., 2014) This can lead to increased GIS, local and systemic inflammation, and nutrient malabsorption (Camilleri, 2019), which can negatively impact performance and recovery.

Exercise-induced damage to the gastrointestinal barrier can be assessed using indirect markers. Intestinal-fatty acid binding protein (I-FABP) is 15-kD protein found exclusively within the mature enterocytes of the intestinal lining. Upon acute damage, I-FABP is rapidly released into circulation and has been shown to correlate with splanchnic hypoperfusion (van Wjick et al., 2011). Continuous endurance exercise has consistently shown to elevate I-FABP and GIS (van Wjick et al., 2011; Pugh et al., 2017; Gaskell et al., 2021), but less evidence is available for team sports. Although studies are

limited, repeated high-intensity sprints, resistance exercise and collision-based training in rugby have been shown to increase I-FABP like that of continuous exercise (van Wjick et al., 2013; Pugh et al., 2017; Chantler et al., 2022). Competitive football is likely to include several of these elements and may therefore elevate gastrointestinal damage and GIS. However, to date no evidence exists on the GI response to an elite football-based activity.

Football is an intermittent sport characterised by high-intensity sprints, separated by low intensity running or active recovery (Bangsbo et al., 2006). Dependent on playing position, football players cover an average of 10-14 km (Di Salvo et al., 2009), while achieving an average heart rate of ~85% age predicted max and an intensity equivalent to 70% $\dot{V}O_{2max}$ (Bangsbo et al., 2006; krustrup et al., 2006). Using this data, it is plausible to suggest that a competitive football match exhibits enough physiological demands to cause GI damage and symptoms.

In team sports, including football, relationships exist between higher training loads, the onset of URS and the reduction in sIgA (Mortatti et al., 2012; Moreira et al., 2011; Cunniffe et al., 2011). In addition to a decline in salivary IgA, a post-exercise decline in immunosurveillance has been reported with prolonged (>5 days) and intensive (>60% VO2max) training periods (Hoffman-Goetz et al., 1990), and alongside a post-exercise decline in cytotoxic T cells may introduce a window of opportunity for infection (Steensberg et al., 2001). Elevated sympathetic nervous system activity and vasoconstriction of the blood flow towards the salivary glands are proposed as contributing factors to exercise-induced declines in sIgA (Chicharro et al., 1998). As both GI and upper respiratory illness are the second highest reason (only behind injury) for professional footballers to be unavailable highlights the need for potential therapies that alleviate this.

Micronutrient deficiency can increase illness risk, and the current guidance states that athletes deficient in vitamin C and D supplements should consume supplements during periods of high exertion to reduce the incidence and severity of upper respiratory tract symptoms (Maughan et al., 2018). Vitamin D holds an important role in our immunity and in the modulation of inflammation

(Larson-Meyer et al., 2010) with almost all immune cells expressing vitamin D receptors (Baeke et al., 2010). Vitamin C is an antioxidant that accumulates in leukocytes and can protect cells from oxidative damage (Washko et al., 1993). Vitamin C can regulate immune function by modulating redox-related cell signalling pathways or by directly protecting important cell structural components. There is some indication that vitamin C may support the ability of neutrophils to migrate towards a site of infection and enhance the chemotaxis capability of neutrophils. Curcumin, found in turmeric, has been shown to reduce inflammation and muscle soreness and accelerate the restoration of muscle function (Abbott et al., 2023; Davis et al., 2007; Tanabe et al., 2015). There is also some evidence that curcumin, vitamin D, and vitamin C can all improve gut barrier functioning and influence gut microbiota (Wang et al., 2017; Lobo de Sa et al., 2021; Traber et al., 2019). Therefore, a multi-vitamin and turmeric containing supplement may be beneficial for reducing GI disruptions and URS in professional footballers.

The aims of this study were 2-fold; 1) to assess the impact of a competitive football match on markers of GI damage in professional male football players, and 2): to investigate the effects of a ready-to-drink supplement containing vitamin C, vitamin D and turmeric on markers of exercise-induced GI damage, and self-reported GIS and URS in professional footballers across a competitive season. It was hypothesised that 1) a competitive football match would cause an increase in plasma I-FABP and 2) that the ready-to-drink supplement would reduce GI damage and discomfort, and alleviate acute illness.

5.3 Methods

5.3.1 Participants and study design

Twenty-three elite male (age: 24.3 ± 2.8 years, body mass: 79.7 ± 6.7 kg, height: 1.83 ± 0.06 m) professional footballers, competing in the English third tier volunteered to take part in this study. The study followed a non-randomised, within groups design between September and March during the 2022/23 season. All participants were outfield players with no history of cardiovascular or gastrointestinal complaints and were not regularly consuming any vitamin C, D, turmeric, probiotic or prebiotic supplements. All participants provided written consent after they were informed verbally and in writing of the nature and requirements of the study. The study was approved by the Nottingham Trent University Human Invasive Ethics Committee (REF: 716), and all procedures conformed to the declaration of Helsinki.

5.3.2 Overview of experimental design

In September 2022, participants completed a 22-day (totalling 484 player days) control period where no supplement was consumed (CON). This was followed by a 116 ± 8 days (totalling 2558 player days) intervention period where they consumed a commercially available 60mL “shot” daily (Immunity Support Vitamin C + D, The Turmeric Co., Cambridgeshire, UK) (SUP). The shot was typically consumed in the morning after breakfast. Each shot contained 17.5 g of raw turmeric root (estimated to contain 700 mg curcumin), 1000 mg of Vitamin C, 3000 IU of Vitamin D_3, and 200 mg of black pepper (estimated to contain 10 mg of piperine). Compliance with supplementation was verbally confirmed by participants prior to each match. Participants reported the incidence of upper respiratory symptoms (URS) and gastrointestinal (GI) distress via a daily questionnaire. The CON period took place in September and consisted of two competitive league matches, whereas the SUP period began in November 2022 and ended in February 2023 and included four competitive league matches. Following each competitive match, a blood sample was collected immediately, 40 h and 64

h after. Global positioning system (GPS, Vector, Catapult, Australia) was used to monitor common performance measures (total distance, high speed distance, accelerations, decelerations) during each match.

5.3.3 Blood collection and analysis

Capillary blood samples were collected from the fingertip in a 300µ EDTA microvette tube (Microvette®, Sarstedt, Nümbrecht, Germany) for the assessment of intestinal fatty acid binding protein (I-FABP). Immediately post-match samples were collected where the match took place, with baseline samples collected at the clubs training facility. Samples were immediately stored on ice (maximum of 30 min) and then centrifuged (13,000× g, 10 min, 4◦C). Plasma was then separated, aliquoted and stored at -80^{o}C until analysis. Baseline and post-match samples were analysed using the commercially available enzyme linked immunosorbent assay (Human I-FABP, Hycult Biotech, Amsterdam, Netherlands). The intra-assay coefficient variation was 1.7%.

5.3.4 Upper respiratory symptoms

To assess the presence of upper respiratory symptoms (URS), participants completed the Jackson scale questionnaire daily (Jackson et al., 1958). Participants were instructed to state the presence and severity of 8 separate symptoms (headache, chilliness, sneezing, sore throat, malaise, cough, nasal discharge, and nasal obstruction) over each 24-hour period. Each symptom was rated on a scale of 0 to 3 (0—not present, 1—mild, 2—moderate, 3—severe). All scores from the eight symptoms were summed to provide an overall score for that 24-hour period. A URS episode was defined by either, any period lasting ≥ 3 days with a cumulative score of at $\geq$ 14 with the presence of nasal discharge, or any ≥ 3-day period at which there is a cumulative symptom score < 14 but there is a subjective impression of having a cold (Martineau et al., 2015). If URS returned within one week it was regarded as the same episode (Martineau et al., 2015).

5.3.5 Gastrointestinal symptoms

To assess GI discomfort, participants used a standardised questionnaire previously demonstrated to detect changes in GI symptoms in athletes (Pfeiffer et al., 2012). Participants rated the presence of global GI symptoms over the previous 24-hours using a subjective analogue scale ranging from 0 to 9 (0—"no problem at all", 9—"worst it's ever been"). Participants completed these scales each day on their smart phones.

5.3.6 GPS

The physical demands of match-play and training were monitored using a 10 Hz GPS (S7, Vector, Catapult, Victoria, Australia). This system has previously been demonstrated as a valid and reliable method to assess athlete movements (Johnston et al., 2014). Each player wore a GPS unit positioned between the shoulder blades using a bespoke garment. Each GPS unit was downloaded after each match or training session, and analysed using commercially available software (Open field, Catapult, Victoria, Australia). The physical performance variables assessed included the following; total distance covered (m), high speed (>5.5 m/s) distance covered (m), very high speed (>7.0 m/s) distance covered (m), number of accelerations above 0.5 m/s2 for >0.5 s, and number of decelerations below −0.5 m/s2 for >0.5 s.

5.3.7 Statistical analysis

A Pearson chi-square test (χ 2) was used to assess the observed frequency of URS in CON and SUP. Paired sample t-tests were conducted to assess differences in GI symptoms relative to the days in each trial. The significant main effects were followed-up using Bonferroni-corrected paired t-tests. For plasma I-FABP, the average post-match and baseline-post match absolute and change from baseline values were calculated during the control and supplementation periods (n = 7). Differences between the trials were assessed using a paired sample t-test. A Paired samples t-test was

conducted to assess the differences in GPS metrics between the control and the intervention periods. Significance was accepted at the 95% confidence level ($p < 0.05$). The mean and standard error were used to describe the average and variability of data, unless stated otherwise. The effect sizes were calculated for chi-squared tests using Cramer's V (small = 0.10–0.29, medium = 0.30–0.49, large ≥ 0.50), for t-tests using Cohen's dz (small = 0.20–0.49, medium = 0.50–0.79, large ≥ 0.80).

5.4 Results

5.4.1 Upper respiratory symptoms

There was greater incidence of URS during CON compared to SUP (Chi Sq = χ 2 75.7, DF = 1, p < 0.001, V = 0.16). Illness incidence was reported as 3.9 per 1000 player days during SUP compared to 10.6 per 1000 player days during CON (figure 5.1a). This equated to 94% of time spent without a URS during SUP compared to 83% of time spent without a URS during CON.

5.4.2 Gastrointestinal discomfort

The severity of GI distress was significantly lower during SUP (43 GI discomfort score per 1000 player days) compared to Con (111 GI discomfort score per 1000 player days) period (p < 0.01, dz = 0.62) .

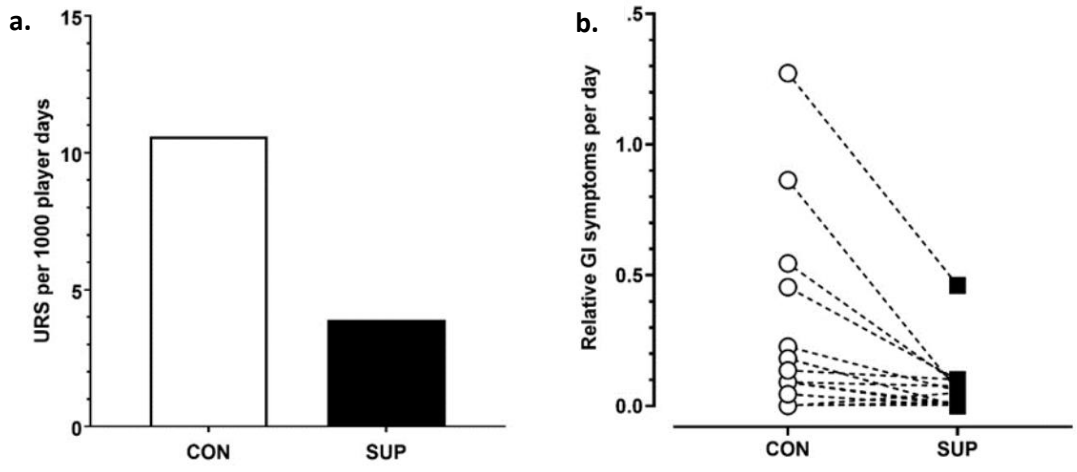

Figure 5.1. Mean upper respiratory symptoms per 1000 player days (panel a) and individual player responses to gastrointestinal symptoms per day (normalized over time) (Panel b) in the control period (CON) and supplementation period (SUP).

5.4.3 Plasma I-FABP

The mean concentration of I-FABP immediately post-match was lower during SUP than CON (SUP: 735 ± 275 pg/mL; CON: 1821 ± 1280 pg/mL; p < 0.05, dz = 0.98) (figure 2).

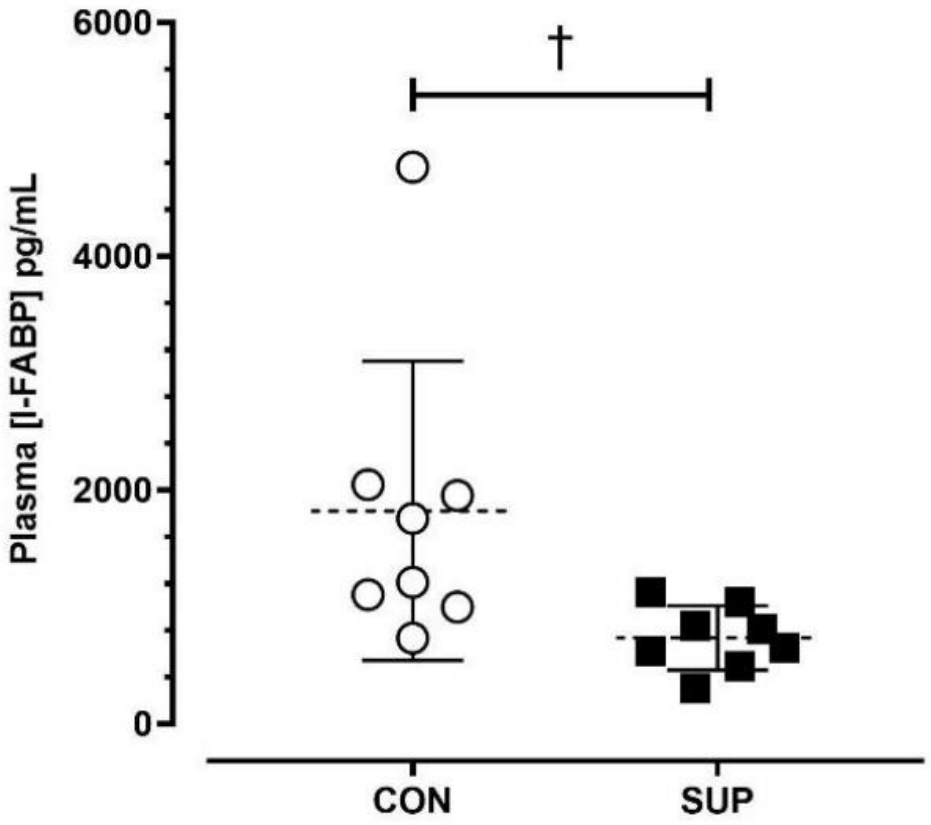

Figure 5.2 Absolute plasma concentrations of intestinal fatty-acid binding protein (I-FABP) immediately post-match in the control period (CON) and supplementation period (SUP). † indicates a difference between trials ($p < 0.05$). Values are means with error bars representing standard error.

5.4.4 GPS

There was no difference between trials for total distance covered (CON: 9845 ± 1026 m per match; SUP: 9883 ± 437 m per match; $p = 0.95$), high speed distance covered (CON: 633 ± 76 m per match; SUP: 534 ± 90 m per match; $p = 0.19$), number of accelerations above 0.5 m/s2 for >0.5 s (CON: 28 ± 3 n per match; SUP: 26 ± 2 n per match; $p = 0.40$), or number of decelerations below −0.5 m/s2 for >0.5 s (CON: 39 ± 5 n per match; SUP: 41 ± 4 n per match; $p = 0.56$).

5.4.5 Summary of Findings

- **A 90-min football match at professional level increases plasma I-FABP concentrations.**
- **A combined supplementation of turmeric, vitamin D_3 and vitamin C alleviates the increase in I-FABP following a 90-min football match.**
- **There was a lower incidence of URS when players were on SUP, this equated to 94% of days without a URS on SUP compared to 83% on CON.**
- **GI discomfort was lower during SUP than CON as shown by severity scores, 43 GI discomfort score per 1000 player days) compared to Con (111 GI discomfort score per 1000 player days.**

5.5 Discussion

The main findings of this study were that; 1) a competitive 90-minute game of football induced gastrointestinal damage as shown by elevations of I-FABP in professional footballers, and 2) that a ready-to-drink supplement containing 17.5g raw turmeric root (700 mg curcumin), 1000 mg of vitamin C, 3000 IU of vitamin D_3, and 200 mg black pepper (10 mg of piperine) alleviated GI damage, URS and GIS. These findings suggest that this combination supplement could be a suitable method for reducing illness and strengthening the epithelial barrier of the small intestine in elite male footballers.

5.5.1 Plasma I-FABP response to 90-min football match

Continuous, interval and resistance-based exercise induces GI damage as shown by elevations in circulatory I-FABP (van Wjick et al., 2011; Pugh et al., 2017; Hart et al., 2022). High I-FABP at rest is associated with various gastrointestinal complications (Thumser et al., 2014), while an acute rise following intense exercise are proposed to disrupt nutrient absorption and the onset of GI symptoms (Lang et al., 2006; van Wjick et al., 2013). To date, only one know study has assessed I-FABP concentrations in teams sports. Chantler et al. (2022) reported elevated I-FABP in addition to a greater LR ratio following a short bout of collision-based rugby union training. We have shown for the first time that football match-play induces significant GI damage in elite male footballers. Although the mechanism is not entirely understood, it is likely that splanchnic hypoperfusion, as seen during continuous exercise, is the main contributor. This novel finding shows that athletes who perform intermittent sports experience the same GI stress to that of endurance athletes and that there may be similar complications to GI function, like the onset of discomfort.

5.5.2 Effects of SUP on plasma I-FABP and mechanisms of turmeric

In addition to showing the impact of competitive football on circulating I-FABP, we found an attenuation in the post-match increase of I-FABP during the supplement period when compared to control (SUP average I-FABP 735 ± 275 pg/mL vs CON average 1821 ± 1280 pg/mL). This level of improvement is like that found in a study that used a smaller dose of curcumin in runners (Szymanski et al., 2018). In 2018, Szymanski and colleagues assessed the impact of a 3-day curcumin supplementation on markers of GI damage and inflammation following a 60-min run at 65% $\dot{V}O_{2max}$ in 37^{o}C heat. Despite using a significantly shorter intervention period and smaller dose (500 mg/day) to the present study, GI damage was still attenuated post exercise. The authors reported a greater pre to post and 1 hour post increase in I-FABP in the placebo group compared to curcumin (pre to post: 87% vs 58%; pre to 1h post: 33% vs 18%) (Szymanski et al., 2018). The authors speculate that the attenuation of I-FABP immediately post exercise following the curcumin supplementation could be due to several mechanisms. One of which is the reinforcement of intestinal defences against gram-negative bacteria. In murine models, curcumin has been shown to downregulate the production of IL-17 following retinal ischemia-reperfusion injury and IL-1β following LPS-induced sepsis and liver failure (Zhange et al., 2015; Zhong et al., 2016). As intense exercise significantly elevates core temperature, it is likely that LPS-induced inflammation is heightened via the secretion of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) from liver Kupffer cells (Jiang et al., 1999). Neutrophils orchestrate the host defence against gram-negative bacteria, with intestinal cells releasing IL-1β and IL-17 via the stimulation of neutrophil chemotaxis (Mei et al., 2012; Nakagawa et al., 2016; Rosales et al., 2016). Furthermore, it is plausible that in the present study curcumin was absorbed from the gastrointestinal tract in the portal circulation, reducing LPS stimulation and attenuating GI damage as shown by lower concentrations of I-FABP.

Another possible mechanism at which curcumin may attenuate GI damage is via reductions in LPS-stimulated IL-1β-induced production by intestinal epithelial cells and through reductions in IL-1β-

induced activation of p38 MAPK. Through this pathway, curcumin was shown to reduce the expression of myosin light chain kinase, a key mediator of intestinal integrity, resulting in a better maintenance of the tight junction complexes within the intestinal barrier (Wang, Ghosh & Ghosh, 2017). It is also possible that curcumin may have influenced changes within the gut microbiota. Peterson et al. (2018), reported a significant change in the gut microbiota following an 8-week supplementation with either turmeric or curcumin compared to placebo. Specifically, they found an average increase of 69% in detected species in the curcumin group, with notable increases in Clostridium spp., Bacteroides spp., Citrobacter spp., Cronobacter spp., Enterobacter spp., Enterococcus spp., Klebsiella spp., Parabacteroides spp., and Pseudomonas spp. It is well documented that the gut microbiota plays a key role in the maturation of the immune system and intestinal barrier function (Smith, McCoy & Macpherson, 2007; Hooper et al., 2001). Indeed, disruptions in the composition of the gut microbiota (dysbiosis) is consistently proposed to contribute to the pathogenesis of celiac, irritable bowel syndrome and inflammatory bowel disease (Cerf-Bensussan & Gaboriau-Routhiau, 2010).

The impact of dysbiosis on intestinal barrier integrity has been highlighted in murine models. Studies using germ-free mice have shown that a lack of gut microbiota impairs the morphology and structure of the GI barrier (Smith et al., 2007; Hooper et al., 2001). In germ-free mice, the absence of gut bacteria reduced the intestinal surface area and shorter ileal villi than those born in conventional conditions (Gordon & Bruckner-Kardoss 1961; Abrams et al., 1963; Meslin et al., 1999). This was accompanied by a lower rate of turnover of ileal and Preyer's patches in germ-free mice (Abrams et al., 1963). Such impairments in cell renewal can reduce the proliferation and regenerative ability within the intestinal epithelia, highlighting the importance of gut bacteria on GI barrier integrity. Additional studies in germ-free mice also show the importance of gut bacteria on mucus production. Germ-free mice have lower numbers of mucin-secreting goblet cells and display a thinner, less stable, and compact mucus layer than colonised animals (Meslin et al., 1999; Szentkuti et al., 1990; Petersson et al., 2011). Mucins are essential in preventing luminal bacteria from adhering or

penetrating the GI barrier. Although it is unlikely that the participants within the present study were in a state of dysbiosis, the importance of gut bacteria on the GI barrier integrity is evident. Moreover, it can be speculated that curcumin altered the gut microbiota in favour of commensal bacteria that contribute significantly to the maintenance of GI barrier integrity and the attenuation of I-FABP.

5.5.3 Vitamin D_3 and GI barrier integrity

In addition to curcumin, the high dose of vitamin D may have contributed to the attenuation of GI damage. Vitamin D plays a pivotal role in the maintenance of GI barrier integrity by regulating proteins associated with intestinal epithelial and tight junctions (Kong et al., 2008). Specifically, vitamin D receptors are known to transcriptionally regulate claudin-2, 5, 12 and 15 (Chatterjee et al., 2021; Zhang et al., 2021). Reductions in vitamin D receptors within intestinal epithelial results in lower protein levels of claudins and thus, loss of GI barrier integrity. To initiate transcription, 1,25-dihydroxy vitamin D (the active form of vitamin D) must first bind to a vitamin D receptor. The vitamin D receptor heterodimerizes with the retinoid X receptor in nuclei and then binds to the vitamin D-response element in the promotor of the target gene, activating gene transcription (Kato, 2000). Indeed, evidence from in vitro shows that the expression of vitamin D receptors correlates with the expression of intestinal tight junction zonula occludins, occludins and claudins (Liu et al., 2017; Wang et al., 2019; Palmer et al., 2001). Little evidence is available for the role of vitamin D and vitamin D receptors in vivo, however in cirrhotic rats, vitamin D_3 significantly attenuated bacterial translocation and permeability (Lee et al., 2021). This finding was in parallel with upregulated occludin and claudin in the small intestine and colon. Furthermore, it is possible that in the present study that the vitamin D_3 component of the supplement had increased the expression of key structural proteins within the GI barrier.

5.5.4 Impact of SUP on GI and upper respiratory illness

Acute illness is the second most common reason for an athlete to receive medical attention (Palmer-Green et al., 2015). In the present study, upper respiratory illness was reported as 3.9 (SUP) and 10.6 (CON) per 1000 player days. Previously, illness has been shown to occur in 7-17% athletes at major sporting events (Schwellnus et al., 2016). In professional football, illnesses have an incidence of 0.6 and account for 2.5 days lost per 1000 playing hours (Sprouse et al., 2020). The incidence in the current study is greater than that reported previously, which may be due to transmission within the squad. Although, acute illness has less influence on availability than physical injury, the fact that it can impair performance (Jaworski & Carrie, 2019) highlights the need to mitigate its occurrence.

In the current study, the combination of vitamin C, vitamin D_3 and turmeric root was able to reduce the incidence of URS. To our knowledge, this is the first time that a combined supplement with these elements has improved respiratory illness in athletes. The combined nature of the supplement makes it difficult to identify the exact mechanisms that underpin these therapeutic effects. Vitamin D deficiency has been associated with a compromised immune system (Lucas et al., 2014). Indeed, a cross sectional study found that insufficient vitamin D_3 concentrations increased the likelihood of duty absence due to respiratory illness in military personnel (Laaksi et al., 2007). Similarly in athletes, lower vitamin D concentrations were apparent in those that reported a positive respiratory infection (Cox et al., 2008), while four-weeks of 5000 IU/day vitamin D_3 reduced daily upper respiratory tract infection severity in taekwondo athletes during the winter period (Jung et al., 2018). Although the exact mechanisms are unclear, it is possible that vitamin D supports the physical barrier within the respiratory system, which may contribute to the therapeutic effects on URS. Specifically, vitamin D can upregulate the production of tight-junction proteins that can strengthen the barrier and protect against the infiltration of bacteria and viruses (Chen et al., 2018). Vitamin C is an essential micronutrient involved in various cellular functions of both the innate and adaptive immune systems. A Cochrane review containing 29 trial comparisons found that vitamin C reduced both the duration and severity of the common cold (Hemila & Chalker, 2013). In ultra-marathon runners, a 600mg/day of vitamin C for 21 days halved the incidence of a cold and flu in the 14 days

after an ultra-marathon event (Peterson et al., 1993). Vitamin C may contribute to the reduction in illness via its anti-inflammatory properties. Vitamin C can protect cells from oxidative damage, alleviating neutrophil oxygen radical production, cytokine production and inflammation (Dwenger et al., 1992; Schwager & Schulza, 1998; Bijur et al., 1999).

Like vitamin C and D, curcumin, a key ingredient of turmeric is also shown to have antioxidant, anti-inflammatory and anti-viral propeties (Zielinska et al., 2020). Curcumin can modulate immune function by promoting the activation of immune component cells, reduce excessive activation of inflammation, and enhance endogenic immune activity to protect against pathogens (Kahkhaie et al., 2019). Specifically, curcumin can supress intracellular NF-kB, MAPKs and JAKs/STATs pathways regulating the expression and secretion of key inflammatory mediators, IL-1B, TNF-a, IL-2, IL-6, and IL-10 (Yaw-Syan Fu et al., 2021). Indeed, 180 mg/day of curcumin for 7 days attenuated the IL-8 response in the 12h following maximal isokinetic exercises (Tanabe et al., 2019). While a larger dose (2800mg/day) was shown to reduce circulatory CRP in elite footballers (Clayton et al., 2023). It is plausible that the 700mg dose of curcumin within the supplement contributed to the suppression of inflammatory mediators and attributed to the reduction in URS in the present study. Another possible mechanism is that curcumin provided anti-viral effects. Curcumin can combine with viral coat proteins, virus -specific enzymes or RNA polymerase and abolish virus replication, minimising infection development and damage to cells (Yaw-Syan Fu et al., 2021). Nevertheless, due to the nature of supplement used in the present study it is unclear whether improvements are orchestrated through the combination or just one of the ingredients.

5.5.5 Practical implications

Overall, these findings show promise in the use of a combined supplementation of turmeric (curcumin), vitamin D and C for maintaining GI integrity and reducing acute illness in elite footballers. The significance of these findings is particularly important within the context of elite football, given the various stressors faced by players. The frequent travel (Schwellnuss et al., 2012),

high workload (Budgett 1990), physical contact (Turbeville et al., 2006), poor sleep and prolonged training (Wentz et al., 2018) have all been linked to GI and URS disturbances. Traditional vitamin supplementation in the form of tablets has often exhibited low adherence rates (Osterberg & Blaschke, 2005) with excessive amounts manifesting unwanted side effects such as GI distress (Jacob 2002). Nevertheless, in the present study, participants anecdotally highlighted the user-friendly nature of the 60mL shot, describing it as “easy to consume”. There were also no reports of additional GI distress when taking the supplement. This evidence shows that in addition to the combined supplement being beneficial for mitigating acute illness and GI complications, it is also adherable within elite team sports.

5.5.6 Strengths and limitations

This study has several strengths and limitations. One significant strength is that it was conducted within an applied environment with elite footballers, it is also to our knowledge, the first study to assess GI damage during a professional team-sport fixture. Moreover, it is one of the first studies to investigate a supplement of this nature across the course of a competitive season in elite sport. The nature of the study meant it was impossible to blind participants to the study intervention, which may have influenced the findings. There is some evidence that the placebo effect can influence illness responses, with reports of an 85% reduction in cough symptoms (Eccles, 2002). Nevertheless, future studies should aim to incorporate a suitable placebo to mitigate the risk. Another limitation is that the comparison between control and SUP did not occur during the same time of year. There is a known seasonal variance in URS risk and incidence which may have influenced the impact of the intervention. The omission of a control group during the intervention period makes it more difficult to compare as it is unclear whether a control group would have experienced fewer or greater URS episodes. The differences in the length of the control and supplementation period also meant we could not measure the duration of illness. Instead, we opted to characterise illness as a ratio (illness per 1000 player days). This method is used elsewhere (Dvorak et al., 2011) and did still show positive

effects, but we may have not seen the full benefits of the supplement regarding illness recovery. The applied nature of the study limited the controlling of diet and physical activity. We could not control when and what the participants ate and could not obtain a detailed supplementation history for each player. However, as the participants were all part of the same squad, it was unlikely that significant variations occurred between players across the course of the season. We did instruct participants to maintain their usual dietary and supplementation patterns with the exception of additional vitamin C, D and turmeric consumption. Although it is recommended that future studies should adopt more vigorous control conditions, our design allowed 'real life' responses and did not disrupt coaches or participants during training and competition. The improve our knowledge on this combined supplement, future studies should conduct the supplement over longer periods, use greater controls and explore specific dosing strategies.

5.5.6 Conclusion

In conclusion, the main findings of the present study were two-fold, one being that a competitive match in professional football causes GI damage as shown by elevated circulatory I-FABP. And secondly, that the daily consumption of a turmeric root, vitamin C and vitamin D_3 combined supplement attenuated the post-match elevation in I-FABP, while reducing daily GI distress and upper respiratory symptoms in elite footballers. The exact mechanisms are unclear, but the fact that GI damage was reduced indicates a possible mechanism. These findings suggest that a combined turmeric root, vitamin C and vitamin D supplement may be beneficial for maintaining GI health and alleviate the burden of illness in elite male footballers.

6. Chapter 6 – Effects of 24-week prebiotic intervention on self-reported upper respiratory symptoms, gastrointestinal symptoms, and markers of immunity in elite rugby union players.

Note – the data presented here has been peer-reviewed and published (Parker et al., 2023). All data here in Chapter 6 was collected and analysed by Connor Parker.

Parker, C., Hunter, K. A., Johnson, M. A., Sharpe, G. R., Gibson, G. R., Walton, G. E., ... & Williams, N. C. (2023). Effects of 24-week prebiotic intervention on self-reported upper respiratory symptoms, gastrointestinal symptoms, and markers of immunity in elite rugby union players. European journal of sport science, 23(11), 2232-2239.

Parker, C., Hunter, K. A., Johnson, M. A., Sharpe, G. R., Gibson, G. R., Walton, G. E., ... & Williams, N. C. (2023). Effects of 24-week prebiotic intervention on self-reported upper respiratory symptoms, gastrointestinal symptoms, and markers of immunity in elite rugby union players. *European journal of sport science*, *23*(11), 2232-2239.

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6.1 Abstract

Objectives Elite rugby union players face numerous physiological and psychological stressors which can increase upper respiratory and gastrointestinal illness risk, which in turn can compromise training and competitive performance. This study aimed to investigate the effect of a daily prebiotic supplementation on upper respiratory symptoms, gastrointestinal symptoms, and markers of immune function in elite rugby union players. **Methods** 33 elite rugby union players were randomly assigned to consume a prebiotic (2.9g/day galactooligosaccharide) or placebo (2.9g/day maltodextrin), daily for 168 days under double-blind conditions. Participants completed daily and weekly questionnaires for self-reported upper respiratory and gastrointestinal symptoms respectively. Blood and saliva samples were collected at 0, 84, and 168 days for assessment of plasma TNF-α and CRP, and saliva IgA respectively. **Results** The prebiotic group experienced a 2-day reduction in upper respiratory symptom duration (7.4 ± 2.8 vs 9.8 ± 4.1 days) (p = 0.045). Gastrointestinal symptom severity (AUC: 50 vs 149) ($p < 0.001$) and incidence were lower in the prebiotic group compared to the placebo group (symptom free weeks: 11.0 ± 5.2 vs 7.0 ± 4.9)(P = 0.041) respectively. Salivary immunoglobulin A secretion rate was 42% greater in the prebiotic group compared to the placebo group at day 168 (P = 0.004), no differences in CRP and TNF-α were found ($P > 0.05$). **Conclusion** A 168-day dietary prebiotic intervention reduced the duration of upper respiratory symptoms and reduced the incidence and severity of gastrointestinal symptoms in elite rugby union players. These findings suggest that seasonal prebiotic interventions may be beneficial for reducing illness in elite rugby union players, improving their availability to train and compete.

6.2 Introduction

In elite sport, the presence of a gastrointestinal (GI) or upper respiratory illness can be detrimental to training and competition (Parry & Dust, 2006; Raysmith & Drew, 2016). Concerningly, GI and upper respiratory illness are the most common non-injury related reason for an athlete to require medical attention (Engebretsen et al., 2013; Soligard et al., 2018). Typically, GI symptoms (GIS) are categorised into upper and lower regions, including symptoms such as bloating, belching, flatulence, nausea and diarrhoea (Peters & Bateman, 1983; Drew et al., 2017; Hellard et al., 2015; Svendsen et al., 2016; Wentz et al., 2018). Depending on the mode, intensity and duration of exercise, GIS are reported by 30-90% of athletes (De Oliveira, Burini, & Jeukendrup, 2014). The aetiology of GIS during exercise is complex and likely multifactorial. The redistribution of blood flow and splanchnic hypoperfusion is regularly proposed as a key contributor to the onset of GIS, though dietary intake, time of day, sleep, medication, and the external environment may also contribute (Costa et al., 2020; Gaskell et al., 2020; Pals et al., 1997; Smith et al., 2021; Wilson et al., 2020; Wilson et al., 2015).

Upper respiratory symptoms (URS) are commonly associated with common colds and influenza and include symptoms such as coughing, sneezing, sore throat & nasal congestion. In sport, upper respiratory illness account for 35-65% of non-injury related problems (Fricker et al., 1999). Heavy exercise, psychological stress, poor sleep, and long-haul travel are all linked to the high prevalence of URS in athletes (Fitzgerald 1988; Wentz et al., 2018; Drew et al., 2017; Svendsen et al., 2016).

Most data exploring the incidence of GIS and URS in elite sport has focused on endurance events and Olympic competition rather than team-based sport. Team sport athletes follow physiological and psychologically demanding training schedules, with frequent competitive matches, limited recovery time, and regular international travel. Collectively, these stressors may impair immunity and increase the risk of acute illness which can be detrimental to performance. Indeed, the presence of GIS have been cited to impair performance in 14% of male team sport athletes (Wilson, Fearn & Pugh, 2022).

High incidences of URS are also commonly reported at major tournaments in both football and rugby union (Dvorak et al., 2011; Theron et al., 2013; Schwellnus et al., 2012). This coupled with our findings in chapter 5 clearly show that elite team-sport athletes are at similar risk of infection and GI disturbances to endurance-based athletes.

Rugby union is a unique sport which includes high intensity intermittent actions, high training volumes, collisions, limited recovery time and regular travel. This in addition to large squads, close contact and the sharing of equipment and facilities may increase the risk of infection and transmission. Indeed, a recent study observed disruptions to the GI tract following a collision-based rugby training session (Chantler et al., 2022), while another found that 50% of rugby players experience GIS on a weekly basis (Chantler et al., 2024). This is accompanied by an average of four URS episodes across the season, with the greatest incidences reported during pre-season and winter (Cunniffe et al., 2009; Tiernan et al., 2020; Keaney et al., 2021). Therefore, identifying interventions that may help reduce these clinical presentations, or accelerate illness recovery to allow return to play is imperative for optimising rugby players health and team performance.

The profile, genetic material, and functional activity of the gut microbial community (the gut microbiome) have a substantial influence on systemic immunity (Roberfroid et al., 2010). Manipulation of the gut microbiome is possible through dietary intervention, most commonly through pro- or prebiotic dietary supplements. This may provide a potential strategy to help reduce URS and GIS in team sport athletes. Probiotic supplementation has been shown to reduce URS incidence in active runners (Cox et al., 2010; Gleeson et al., 2011; Strasser et al., 2016). This improvement was attributed to a better maintenance of salivary immunoglobulin A (sIgA) (Gleeson et al., 2011), an antibody which provides the initial barrier of defence against invading pathogens. Furthermore, a 2015 Cochrane meta-analysis concluded that probiotics reduced the number and duration of URS episodes in adults and children (Zhao et al., 2022). Similarly in elite rugby union, the use of a multi-strain probiotic showed a trend for ~2 day reduction in duration of URS (Haywood et al., 2014).

Currently, the variety of probiotic strains used across different studies creates uncertainty as to which may be most beneficial for athlete health.

Prebiotics are a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017). Galactooligosaccharides (GOS) are a prebiotic derived from the action of the enzyme β-galactosidase on lactose and provide an alternative to probiotics. Moreover, prebiotics tend to act at the genus level thereby overcoming species variability that exists with probiotics. Bimuno-galactooligosaccharides (B-GOS) have been shown to increase the count and activity of the genus *Bifidobacterium* (Depeint et al., 2008; Vulevic et al., 2008) and elicit immunomodulatory effects as shown by reductions in proinflammatory cytokines (C-reactive protein and interleukin-1β) in elderly, overweight, asthmatic, and healthy individuals (Vulevic et al., 2013; Vulevic et al., 2015; Williams et al., 2016). GOS has also previously been shown to reduce the number of URS days, and severity of GIS in a student cohort (Hughes et al., 2011) and reduce incidence of travellers' diarrhoea (Drakoularakou et al., 2010; Hasle et al., 2017). Whether similar improvements can be replicated in elite rugby union players is currently unknown.

The aim of this study was to assess the effects of a 168-day B-GOS supplementation on the severity, duration and incidence of URS and GIS, sIgA, and plasma concentrations of C-reactive protein and TNF-α in elite rugby union players during a competitive season. It was hypothesised that B-GOS would reduce URS, GIS, TNF-α, CRP and enhance sIgA.

6.3 Methods

6.3.1 Study Design and Participants

The study was a randomised, double-blind, placebo-controlled trial over 168 days during a regular rugby union season in the Gallagher English Premiership. Forty-one healthy, elite rugby union players (age 23 ± 4 years; body mass 103 ± 13 kg; height 186 ± 7 cm) from a single club volunteered to participate in the present study. Participants were non-smokers, had no history of gastrointestinal illness (e.g. irritable bowel syndrome, inflammatory bowel disease, lactose intolerance and chronic constipation or diarrhoea) and were not regularly consuming foods enriched with probiotics, prebiotics, or vitamins. They were matched into pairs based on body mass and playing position before randomly being allocated an intervention. All data were collected between September 2019 to February 2020 in the English autumn and winter months (temperature range -4 to +25^{o}C). The study duration was originally scheduled for 252 days but was terminated at 168 days due to the Coronavirus-19 pandemic. The study was conducted in accordance with the Declaration of Helsinki, Human Tissue Act 2004 and approved by the Nottingham Trent University human ethics committee (ethical protocol 612, approved 23rd May 2019). All participants were informed, both verbally and in writing, of the nature of the study before providing written consent to participate.

During the study, a typical week for participants included four to five training days (~5 hours per day), one competitive match and at least one day of rest. Training included resistance, skills, fitness, tactics, and match play exercises. Players not named in the competitive matchday squad trained an extra day. Participants did not follow individualised diet plans but were provided meals onsite during training days. Similarly, no meal plan was provided when away from the training ground. However, individuals were instructed to avoid any foods and supplements enriched with probiotics (i.e. fermented products), prebiotics (i.e. foods containing prebiotic fibres), and vitamins. In addition, players were asked to report the use of prescribed and/or over the counter cold and flu remedies. Before each data

collection visit, players were asked to arrive following an overnight fast, and to have avoided using mouthwash as this could impact the saliva collection

6.3.2 Supplementation

Players were randomised to consume either 2.9g/day of the commercially available B-GOS (Bimuno, Clasado Biosciences Ltd, Reading, UK) or 2.9g/day placebo (maltodextrin) provided as a powder in single dose sachets (Clasado Biosciences Ltd, Reading, UK). Both supplements were identical in taste and colour and were blinded at the site of manufacture (Clasado Biosciences Ltd). The research team, club staff, and participants remained blinded until all statistical analysis was complete. Participants were provided with supplement pre-mixed in water at the training ground and consumed under observation by a member of club staff. On rest days, players were instructed to mix the sachet into water and consume at breakfast. Participants returned used and unused sachets to assess supplement adherence.

6.3.3 Daily upper respiratory symptoms

To establish the presence of URS, participants completed the Jackson questionnaire daily (Jackson et al., 1958). The presence of 8 symptoms (headache, chilliness, sneezing, sore throat, malaise, cough, nasal discharge, nasal obstruction) were rated on a scale of 0-3 (0-none, 1-mild, 2-moderate, 3-severe). Total symptom scores for each day were summed to give a total Jackson symptom score. An episode of URS was defined using the Jackson criteria as applied by (Martineau et al., 2015) with an episode defined by any period lasting ≥ 3 days with a Jackson score ≥ 14,or a symptom score < 14 with a subjective impression of having a cold for at least 3 days the presence of nasal discharge. If URS returned within one week it was regarded as the same episode (Gleeson et al., 2011).

6.3.4 Weekly gastrointestinal symptoms

To assess the presence of GIS, participants completed a GIS tool at the end of each week (Gaskell et al., 2019). Participants were educated and advised to rate the presence each symptom over the past week using the 10-point visual analogue scale, with 1-4 indicative of mild GIS, 5-9 indicative of severe GIS, and 10 indicating extremely severe GIS. If no specific GIS was reported, this would be rated as zero. The presence of either regurgitation, projectile vomiting or defaecation were rated as either 0 or 10 as these are extremely severe or not present at all. All symptom scores were summed to give a weekly total and incidence. Symptoms associated with the upper and lower GI tract were also separated to give total symptom scores for both upper and lower regions as previously described (Gaskell et al., 2019). To evaluate whether there were any between-group differences when starting the study, players reported GI symptoms for the week prior to day 0 measurements and the start of the intervention.

6.3.5 Collection and analysis of sIgA

At day 0, 84 and 168 all participants provided a saliva sample to determine sIgA. Participants rinsed their mouth using plain water and remained seated for 10-min. An unstimulated, passive saliva sample was produced, whereby participants were instructed to tilt their head forward and release saliva into the collection tube for 2-mins. Samples were immediately frozen at -20^{o}C and then at -80^{o}C within 48 hours until analysis. Upon analysis, samples were fully thawed at room temperature and sIgA concentration was determined by enzyme-linked immunosorbent assay (ELISA) (Salimetrics, Philadelphia, PA). The sIgA, intra- and inter-assay variation was 3.2% and 7.3% respectively, and the minimum detectable level of the assay was 2.5 µg/mL which all samples exceeded. sIgA secretion rate was calculated by multiplying the concentration of sIgA (µg/mL) by the flow rate (volume/duration), resulting in a concentration measure per unit of time (µg/min) as per manufacturer's instructions. In

healthy, illness free athletic populations, mean sIgA secretion rate was reported to be 80.3 µg/min (Gleeson et al., 2012).

6.3.6 Collection and analysis of blood biomarkers of systemic inflammation

All participants provided a blood sample at day 0 (pre intervention), 84 and 168 to determine TNF-α and CRP concentrations. Samples were drawn from the antecubital vein in two 10ml vacutainers, one containing a heparin and one containing a EDTA anticoagulant (BD Vacutainer®). Samples were then centrifuge with the plasma immediately frozen at -80^{o}C until further analysis. TNF-α was assessed using a high sensitivity ELISA and CRP was assessed using a regular ELISA protocol (R&D systems). For TNF-α, the intra- and inter-assay variation was 7.5% and 5.8% and the minimum detectable level of the assay was 0.022 pg/ml which all samples exceeded. For CRP, the intra- and inter-assay variation was 4.4% and 7.8% and the minimum detectable level of the assay was 0.022 pg/ml which all samples exceeded.

6.3.7 Statistical Analysis

Statistical analyses were performed using the statistical package for social sciences (IBM SPSS version 26, New York, United States). All data were checked for normal distribution using a Shapiro-Wilk test. Between-group comparisons for body mass, height, age, average weekly workload, and competitive minutes played were conducted using an independent t-test. Between-group comparisons were conducted for the area under the curve (AUC) of daily URS and weekly GIS over the 168 days using a Mann-Whitney U test to assess differences in symptom severity. Between group differences in URS incidence were assessed using a Mann-Whitney U test. Between group differences in URS episode duration and GIS free weeks were assessed using an independent t-test. CRP, TNF-α and sIgA secretion rate were evaluated using a Mixed-model analysis of variance (ANOVA). All significant interactions and main effects were assessed further with pairwise comparisons using Bonferroni corrections. Data evaluated using parametric tests are presented as mean ± standard deviation. Data presented using

non-parametric tests are presented as median (interquartile range). Statistical significance was set at $P < 0.05$.

6.4 Results

6.4.1 Player Characteristics

Thirty-three participants (n = 17 Placebo, 16 B-GOS) from the original 41 completed the full 168-day supplementation period. Eight participants were removed due to noncompliance with taking the supplement. Supplement adherence of the remaining 33 participants did not differ between groups (B-GOS 80.4 ± 13.9 % vs placebo 78.3 ± 14 %; *P* = 0.73). Body mass (B-GOS 103.4 ± 14.0 kg vs placebo 105.2 ± 13.2 kg), height (B-GOS 186.9 ± 9.4 cm vs placebo 186.6 ± 7.3 cm) and age (B-GOS 22.4 ± 3.3 years vs placebo 24.5 ± 5.2 years) was not different between groups. There were no between group differences in average weekly workload (B-GOS 671 ± 338 sRPE vs placebo 675 ± 340 sRPE) and competitive minutes played (B-GOS 657 ± 278 min vs placebo 597 vs 261 min) (P > 0.05). No players reported the use of cold and flu remedies throughout the duration of the study.

6.4.2 Upper respiratory Symptoms (URS)

A Mann-Whitney U test revealed no differences in the URS incidence rate between B-GOS (1.0 ± 1.4) and Placebo (1.0 ± 1.0) (*P* = 0.641). The duration of individual URS episodes was shorter in the B-GOS group (7.4 ± 2.8 days) compared to the placebo group (9.8 ± 4.1 days) (*P* = 0.045) (Table 1). There was no difference in AUC of the daily symptoms scores between the two groups (*P* = 0.77).

Table 6.1: Overview of self-reported URS data

	B-GOS				Placebo				
	Mean	**SD**	**Median**	**Range**	**Mean**	**SD**	**Median**	**Range**	**P Value**
Episode Duration (days)	7.42	2.83	7.00	(4-15)	9.82	4.05	10.00	(4-17)	0.045
Incidence (episode per person)	1.00	1.44	1.00	(0-5)	1.00	1.00	1.00	(0-3)	0.641
Severity (symptom score per episode)	43.47	27.41	35.80	(14-118)	59.97	34.17	50	(15-138)	0.118

6.4.3 Gastrointestinal Symptoms (GIS)

No differences in GIS over the previous 7-days prior to the start of the intervention (day 0) were evident between the two groups ($P = 0.53$). A Mann-Whitney U test revealed the AUC of total weekly symptom scores was lower in the B-GOS group (50 [10.5-139.5]) compared to the placebo group (149 [69-208]) ($P = 0.03$) (Figure 1a). AUC for weekly upper GIS scores was lower in the B-GOS group compared to the placebo group ($P < 0.001$) (Figure 1b), but no differences were found for lower GIS ($P = 0.113$). The number of symptom free weeks for total GIS ($P < 0.041$) and upper GIS ($P < 0.002$) was higher in the B-GOS group compared to the placebo group, whereas no differences were evident for lower GIS ($P = 0.151$) (Table 2).

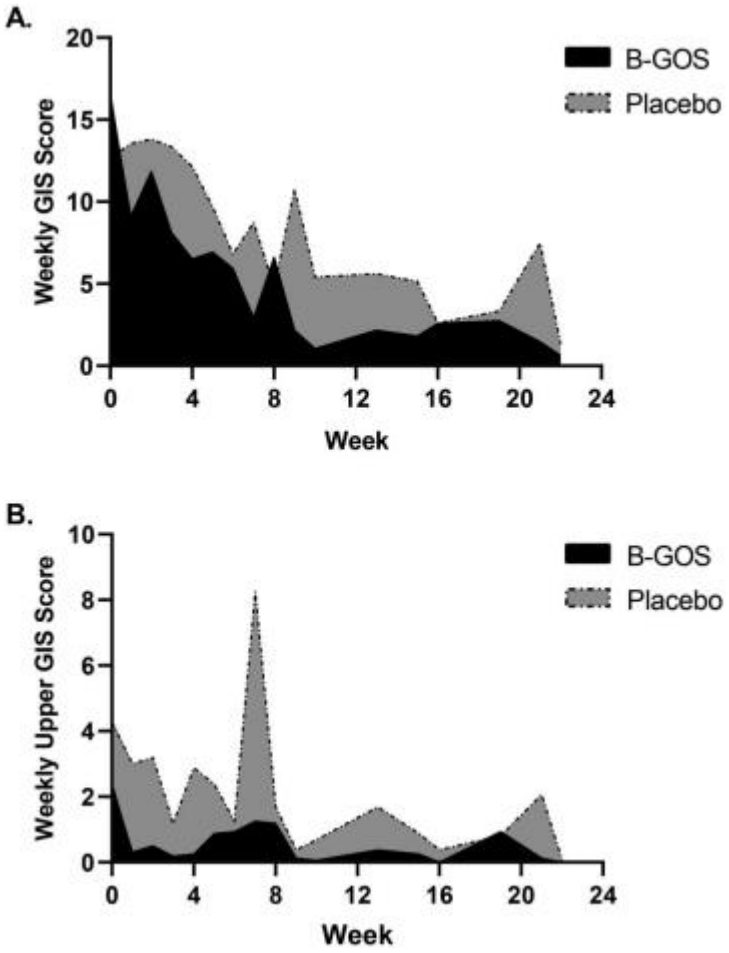

Figure 6.1A. Weekly GIS scores reported during 24-week study (B-GOS n = 16, Placebo n = 17). AUC analysis revealed between-groups differences in total GIS scores ($P = 0.03$). **Figure 6.1B.** Weekly upper GIS scores reported during 24-week study (B-GOS n = 16, Placebo n = 17). AUC analysis revealed between-groups differences in upper GIS scores ($P < 0.001$).

Table 6.2: Symptom free weeks for GIS during 24-week study.

Symptoms	B-GOS	Placebo	Mean Difference	P-values
	Mean (SD)	Mean (SD)	95% Conf. Interval	t-test
Total GIS free weeks	11 (5.2)	7 (4.9)	4 (0.2; 7.5)	0.041
Upper GIS free weeks	15 (2.7)	11 (3.6)	4 (1.5; 6.1)	0.002
Lower GIS free weeks	11 (5.0)	9 (4.9)	3 (-1.0; 6.3)	0.151

6.4.4 Systemic inflammation

There was no trial x time interaction for plasma CRP (pooled data, B-GOS 1503.27 ± 1356.32 ng/mL vs Placebo 1742.67 ± 1486.77 ng/mL) and TNF-α (pooled data, B-GOS 0.82 ± 0.25 pg/mL vs Placebo 0.90 ± 0.33 pg/mL) (P > 0.05).

6.4.5 Salivary Immunoglobulin A

There was a main effect of time for sIgA secretion rate (p = 0.009) but not group (p = 0.942). There was a significant trial x time interaction for sIgA secretion rate (*P* = 0.001). No differences were observed in sIgA secretion rates at day 0 or 84, but sIgA secretion rate was higher in B-GOS (129.23 ± 38.15 µg/min) than Placebo (90.06 ± 33.45 µg/min) at day 168 (*P* = 0.004) (Figure 6.2).

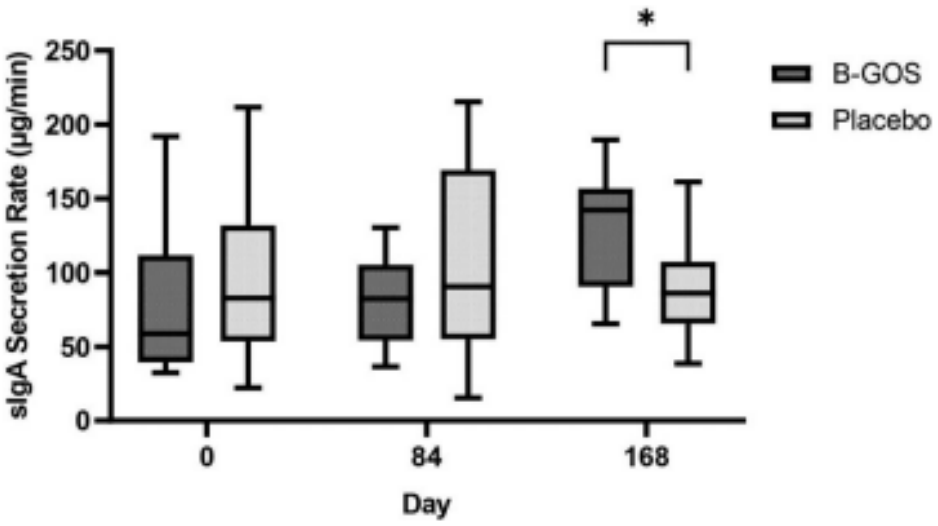

Figure 6.2 Saliva IgA secretion rate before and after 12 and 24 weeks of the study**.** (B-GOS n = 16, Placebo n = 17), data presented as mean ± SD. Asterisk (*) denotes significant difference between groups (*P*<0.05).

6.4.6 Summary of Findings

- **The duration of URS episodes 2.5 days less in the B-GOS group than Placebo group, but there were no differences in incidence or severity.**
- **The severity of GIS over the course of the 168-day supplementation as shown by AUC was lower in B-GOS than Placebo.**
- **Players in the B-GOS group experienced more weeks without the presence of GIS than those in the Placebo group.**
- **sIgA secretion rate was higher in B-GOS than Placebo after 168 days of supplementation.**

6.5 Discussion

The main findings of this study were that daily supplementation with B-GOS reduced the duration of URS and incidence of GI symptoms in elite rugby union players over a 168-day period. Furthermore, B-GOS increased sIgA secretion rate at 168 days when compared to the placebo group. These findings suggest that B-GOS can potentially modulate the immune and GI system, suppressing URS and GI discomfort experienced by elite rugby union players.

6.5.1. Impact of Prebiotic B-GOS on URS and GIS

Illness can have detrimental effects on athlete training availability and match preparation (Cunniffe et al., 2009; Tiernan et al., 2020; Keaney et al., 2021). The influence that the gut microbiome can have on the immune system has encouraged research into the potential use of dietary interventions to enhance immune function and mitigate illness risk. To our knowledge this is the first study to assess the effect of a prebiotic dietary intervention on URS and GIS in an elite-athletic population. The 2-day reduction in URS episode duration is relevant for athletes and coaches, potentially aiding a quicker return to play following an URS episode. Our findings are similar to Hughes et al who observed reductions in GIS incidence and the percentage of days with URS in academically stressed students following a daily dose of either 2.5g or 5g of GOS over 8-weeks (Hughes et al., 2011). In addition, probiotic interventions in similar athletic cohorts have also reported reductions in URS incidence and duration (Cox et al., 2010; Gleeson et al., 2011; Strasser et al., 2016; Haywood et al., 2014). Haywood et al also observed a 2-day reduction in URS episode duration and GIS incidence in elite rugby union players following use of a multi-strain probiotic (Haywood et al., 2014). However, not all studies have reported improvements in URS following probiotic supplementation (West et al., 2011; Gleeson et al., 2012). Differences between previous studies could be due to variations in URS incidence and chosen probiotic strains. One strength of using prebiotic dietary interventions such as B-GOS in comparison

to probiotics, is that prebiotics have been consistently shown to reach the gut undigested and increase the number and activity of beneficial bifidobacterial, alongside the production of beneficial metabolites (SCFA) (Depeint et al., 2008; Vulevic et al., 2008; Vulevic et al., 2013).

6.5.2. Mechanisms of Action

The mechanisms by which prebiotics reduce URS and GIS is likely to involve the increase of short chain-fatty acid (SCFA) producing bacteria, such as *bifidobacteria*. Indeed, elevated faecal SCFA concentrations have been accompanied by enhanced gut epithelial integrity and mucosal immunity (Mariadason et al., 1997; Hernot et al., 2009). B-GOS has previously been shown to encourage the growth of *bifidobacteria* in the human gut and subsequently confer numerous health benefits such as reduced systemic inflammation and improved immune response in elderly and overweight populations (Vulevic et al., 2008; Vulevic et al., 2013). In the current study B-GOS increased sIgA secretion rates at day 168 when compared to placebo. This finding is consistent with the notion that positive manipulation of the gut microbiome may support mucosal immunity and salivary IgA production. Sixteen weeks use of the probiotic *Lactobacillus casei* Shirota maintained salivary IgA during the winter season and reduced URS incidence in active runners (Gleeson et al., 2011). However, our findings contrast with a 12-week supplementation of prebiotic B-GOS in obese individuals, which showed an increase in faecal IgA but not saliva (Vulevic et al., 2013). Nevertheless, the intervention period in that study was only 12-weeks, thus it is possible that changes in sIgA occur between 12 and 24 weeks.

6.5.3. Limitations

It should be noted that the total number of URS episodes across both groups in the present study were lower than those previously reported for the winter season. Elite rugby union players have been reported to have experienced four URS episodes per season (Cunniffe et al., 2009), with the greatest incidence during winter. We reported significantly lower rates with higher incidences in the early part

of the season. This may explain why B-GOS had little influence on URS incidence and severity. However, it does suggest B-GOS can still improve URS duration even when incidence rates are low. It was also found that participants continued to train despite showing URS. This has been seen elsewhere in a similar cohort and may be because the participants were required to train when at the training site (Cunniffe et al., 2009). They may also fear the possibility of being deselected from the upcoming match. However, it should also be considered that the symptoms could be non-infection related. These are limitations of collecting self-reported data, and future research should determine infections using molecular testing. Similarly, despite no participants reporting the usage of additional URS treatments (e.g. cortisone nasal spray), it was not possible to track whether participants used additional treatments outside of the club. Another limitation was the frequency of sIgA and cytokine measurements. It is possible that B-GOS reduced the duration of URS through enhancing sIgA secretion rate. However, it was beyond the scope of the present study to examine weekly changes in sIgA and their association with URS. This should be considered for future research.

6.5.4. Conclusion

In conclusion, over a 168 day supplementation period the prebiotic B-GOS reduced the duration of URS, incidence and severity of GIS and enhanced sIgA secretory rate in elite rugby union players. These findings suggest that prebiotic use may have the potential to modulate immune function and reduce illness, which may improve an athlete's availability to train and compete. The mechanisms by which B-GOS reduces URS and GIS requires further exploration.

7. Chapter 7 – Effects of a 6-week prebiotic intervention on indirect markers of gastrointestinal damage, gastrointestinal symptoms and self-reported upper respiratory symptoms following a simulated football match in the heat.

7.1 Abstract

Introduction: It is becomingly increasingly common for team-based athletes to perform in hot climates which may increase the likelihood of gastrointestinal and respiratory illness. The aim of the current study was to investigate whether a 6-week prebiotic intervention can alleviate gastrointestinal damage, enhance immunity, and reduce upper respiratory symptoms following football specific exercise in the heat. **Methods:** Twenty-six male team sport athletes were randomised to receive 2.8 g/d of either Bimuno-galactooligosaccharide (B-GOS; n=13) or a maltodextrin placebo (PLA; n=13) for 42 days in a double-blind parallel group design. At day0 and day42 of each intervention participants completed the football specific intermittent treadmill protocol (FSITP) in 33^{o}C and 50% relative humidity. Blood, saliva and GI symptoms were collected at day 0 and 42 for the assessment of plasma I-FABP, LBP, salivary IgA and GI discomfort. Participants also completed daily and weekly questionnaires for self-reported upper respiratory and gastrointestinal symptoms over the 42 days. **Results:** At D42 following the FSITP the Peak Δ in I-FABP was 35.42% lower in the B-GOS group than the placebo (χ^2 (1) = 3.995, P = 0.046). There was increase in SIgA secretion over the 42 days in the B-GOS group compared to fall in placebo group (B-GOS: +74.00 ± 204.40 μg·min^{-1}; Placebo: -106.19 ± 124.60 μg·min^{-1}; P = 0.016). During exercise, there was a greater reduction at day 42 in the total GIS score in B-GOS than PLA (B-GOS: -16.7 ± 96.3 %; PLA: 13.3 ± 66.9 %; P = 0.021). There was no between-group difference in URS incidence, but the mean duration of episodes was in B-GOS group (3.4 ± 5.1 days) compared to PLA (9.0 ± 5.9 days; P = 0.025) and the severity (B-GOS: 12 ± 16.5; PLA: 34.5 ± 22.5; P = 0.029) was lower in B-GOS than PLA. **Conclusion** 6-week of a prebiotic B-GOS intervention can improve GI barrier resistance and reduce GIS and better maintain sIgA secretion rate in response to football specific activity in the heat; whilst also reducing the duration and severity of slef-reported upper respiratory illness in team sport athletes.

7.2 Introduction

Exercise in the heat can acutely compromise GI and immune function as shown by the onset of gastrointestinal discomfort and decreases in the secretion rate of the primary antibody in saliva, immunoglobulin A (sIgA) (Yeh et al., 2013; Laing et al., 2005). Although, the exact mechanisms of exercise-induced gastrointestinal symptoms (GIS) are not clear, it is likely that splanchnic hypoperfusion and intestinal ischemia contribute (van Wjick et al., 2011). Splanchnic blood flow is reduced by 80% after 60-mins of submaximal exercise (Reher et al., 2001), disrupting intestinal barrier integrity and increasing permeability (Doklandy et al., 2016; Costa et al., 2017). Endotoxins can then translocate across the intestinal lumen, activating CD-14 and toll-like receptor 4, initiating both local and systemic inflammation (Ducharme et al., 2022). This can lead to GIS and nutrient malabsorption (Camilleri, 2019), which can interrupt performance and recovery.

Reductions in sIgA are commonly associated with an increased susceptibility to upper respiratory illness in athletes (Gleeson et al., 1999; Nieman & Nehlsen-Cannarella, 1991). sIgA secretion provides the initial line of defence against pathogens in the oral cavity (Dowd, 1999), with saliva flow rate considered the most influential factor (Walsh et al., 1999). Subsequently it is most appropriate to report sIgA in relation to its secretion rate (flow x concentration) to represent total sIgA availability and to correct for any drying effect during exercise (Walsh et al., 1999). Prolonged exercise in ambient and hot environments have been shown to reduce sIgA secretion rate (Walsh et al., 2002; Laing et al., 2005), with an increase in sympathetic nervous system activity and vasoconstriction of the blood flow to the salivary glands proposed as the mechanistic cause (Chicharro et al., 1998). Exercising in the heat is reported to induce a greater norepinephrine response and higher sympathetic activity than when performed in thermoneutral conditions (Galbo et al., 1979), possibly leading to a greater reduction in sIgA secretion rate and a higher risk of URS.

It is evident that an acute bout of continuous or intermittent exercise increases gastrointestinal damage, symptoms, and Immunosuppression (Yamauchi et al., 2011; Pugh et al., 2017; Mckenna et

al., 2022, Pugh et al., 2019; Yeh et al., 2013, Gaskell et al., 2020). Recent literature suggests that intermittent, invasion-based, team sports can also compromise gastrointestinal and immune function. Chantler et al. (2022), observed elevated gastrointestinal damage and permeability as shown by high plasma intestinal fatty-acid binding protein (I-FABP) and the urinary lactose/rhamnose ratio following a bout of collision-based rugby training. On average, rugby union players also experience four cold and flu episodes per year, hindering their availability for training and competitive matches. Elite football players experience similar illness burdens (Parry & Dust, 2006) and it was shown in a previous study shown within chapter 5 of this thesis that I-FABP increased from baseline following a competitive match (Clayton et al., 2024). This is not a surprise as soccer is an intense activity characterised by high-intensity sprints, separated by low intensity running (Bangsbo et al., 2006), with elite players covering 10-14km per match, achieving a heart rate of ~85% age predicted max (Di Salvo et al., 2013; Bangsbo et al., 2006). Concerningly, it is increasingly common that team-based sports are performed in hotter climates. Exercising in hotter environments provides further stress to the gastrointestinal tract than cooler environments, leading to greater damage and a higher risk of upper respiratory illness (Yeh et al., 2013; Snipe et al. 2018). Furthermore, it is imperative to find solutions that can reduce the likelihood of illness and discomfort during such performances.

The human gut microbiota harbours trillions of bacteria which contribute significantly to gastrointestinal integrity and function (Eckburg et al., 2005). Recent research has postulated that manipulation of the diet to help promote beneficial bacteria within the gut could reduce gastrointestinal perturbations during exercise. One common method used to manipulate the gut microbiome is using probiotics. Probiotics are live ingestible bacteria that when consumed can confer numerous health benefits (WHO, 2001). Indeed, probiotics are reported to reduce gastrointestinal symptoms, upper respiratory illness, and improve exercise performance (Gleeson et al., 2011; Pugh et al., 2019, Haywood et al., 2013; Cox et al., 2010, shing et al., 2014). There are, however, numerous studies that fail to demonstrate any positive effects in sport and exercise or GI

damage (Carbuhn et al., 2018; Cox et al., 2007, Shing et al., 2014; West et al., 2012). For example, a recent review observed that only 3 out of 14 studies have shown improvements in performance following a probiotic intervention (de Pavia et al., 2023). The equivocal findings of probiotics may be due to the wide variety of probiotic strains used with some unable to reach the small intestine where it can aid the proliferation of commensal bacteria.

An alternative method to alter the gut microbiota is through the use of prebiotics. Prebiotics are substrates that selectively utilised by host microorganisms conferring a health benefit (Gibson et al., 2017). One benefit of using prebiotics rather than probiotics is its ability to act at the genus level, which bypasses the species variability of probiotics. The commercially available Bimuno-galactooligosaccharide (Bimuno®) (B-GOS) has been shown to increase the count and activity of the genus Bifidobacterium (Depeint et al., 2008; Vulevic et al., 2008) and reduce systemic inflammation in elderly, asthmatic and healthy individuals (Vulevic et al., 2013; Vulevic et al., 2015; Williams et al., 2016). Recently, in a study within chapter 6 of this thesis we showed that a 24-week intervention with B-GOS reduced the duration of URS, and the severity and incidence of GIS in elite rugby union players during a competitive playing season (Parker et al., 2023). Although the mechanisms were unclear, there was a greater maintenance of sIgA secretion rate, implying that B-GOS may have influenced immune function. Although these findings are extremely novel and potentially significant for athlete availability. The design of the study did not allow for the assessment of gastrointestinal functioning, immunity, and symptoms in response to a specific exercise stressor. As acute exercise, particularly when performed in the heat can compromise GI and immune function, assessing B-GOS effectiveness when against a direct stressor is imperative.

Furthermore, the aim of this study was to assess the effects of a 6-week B-GOS supplementation on markers of exercise-induced GI damage, GIS, sIgA and upper respiratory illness following football specific exercise in 33^{o}C heat. It was hypothesised that when compared to a placebo supplement, B-

GOS supplementation will alleviate exercise-induced GI damage, GI discomfort and respiratory illness while enhancing sIgA concentrations.

7.3 Methods

7.3.1 Participants and study design

Thirty recreationally active team sport-based males [age 23.6 ± 3.2 years, height 182.2 ± 6.9 cm, body mass 79.83 ± 9.28, $\dot{V}O_{2max}$ 53.6 ± 4.7 $mL.kg^{-1}.min^{-1}$] participated in the current study using a double-blind, randomised, placebo-control design. All participants were non-smokers, had no history of gastrointestinal illness (e.g. irritable bowel syndrome, inflammatory bowel disease, lactose intolerance and chronic constipation or diarrhoea) and were not regularly consuming foods enriched with probiotics, prebiotics, or vitamins. To be able to participate, participants were required to perform at least 3h of team sport activity (e.g. soccer, rugby, hockey) or intermittent exercise per week. Once informed of the nature and risks associated with the study, participants provided their written informed consent. The study was approved by the Nottingham Trent University Ethics Committee and was performed in accordance with the declaration of Helsinki.

7.3.2 Overview of experimental design

All data was collected at Nottingham Trent university with each participant visiting the laboratory on 4 separate occasions. Each participant completed a baseline and familiarisation visit prior to experimental trials. The familiarisation and 2 experimental trials were performed in an environmental chamber (TISS series 201003-1, TIS services UK) set at 33°C and 50% relative humidity. Each experimental trial was separated by 6-weeks (42 days), whereby each participant was randomly allocated the prebiotic or placebo supplement to take daily. Each visit was completed at the same time of day and participants were instructed to arrive 2h post prandial and having consumed 500ml of water 2h prior to arrival. In the 24h preceding each experimental trial

participants replicated the same diet and were instructed to avoid any exercise, alcohol, and caffeine.

7.3.3 Baseline testing and familiarisation

During the first visit, participants performed a speed lactate test and an incremental exercise test to failure to obtain their maximal lactate steady state (MLSS) and maximal oxygen uptake ($\dot{V}O_{2max}$). Following a 5-min rest, a baseline capillary sample was collected from the fingertip for the analysis of blood lactate [La^B] (Biosen lactate analyser, EKF Diagnostics, Germany). Body mass, height, and resting heart rate (HR) (Beurer PM62, Beurer, Germany) were then collected. To start the speed lactate test, participants began running on a motorised treadmill (HP Cosmos, Germany) at a speed of 9km·h at a 1% gradient for 3 min. At the end of each 3 min stage the participant would straddle the treadmill and capillary blood sample would be collected (MacLeod et al., 2018). The speed would then increase by 1km·h and the test would continue until the MLSS threshold was achieved, which was defined as the fastest speed with less than a 1 mmol.L increase in [La^B] above the preceding levels (Astrand et al., 2003). After a 10 min rest, participants started the $\dot{V}O_{2max}$ test. Running initiated at their MLSS at a 1% gradient, with an increase of 1km·h^{-1} every minute until task failure. Ventilatory and pulmonary variables were measured breath-by-breath throughout the maximal test using a metabolic cart (Vyntus CPX, Vyaire Medical Inc, United Kingdom), which was calibrated with the known gases (O_2 = 15.96%, CO_2 = 4.99%). Participants wore a facemask for the entirety of the test (Hans Rudolph 7450, Hans Rudolph Inc, USA) and expired air was sampled using a DVT flow, and O_2 and CO_2 sensors. Ten second averages for the ventilatory and pulmonary variables were calculated by the system, with $\dot{V}O_{2max}$ considered the highest $\dot{V}O_2$ value over any 10s average. Together the results from the speed lactate and $\dot{V}O_{2max}$ test provided the speeds used for various stages of the football specific interval training protocol (FSITP). At least one week after the preliminary visit, participants completed the familiarisation trial and performed one half of the FSITP in 33°C.

7.3.4 Experimental visits

Participants arrived at the laboratory and provided a urine sample to measure urine osmolality (Pocket-Pal Osmo-Osmocheck™, 4595-E04, Vitech Scientfic Ltd, Horsham, UK). Following this, nude body mass (GFK 150 AEADAM digital scale, Vitech scientific Ltd) was measured in private. Participants then rested for 10-min before a venous blood, capillary blood and saliva sample was collected. Resting HR and core temperature (T_{core}) was then collected. T_{core} was collected via an ingestible telemetric pill (e-celsius BodyCap, France) which was ingested 6h prior to the trial. Participants then completed the 90-min FSITP in the heat chamber (33°C and 50% RH). The FSITP was made up of 2 x 45 min blocks of activity, separated by a 15 min rest period to replicate half-time. A 3 min water break was provided half-way through each half where participants could drink water ad libitum. The amount of water consumed was recorded and repeated for the subsequent trial. Throughout the FSITP, HR, T_{core} and rating of perceived exertion (RPE) (Borg et al., 1982) were recorded every 5 min. Another venous and capillary blood sample was collected at half-time. Thermal sensation and fatigue were assessed using subjective questionnaires every 15 min (Young et al., 1987; Micklewright et al., 2017). Immediately after completion of the FSITP, nude body mass, urine, venous, capillary, and saliva samples were collected. A final venous blood sample was collected after 60-min of rest. Each participant completed a gastrointestinal symptom questionnaire before, at half-time and on completion of the FSITP (Gaskell et al., 2019). Once completing the first experimental trial, the participant would begin the 6-week supplementation with either B-GOS or placebo. At 6-weeks, the participant would return and repeat the experimental trial.

7.3.5 FSITP

The entire FSITP protocol was performed on a motorised treadmill at a gradient of 1%. The treadmill speeds were customised and determined by the participants speed lactate and $\dot{V}O_{2max}$ results. A total

of 7 different speeds were incorporated into the protocol to simulate the intensity, accelerations, and decelerations of a football match. The speeds included stationary (0km·h), walking (4km·h), jogging (speed before MLSS), low speed (85% $\dot{V}O_{2max}$), moderate speed (100% $\dot{V}O_{2max}$), fast run (21 km·h) and sprint (25 km·h).

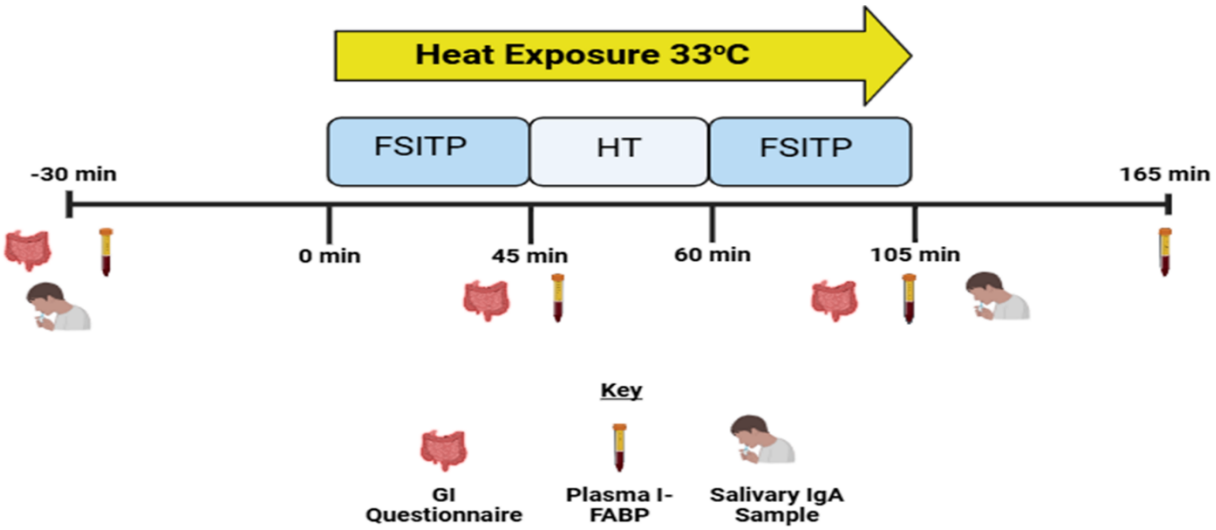

Figure 7.1 Schematic of main experimental trial on day 0 and 42. FSITP, Football Specific Intermittent Treadmill Protocol; HT, Half-Time; GI, Gastrointestinal; IgA, Immunoglobulin A; I-FABP, Intestinal Fatty Acid Binding Protein.

7.3.6 Supplementation

Participants were randomised to consume either 2.9g/day of the commercially available B-GOS (Bimuno, Clasado Biosciences Ltd, Reading, UK) or 2.9g/day of placebo (maltodextrin). Both supplements were provided as single dose sachets in powdered form and were identical in taste and colour. Supplements were blinded at the site of manufacture (Clasado Biosciences Ltd) and were not revealed to researchers until all data had been statistical analysed. Participants were instructed to consume the supplement mixed in water at breakfast. Each participant returned used and unused sachets to confirm supplement compliance across the 6-week period.

7.3.7 Collection and analysis of blood biomarkers

All participants provided blood samples prior (Pre), at half-time (HT), full time (FT) and 60 min post (Post60) in both experimental trials, either side of the supplementation period to determine plasma concentrations of I-FABP and LBP. Samples were drawn from the antecubital vein in three 6ml

vacutainers, two containing an EDTA and one containing a heparin anticoagulant (BD Vacutainer®). Samples were centrifuged immediately, with plasma aliquoted into clean Eppendorf tubes and frozen at -80°C until further analysis. I-FABP and LBP were assessed using commercially available enzyme linked immunosorbent Assay's (ELISA) (Hycult Biotech, Amsterdam, Netherlands). For I-FABP, the intra- and inter-assay variation was 2.7 % and 3.4%. For LBP, the intra- and inter-assay variation was 3.1% and 4.8%.

7.3.8 Collection and analysis of saliva

Before and immediately post FSITP in both experimental trials, a saliva sample was collected to determine salivary immunoglobulin A (sIgA). After rinsing their mouth with plain water and 10 min of resting, participants produced a saliva sample via the passive drool method. Participants were instructed to tilt their head forward and release saliva into the collection tube for 2-mins. Samples were weighed and immediately frozen in -80°C until further analysis. sIgA concentrations were determined by a commercially available ELISA (Salimetrics, Philadelphia, PA). The intra- and inter-assay variation was 1.7% and 2.6%, respectively. Due to the influence of flow rate on sIgA concentrations, sIgA secretion rate was calculated. sIgA concentration (µg/mL) was multiplied by the flow rate (volume/duration), resulting in a concentration measure per unit of time (µg/min).

7.3.9 Gastrointestinal symptoms during FSITP

To assess the presence and severity of gastrointestinal symptoms (GIS) during the FSITP, participants completed a GIS questionnaire at half-time and full-time (Gaskell et al., 2019). Participants were educated on the questionnaire and were advised to rate the presence of each symptom over each 45-min period using a 10-point visual analogue scale, with 1-4 indicative of mild GIS (not substantial

enough to interfere with exercise), 5-9 indicative of severe GIS (substantial enough to interfere with exercise), and 10 indicating extremely severe GIS (warrants exercise reduction or cessation). If no specific GIS was reported, this would be rated as zero. The questionnaire was adapted to incorporate 14 GIS, categorised as those associated with upper GIS, lower GIS, and other GIS. The rating for each symptom was summed to give a total for each 45-min period. The cumulative score for each 45-min were then summed to provide an overall score during the FSITP. This was repeated for the symptoms exclusive to the upper GI tract, lower GI tract and to those that are associated with neither and are simply categorised as 'other symptoms' (Gaskell et al., 2019).

7.3.10 Upper respiratory and gastrointestinal symptoms during supplementation

To assess the incidence and severity of upper respiratory symptoms (URS) during the supplementation period, participants completed the Jackson questionnaire daily (Jackson et al., 1958). Participants would state whether an upper respiratory illness was present and rated the severity of 8 symptoms (headache, chilliness, sneezing, sore throat, malaise, cough, nasal discharge, and nasal obstruction) over each 24-hour period. Each symptom was rated on a scale of 0-3 (0-none, 1-mild, 2-moderate, 3-severe). Total symptoms scores for each day were summed to give a total Jackson score. A URS episode was defined using the Jackson criteria as applied (Martineau et al., 2015) with an episode defined by any period lasting $\geq$ 3 days with a Jackson score 14 and the presence of nasal discharge, or a symptom score < 14 with a subjective impression of having a cold for at least 3 days. If URS returned within one week it was regarded as the same episode. Participants also stated the use of any over the counter or prescribed medication used during this period and if it impacted exercise.

To determine the incidence and severity of GIS during the supplementation period, participants completed the gastrointestinal symptom rating scale (GSRS) every 7 days. Participants were asked to

rate the presence and severity of 15 GIS during the previous 7 days on a seven-point Likert scale from "no discomfort" to "very severe discomfort" (Svedlund et al., 1988).

7.3.11 Statistical analysis

For I-FABP and LBP, the highest value was calculated from the HT, FT and P60 during each visit. This was then subtracted from the retrospective baseline value to show the peak change (peak Δ) for each visit. A Shapiro wilk test revealed this data to not be normally distributed, therefore a Kruskal-Wallis test was used to assess between group differences. The magnitude of change for sIgA secretion rate following the intervention was calculated by determining the relative change at FT from baseline at day 0 and day 42, the value for day 0 was then subtracted from the corresponding day 42 value. An independent T-test was used to assess between-group differences. GI symptoms during exercise were categorised into four categories (total, upper, lower and other). The symptom scores that were collected at HT and FT were summed to provide an overall score for each category. The percentage change from day 0 to day 42 were calculated for each category and between group differences were assessed using a Mann Whitney U test. The percentage change in GI symptoms during the intervention period at day 0 and day 42 were assessed using a Mann Whitney U test. Between group differences for URS data was determined using independent T-tests. All physiological data (HR, T_{core}, La^-_B, RPE, Thermal sensation, fatigue, urine osmolality and sweat rate) were checked for normal distribution and then analysed using a mixed model repeated measures ANOVA (condition x time) with <0.01, <0.06 and >0.14 considered as a small, medium, and large effect. For all tests, the statistical significance was accepted at the 95% confidence level ($p < 0.05$). The mean and standard deviation were used to describe the average and variability of data, unless stated otherwise.

7.4 Results

7.4.1 Participant characteristics

There was a reduction in body mass following the FSITP in both groups during both experimental visits ($P < 0.05$), but this did not differ between day or group ($P > 0.05$). There were no differences in fluid intake or sweat rate either between day or group ($P > 0.05$). Supplement adherence was high and there was no between group difference (B-GOS: 96± 5 %; Placebo: 97 ± 2 %; $P > 0.05$).

7.4.2 Peak Δ Plasma I-FABP

At D0 a Kruskal Wallis test revealed no between group difference in the peak Δ in I-FABP in the response to the FSITP (χ^2 (1) = 0.622, P = 0.622) with a mean rank of 11.33 for B-GOS and 12.73 for Placebo (figure 1). At D42 a Kruskal-Wallis test revealed that following the FSITP the Peak Δ in I-FABP was 35.42% lower in the B-GOS group to the placebo (χ^2 (1) = 3.995, P = 0.046) with a mean rank of 9.85 for B-GOS and 15.640 for Placebo (figure 1).

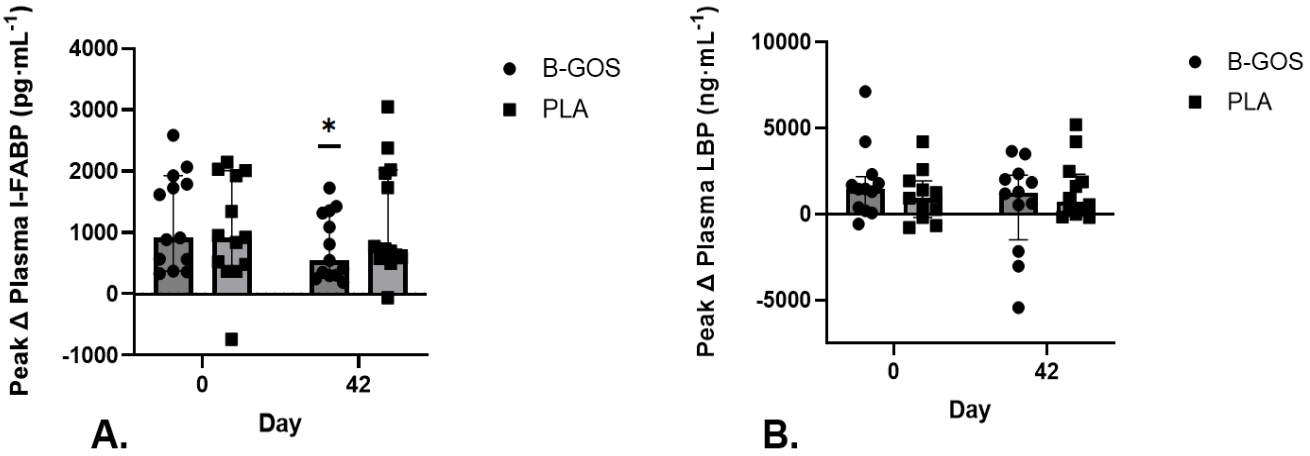

Figure 7.2 A) Peak Δ plasma I-FABP at day-0 and day 42 (B-GOS n =13, Placebo = 13). A Kruskal-Wallis test revealed a significant between-group difference in peak Δ plasma I-FABP at day 42. (B) Peak Δ LBP at day 0 and day 42 (B-GOS n=12, Placebo n=12). A Kruskal-Wallis test revealed no between-group difference in peak Δ plasma LBP at day 0 and 42. Values are

peak Δ for each individual, bars represent the group median ± interquartile range. Asterisk (*) denotes a significant difference between groups during that visit ($p < 0.05$).

7.4.3 Plasma Δ LBP relative to pre intervention

A Kruskall Wallis test revealed no between group differences in the peak Δ plasma LBP at D0 (χ^2 (1) = 0.633, P = 0.426) or D42 (χ^2 (1) = 0.066, P = 0.797) (figure 7.1).

7.4.4 Salivary IgA secretion rate

The D0 to D42 relative change in SIgA secretion was greater in the B-GOS group compared to the placebo group (B-GOS: 74.00 ± 204.40 µg·min^{-1}; Placebo: -106.19 ± 124.60 µg·min^{-1}; P = 0.016) (figure 2).

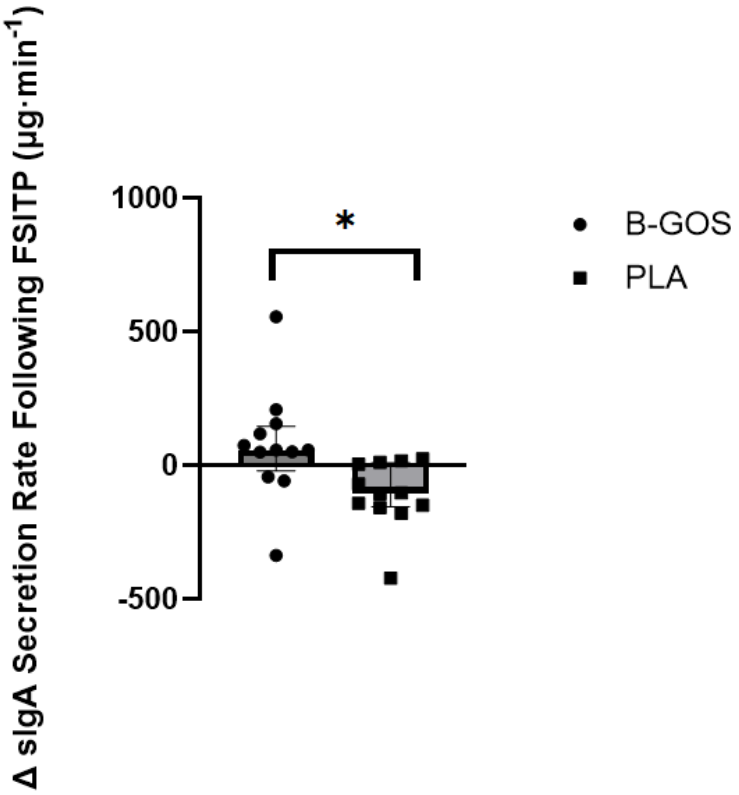

Figure 7.3. The D0 to D42 Δ sIgA secretion rate following FSITP. An independent T-test revealed a significant between-group difference (B-GOS n=12, Placebo n=12). Values are peak Δ for each participant, bars represent the group median ± interquartile. Asterisk (*) denotes a significant difference between groups ($p < 0.05$).

7.4.5 Upper respiratory symptoms (URS)

Throughout the 42-day intervention period there was no difference in the number of URS episodes between B-GOS and Placebo groups (B-GOS: 0.5 ± 0.5; Placebo: 0.8 ± 0.4; P = 0.118). However, the average duration of a URS episode was shorter by 5.7 days in the B-GOS group compared to the Placebo (B-GOS: 3.2 ± 5.0 days; Placebo: 8.9 ± 5.4 days; P = 0.007). The severity of the URS episodes was lower in B-GOS than Placebo (B-GOS: 11.0 ± 16.1; Placebo: 32.3 ± 21.2; P = 0.017). There was no between group difference in the number of training days affected by URS, or number of days of medication (P > 0.05) (table 1).

Table 7.1 Overview of self-reported URS data during the 42-day intervention.

	BGOS		**Placebo**		**P Value**
	Mean	**SD**	**Mean**	**SD**	
Episode Duration (days)	3.2	5.0	8.9	5.4	0.007
Incidence (episode per person)	0.5	0.5	0.8	0.4	0.118
Severity (symptom score per episode)	12.0	16.5	34.5	22.5	0.017
Training Days effected by URS	0.4	1.1	1.3	2.3	0.178
Days of Medication During URS	0.3	0.5	1.2	2.1	0.705

7.4.6 Gastrointestinal symptoms during intervention period

There were no differences in the average weekly GI symptom score between B-GOS and Placebo groups (B-GOS: 4.4 ± 4.1; Placebo: 8.8 ± 8.7; P = 0.114) (figure 7.3). However, there was a significant attenuation in GIS over the 6-weeks in the B-GOS compared to placebo group (B-GOS; -67.1 ± 36.1%; Placebo -4.8 ± 111.1; P < 0.001). There were no differences in the number of symptoms reported across the 6-week intervention period (B-GOS: 26.6 ± 24.5; Placebo: 30.5 ± 25.9; P = 0.428).

7.4.7 Gastrointestinal symptoms during exercise

A Mann Whitney U Test revealed no between group difference in Total GIS during exercise at Day 0 (P = 0.157). There was a reduction in Total GIS at D42 compared to D0 in the B-GOS group but no change following the placebo intervention (B-GOS: -38.9 ± 24.2 %; Placebo: 14.8 ± 65.4 %; P = 0.009). For GIS subcategories, there was a reduction in "other GIS" from day 0 to day 42 in the B-GOS group but not Placebo (B-GOS: -34.6 ± 54.8 %; Placebo: 32.5 ± 96.5; P = 0.006). There was no change for upper GIS (B-GOS: -46.7 ± 33.4 %; Placebo: -16.8 ± 84.1; P = 0.337) or lower GIS (B-GOS: -25.9 ± 64.8 %; Placebo: 36.7 ± 85.9; P = 0.175). There was no change in symptom incidence between Day0 and D42 (B-GOS: -0.5 ± 5.4; Placebo: -1.3 ± 3.9; P = 0.977).

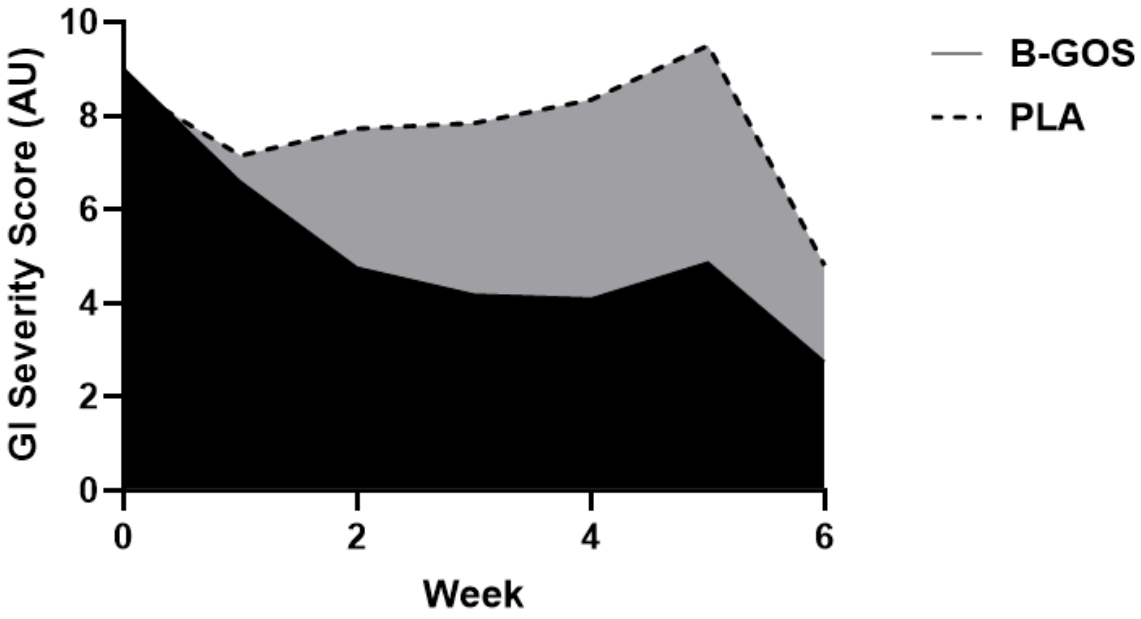

Figure 7.4 AUC for Weekly GIS scores reported during 6-week intervention period (B-GOS n = 13, Placebo n = 13). There was no statistical difference between groups (p > 0.05).

7.4.8 Core temperature

There was a main effect of time for T_{core} ($P < 0.001$, $n^2p = 0.835$) with higher values at all time points compared to baseline in both groups during both visits ($P < 0.001$). There was no main effect of group ($P = 0.669$, $n^2p = 0.008$) and no trial x time interaction ($P = 0.824$, $n^2p = 0.030$) (table 7.2).

7.4.9 Heart rate

There was a main effect of time for heart rate ($P < 0.001$, $n^2p = 0.904$) with higher values at all time points compared to baseline in both groups during both visits ($P < 0.001$). There was no main effect of group ($P = 0.329$, $n^2p = 0.043$) and there was no trial x time interaction ($P = 0.944$, $n^2p = 0.023$) (table 7.2).

7.4.10 RPE

There was a main effect of time for RPE ($P < 0.001$, $n^2p = 0.917$) with higher values at all time points compared to baseline in both groups during both visits ($P < 0.001$). There was no main effect of group ($P = 0.624$, $n^2p = 0.011$) and no trial x time interaction ($P = 0.411$, $n^2p = 0.044$) (table 7.2).

7.4.11 Thermal sensation

There was a main effect of time for thermal sensation ($P < 0.001$, $n^2p = 0.732$) with higher values at all time points compared to baseline in both groups during both visits ($P < 0.001$). There was no main effect of group ($P = 0.169$, $n^2p = 0.001$) and no trial x time interaction ($P = 0.862$, $n^2p = 0.016$).

7.4.12 Fatigue

There was a main effect of time for subjective fatigue ($P < 0.001$, $n^2p = 0.669$) with higher values at all time points compared to baseline in both groups during both visits ($P < 0.001$). There was no main effect of group ($P = 0.879$, $n^2p = 0.011$) and no trial x time interaction ($P = 0.101$, $n^2p = 0.079$).

7.4.13 Lactate

There was a main effect of time ($P < 0.001$, $n^2p = 0.449$) and group ($P = 0.007$, $n^2p = 0.285$) for [La^B]. [La^B] concentrations were higher at HT and FT than baseline during both trials ($P < 0.05$). Pairwise comparisons revealed that [La^B] was lower in B-GOS than Placebo at FT in visit 2 (B-GOS: 1.69 ± 0.62 mmo/L; Placebo: 2.45 ± 0.62; $P = 0.007$). There was no trial x time interaction ($P = 0.187$, $n^2p = 0.069$) (table 7.2).

Table 7.2 Overview of physiological responses during FSITP at D0 and D42 visits in B-GOS and Placebo groups. Data is presented as mean (SD). ∆ Peak core temp is the peak change from baseline value.

	B-GOS (n=13)		Placebo (n=13)	
Outcome	**D0**	**D42**	**D0**	**D42**
HR (bpm)	151.8 (13.4)	151.3 (16.9)	151.2 (11.8)	155.3 (12.4)
∆ Peak Core Temp (°C)	2.1 (0.5)	2.0 (0.5)	2.0 (0.5)	2.1 (0.4)
RPE	12.7 (1.7)	12.5 (2.0)	13.1 (1.6)	13.4 (1.2)
Lactate (mmol.L)	1.9 (0.6)	1.7 (0.6)	2.4 (0.6)	2.5 (0.6)

7.4.14 Summary of Findings

- **There was a pre-post intervention attenuation of plasma I-FABP at immediately post exercise in the B-GOS group but not Placebo.**
- **The duration and severity of URS episodes were lower in B-GOS group than Placebo.**
- **Compared to pre intervention, gastrointestinal discomfort was lower during the FSITP following B-GOS supplementation but not Placebo.**
- **Following 6-weeks of supplementation there was a greater reduction in GI discomfort at rest in B-GOS but not Placebo.**
- **There was a pre-post intervention attenuation of sIgA secretion rate at immediately post exercise in the B-GOS group but not Placebo.**

7.5 Discussion

The aim of the present study was to evaluate the effects of a 6-week prebiotic supplementation on gastrointestinal symptoms, circulatory markers of GI damage and permeability, and upper respiratory illness during simulated football activity in the heat. The current study demonstrated for the first time that supplementing with prebiotics can reduce exercise-induced gastrointestinal symptoms and support gut barrier integrity during intermittent team-sport exercise in the heat. It was also apparent that those supplementing with prebiotics experienced shorter and less severe URS episodes during the intervention period, and that there was a greater maintenance in sIgA post exercise. Collectively, these findings demonstrate that supplementation with prebiotics may be beneficial for team-sport based athletes during match-play and training periods through the alleviation of gastrointestinal symptoms, GI damage, URS and enhanced sIgA.

7.5.1 Reduction of GIS

Acute illness is the second most common reason for an athlete to require medical attention (Palmer-Green et al., 2013). In team sports such as rugby and football, both GI and upper respiratory disturbances contribute significantly to absences and impaired performance (Cunniffe et al., 2009; Parry & Dust 2006; Wilson et al., 2023). Team-based athletes face numerous challenges including high workload, frequent competitive fixtures, psychological stress, environmental stress, poor sleep, and travel, all of which may temporarily depress immune function and increase the risk of acute GIS and URS (Walsh, 2018). Evidence suggests that exercising in hotter ambient temperatures increases the incidence of GIS (Costa et al., 2017). This is a concern for team-sport athletes as it is becoming increasingly common for major tournaments and pre-season periods to be played in such climates. The reduction in self-reported GIS during exercise and the intervention period demonstrates that B-GOS may be a suitable intervention for athletes looking to alleviate such discomfort. This is in accordance with a previous study in chapter 6 of this thesis, where there were reductions in the

severity and incidence of GIS in elite rugby union players following a 24-week supplementation of B-GOS (Parker et al., 2023). The novelty of the present study is that GIS improvements occurred both during a specific period of exercise and during the intervention period.

7.5.2 Impact of B-GOS on plasma I-FABP

In addition to reduced GIS, there was a greater maintenance of GI barrier integrity as shown by an attenuated rise in circulatory I-FABP post-exercise following the 6-week B-GOS intervention. Intestinal epithelial barrier disruption occurs during steady state exercise as blood flow is redistributed away from the splanchnic region to aid skeletal muscle perfusion (Reher et al., 2001), resulting in ischemic stress. I-FABP is a cytosolic enterocyte protein that is abundant in the intestinal mucosa and used as an indirect marker of intestinal damage. Elevated levels of I-FABP have been consistently reported following steady state and more recently, competitive team-sport based exercise (Chantler et al., 2022; Clayton et al., 2024). To date, no known studies have assessed the impact of prebiotics on exercise-induced gastrointestinal damage. In the present study, the post exercise I-FABP concentrations were 20% lower than the pre-intervention trial in the B-GOS group, whereas there was a 10% increase in the Placebo group. This is the first evidence to show that prebiotics can attenuate exercise induced intestinal damage in humans. This supports previous murine work where supplemented GOS attenuated LPS induced intestinal damage and inflammation within the jejunum and ileum (Wang et al., 2021). Specifically, GOS appeared to enhance the epithelial structure by elevating villus height, the villus-to-crypt ratio, and upregulated the gene expression of ZO-1, occludin and claudin-1. Structural changes within the villi may be due to GOS influence on cell maturation and secretion which can amplify intestinal development and nutrient absorption (Hu et al., 2012; Cheng et al., 2018). GOS can also directly modulate goblet cell production and function. Goblet cells are columnar epithelial cells that secrete mucins which provide protection, lubrication and transport between luminal contents and epithelial lining (Bhatia et al., 2014). GOS has previously been shown to upregulate MUC2 gene expression and members of the

trefoil factor family which have a significant influence on mucosal barrier integrity (Bhatia et al., 2014). Therefore, it may be plausible the 6-weeks of B-GOS had similar effects on intestinal morphology within the current exercising cohort.

GOS supplementation is known to be bifidogenic, specifically enhancing bifidobacteria numbers which support the maintenance of gut homeostasis, and to stimulate gut associated immunity (Varasteh et al., 2015). The proliferation of beneficial bacteria may increase competition and inhibit the colonisation of pathogenic bacteria. GOS increases the abundance of various other friendly bacteria including lactobacillus *johnsonii, Lactobacillus_murinus, Lactobacillus_reuteri and Akkermansia muciniphilla* (Wang et al., 2022). Interestingly, *Akkermansia mucinphila* is a key contributor to mucin production, aiding stimulation and thickness which can help epithelial integrity (Zou & Chen, 2020). Alterations in the gut microbiome can upregulate the production of short-chain fatty acids (SCFAs). Butyrate, acetate, and propionate can inhibit the adherence of pathogens, maintain barrier integrity, and modulate immune function (Del Fabbro et al., 2020). Supplementation with GOS prevented the loss of SCFAs during an LPS challenge, which may have attributed the greater barrier resistance reported (Wang et al., 2021). One mechanism at which SCFAs can contribute to gut health is via their interaction with the G protein-coupled receptors 43. G protein-coupled receptors 43 are expressed highly in endocrine L cells where they promote the production of IEC, preserving the integrity of mucosal structure (Fernandez et al., 2016; Oozeer et al., 2013; Dube & Brubaker, 2007; Connor et al., 2016). Although the exact mechanisms are unclear, it is possible that B-GOS strengthen the resistance of the intestinal epithelial cells to intense intermittent exercise in the heat via both direct and indirect pathways that involve GI morphology and the microbiome. This may have then reduced local inflammation and the onset of GIS.

7.5.3 Impact of B-GOS on URS

Alongside reductions in GIS, there was a reduction in the duration and severity of URS in the B-GOS group. This is in accordance with the findings in chapter 6, where the same supplement was able to

reduce the duration of URS in elite rugby union players (Parker et al., 2023). Interestingly, we have shown that improvements are evident as early as 6-weeks, which may be important when athletes are preparing for significant events. Elsewhere, the use of non-digestible oligosaccharides (NDOs) for repressing URS has some attention in non-athletic populations. GOS and FOS are present within human breast milk and have shown to stimulate the growth of bifidobacterial and lactobacilli species (Davani-Davani et al., 2019). In double-blind controlled trials, administration of GOS and FOS reduced the incidence and recurrence of upper respiratory infections in infants during the first 6-12 months of life (Maldonado et al., 2012; Arslanoglu et al., 2008). In a group of stressed students, 2.5g or 5g doses of GOS caused a 40% reduction in the number of days with a cold or flu (Hughes et al., 2011).

Like the effects of GOS on gut barrier integrity, the proposed mechanism for reducing URS are changes in the microbiome and the stimulation of SCFA production. Butyrate has been shown to increase the production of antimicrobial peptides in the lung epithelial cell line VA10 (Steinmann et al., 2009). Acetate is also reported to have protective effects against influenza and pulmonary infections in mice (Sencio et al., 2020). Increasing evidence suggests that NDOs have the potential to be absorbed into the systemic circulation after oral administration, indicating that they may be able to reach the lungs and have direct effects on pathogens within airway epithelial cells (Vazquez et al., 2017; Ruhaak et al., 2014). Such interactions may also impact inflammation, NF-kB activation and cytokine/chemokine production (Mussatto & Mancilha, 2005). For example, GOS has been shown to inhibit M. haemolytica-induced cytokine/chemokine production, TLR-4 expression and the associated MAPK/NF-κB pathway, as well as reduce the LPS (TLR-4 ligand)-induced cytokine/chemokine release in calf primary bronchial epithelial cells (Cai et al., 2021). Specifically, the authors highlighted that M. haemolytica induced NLR pyrin domain containing 3 (NLRP3) inflammasome was the key contributor to the respiratory inflammation. Implying that this could be a potential avenue to target through GOS. To verify these findings in humans, Cai et al. (2021) preincubated human lung epithelial cells with GOS before exposing them to LPS, with the aim of

elevating IL-1β via the production of reactive oxygen species and elevated NLRP3 inflammasome expression. The authors found that GOS ameliorated LPS induced NLRP3 inflammasome expression and NF-kB phosphorylation. These anti-inflammatory properties may help contribute to the reduced burden and improved tolerance of respiratory infections.

7.5.4 Effects of B-GOS on sIgA secretion rate

It is possible that BGOS alleviated the burden of URS through modulating the immune system, specifically the production of IgA. The oral cavity is the first part of the gastrointestinal system (Kiyono & Azegami, 2015) and is thus, the most exposed section to microbial invasion (Diamond, Beckloff & Ryan, 2008). To protect against pathogenic bacteria the oral cavity is constantly covered in saliva which includes antibacterial materials, such as IgA (Dawes et al., 2015). IgA is a major effector in mucosal immunity, preventing viral replication and bacterial attachment to the mucosa (Hanson et al., 1983). Low levels of sIgA are associated with a higher incidence and recurrence of upper respiratory illness in athletes (Gleeson et al., 1999; Neville, Gleeson & Folland, 2008). As saliva flow rate is influential to the absolute concentration of sIgA it is subsequently reported as a secretion rate (Walsh et al., 1999). Exercising in the heat reduces sIgA secretion rate at a greater rate due to a higher norepinephrine response and higher sympathetic activity than when performed in ambient temperatures (Galbo et al., 1979), potentially putting athletes exposed to heat at a greater risk of URS.

In the current study, the pre-post exercise change in sIgA secretion rate from baseline was improved between day 0 and 42 in the BGOS group but not Placebo, indicating enhanced maintenance of sIgA after 42 days of BGOS administration. To our knowledge this is the first time that sIgA has been shown to be maintained immediately post exercise following a prebiotic intervention. This is agreement with Chapter 6, where the same supplement and dose was observed to increase sIgA secretion rate at rest in elite rugby union players after a 168-day intervention (Parker et al., 2023). The exact underlying mechanisms of the improvements in sIgA secretion rate study were not directly

measured. However, it is possible that B-GOS results in an increased production of SCFAs and/or IgA within the intestine. Indeed, BGOS was shown to increase faecal IgA in obese individuals after 12 weeks of supplementation (Vulevic et al., 2013), indicating higher levels of intestinal IgA. In a murine model, the administration of fructooligosaccharide increased IgA flow rate of the saliva per weight of submandibular tissue (Yamamoto et al., 2016). This was correlated with SCFA in the caecal digesta (Yamamtoto et al., 2016), suggesting a link between SCFA production in the intestine and salivary IgA.

One way that intestinal SCFAs may promote sIgA production is through the activation of the sympathetic and autonomic nervous system. SCFAs are signalling molecules that bind to specific G-protein-coupled receptors free fatty acid receptor FFA3 and FFA2 and activate the sympathetic nervous system (Kimura et al., 2013). Carpenter et al. (1998) observed that sIgA secretion is upregulated by nerve impulses and the sympathetic nerves. Furthermore, as it is well reported that BGOS has bifidogenic effects and increases intestinal SCFA concentrations, it is plausible to suggest that the administration of BGOS may have activated the sympathetic nervous system which resulted in a greater secretion of sIgA.

7.5.5 Strengths and limitations

This is the first study to explore the effects of a prebiotic supplement on exercise-induced GI damage, symptoms, and immunity during an intermittent heat exercise model. It is also the first to examine GI responses to intermittent team-based exercise in the heat and a prebiotic intervention. Our findings are promising as they support those found in the field. One benefit of replicating a football match in a laboratory environment meant distance, intensity, ambient temperature, and water intake were matched at day 0 and day 42. On contrary, a limitation of this is that we were unable to simulate movements specific to competitive football matches, including changes in direction, jumping and contact. However, the elevations in the present chapter were similar to those in chapter 5 following a competitive soccer fixture (Clayton et al., 2024) it is likely that the elevation

is representative to an actual soccer match. As GIS and URS were self-reported throughout the study it is possible that some symptoms were non-infection related. Although the methods of assessment have been validated to infection, this cannot be guaranteed. Another limitation of the current study was the lack of inflammatory markers, and the assessment of the microbiome and SCFA production. It is possible that B-GOS improved intestinal barrier integrity and reduced GIS and URS via pathways that involved these elements. Furthermore, as we have shown that B-GOS can reduce acute respiratory and GI symptoms in both field and lab-based studies future research should be conducted to establish the underpinning mechanisms.

7.5.6 Conclusion

In conclusion, a 6-week supplementation with prebiotic B-GOS reduced exercise-induced GI damage and GIS during a simulated soccer match in the heat. This was accompanied with a greater maintenance of sIgA immediately post exercise. In accordance with previous literature, B-GOS also reduced URS severity, duration, and GIS during the supplement period. These findings suggest that prebiotic B-GOS may have the potential to enhance GI barrier integrity during exercise, modulate immune function and reduce the burden of acute illness which may improve athlete availability. Future research should explore the possible underpinning mechanism of B-GOS on the alleviation of illness and exercise-induced GI damage.

8. Chapter 8 - General Discussion

8.1 Introduction

It is evident that athletes and exercisers can experience disturbances in GI and respiratory function. Such experiences can have a detrimental effect of performance, resulting in the need to decrease exercise intensity or bring exercise to a halt. Performing basic exercise in extreme environments can amplify the GI response. It is well evidenced that the added stress of exercising in the heat induces greater GI damage and symptoms than a cooler equivalent. Yet, limited research has investigated the impact of alternative extreme environments. Given the mechanisms at which GI damage occur, it is plausible that hypoxic environments can have a similar effect to the heat. Therefore, it is imperative that the impact of exercising in hypoxia on GI damage and symptoms is investigated. It is becoming increasingly common for team-based athletes to perform in both hot and hypoxic climates. Team-based athletes also face numerous stressors that can increase the risk of acute respiratory illness, GI discomfort and disruptions to GI integrity. Despite this, little research has 1: explored GI issues within team sports; and 2) been produced in identifying nutritional interventions that can alleviate these concerns. As previously discussed, the gut microbiome has a significant influence on GI functioning and the human immune system. Furthermore, nutritional interventions that target the gastrointestinal tract may alleviate the burden of acute illness and GI disturbances in athletic populations.

These questions helped form the rational for this research thesis. A summary of the key findings is below.

Chapter 4 explored the impact of exercising in normobaric hypoxia on markers of gastrointestinal damage and symptoms. In this study running speed was lower when exercising in hypoxia to ensure intensity was matched between the two environments. Despite this, exercising in normobaric hypoxia induced greater gastrointestinal damage as shown by greater plasma I-FABP concentrations. This was accompanied by greater intestinal permeability as indicated by higher plasma LBP

concentrations and more GI discomfort. This suggests that moderate-vigorous exercise in oxygen deprived environments may place greater stress on the GI tract and result in worsened discomfort.

Chapter 5 investigated the potential of a turmeric, vitamin D and vitamin C containing beverage on reducing URS, GI discomfort and GI damage in professional footballers during a competitive season. This study showed for the first time that this combination attenuated GI damage as shown by lower plasma I-FABP concentrations immediately post a 90-minute competitive football match when compared to control. This was in conjunction with a lower incidence of URS and a lower severity in GI discomfort. This suggests that a supplement containing turmeric, vitamin D and C can attenuate exercise induced GI damage and reduce the burden of acute illness in professional footballers.

Chapter 6 assessed the potential of a prebiotic B-GOS supplementation on respiratory and gastrointestinal illness in elite rugby union players over 24 weeks. This study showed for the first time that a daily dose of B-GOS reduced the duration of URS and lowered the severity and incidence of GI discomfort during a competitive rugby union season. This was accompanied by enhanced sIgA at 24-weeks, indicating improved immunity. Together these results suggest that favourable manipulation of the commensal gut bacteria through B-GOS may ameliorate acute respiratory and GI illness in elite rugby union players. Improving player availability for both training and competition.

Chapter 7 aimed to confirm the results of chapter 6 and further investigate whether these improvements also occur during an actual bout of intermittent team-based exercise in a hot environment, where GI responses are amplified. This study confirmed that the same daily dose of B-GOS for a shorter duration (6-weeks) reduces the length and severity of URS. It was also revealed for the first time that the daily supplementation of B-GOS can also improve GIS during a simulated football match treadmill protocol and lessen exercise induced GI damage and immunosuppression during exercise in the heat. This was shown by lower concentrations of plasma I-FABP and improved sIgA secretion rate. These findings provide further evidence that the dietary manipulation of the gut

microbiota through prebiotic B-GOS can protect against acute illness and enhance the gut barrier and immune response to intense exercise in the heat.

8.2 Experimental findings and recommendations

The purpose of this thesis was to investigate whether nutritional interventions that target the gut barrier and its resident microbes can alleviate exercise induced gastrointestinal damage, symptoms and the burden of acute URS. Evidence suggests that exercising in the heat and hypoxia can worsen gastrointestinal disturbances and URS, therefore a large section of this thesis focuses on exercise in such climates. In chapter 4, the impact that exercising in normobaric hypoxia has on gastrointestinal damage and symptoms was assessed. Gastrointestinal discomfort is prevalent when ascending to high altitude (Anand et al., 2006). Recent work in laboratory settings showed that running and cycling in normobaric hypoxia can exacerbate gastrointestinal damage and symptoms when compared to exercise at sea level (Hill et al., 2020; McKenna et al., 2022). Nevertheless, in both studies, exercise in hypoxia was conducted at the same absolute intensity as normoxia. This means that the participants were likely exercising at a greater intensity in hypoxia than normoxia which may have explained the exacerbated disturbances. In chapter 4, markers of gastrointestinal damage, permeability and subjective symptom scores were assessed during 60-mins of treadmill exercise in normoxia and normobaric hypoxia but when intensity was relative to the respective environment.

Interestingly, despite absolute running speed being lower in normobaric hypoxia, greater damage as shown by higher pre to post Δ concentrations of I-FABP immediately post exercise (99% vs 52%). A smaller but albeit significant pre to post Δ increase was observed for Claudin-3 after exercise in normobaric hypoxia but not normoxia (9% vs -1%), indicating disruption to the tight junction protein structure of the intestinal barrier because of hypoxic exposure. Elevated levels of LBP are associated with a compromised intestinal barrier and heightened permeability (Schumann et al., 1990). Similarly to I-FABP and Claudin-3, pre to post Δ plasma LBP was higher immediately post exercise (37% vs 9%) and at 60-min post (46% vs 4%) exercise in normobaric hypoxia than normoxia.

Collectively these findings suggest that the disturbances to the intestinal barrier during hypoxia exercise are due to the environment rather than the higher exercise intensity. One possible consequence of substantial intestinal damage is the onset of gastrointestinal discomfort (Costa et al., 2017). To the author's knowledge this is the first-time gastrointestinal discomfort has been shown to be heightened during hypoxic exercise once the intensity has been accounted for. No correlations were shown between any markers of GI damage or permeability and GI discomfort which suggests there may be another cause for the heightened incidence.

The outcomes of Chapter 4 highlight the negative impact that an acute bout of moderate intensity exercise can have on the GI system. It also confirms that exercise in hypoxia can exacerbate the issue. Further to this, Chapters 5 and 7 showed that competitive intermittent team-based invasion sports and a simulated version on a treadmill is sufficient to induce similar perturbations to the GI tract. Chapter 7 specifically showed that this also occurs when performed in the heat. Intense bouts of exercise can cause temporary immunosuppression. As discussed previously, this is one of the risk factors that are associated with the high incidence of acute URS and GI discomfort in elite athletes (Peake et al., 2017; Smith et al., 2011; Nieman et al., 2019). In team sports, acute URS and GI illness have been cited as a major contributor to player absences (Divorak et al., 2010; Cunniffe et al., 2009). Furthermore, therapeutic strategies to target this and to help alleviate the burden of URS and GI discomfort in team sports are warranted.

Nutritional interventions that can influence gastrointestinal function and the immune system have received considerable interest. Turmeric contains a lipophilic polyphenol called curcumin which has traditionally been used to help prevent metabolic, autoimmune, neurological and cardiovascular diseases (Gupta et al., 2013; Prasad et al., 2014). In more recent times, turmeric and curcumin has been shown to modulate the gut microbiome in both rodents and humans (Zhang et al., 2017; Peterson et al., 2018). A short-term turmeric intervention, whereby participants supplemented with 500mg/day of curcumin was proven to reduce exercise induced gastrointestinal damage following a

60-min treadmill run at 65% $\dot{V}O_{2max}$ (Syzmanski et al., 2017). This implied that through the modulation of the gut, turmeric and curcumin supplementation may support the integrity of the intestinal barrier in response to exercise.

In addition to turmeric, vitamin D is proposed to have numerous health benefits on the host. Vitamin D deficiency has been negatively associated with URTI in British adults (Berry et al., 2011), which is likely due to its influence on the innate and adaptive immune systems via its VDR actions (Barker et al., 2014). Indeed, a recent meta-analysis observed that a daily dose of 400-1000 IU of vitamin D was protective against URTI in the general population (Jolliffe et al., 2021). Interestingly, several studies have reported alterations in the gut microbiome during vitamin D supplementation (Bashir et al., 2016, Canteral et al., 2015, Kanhere et al., 2018; Shi et al., 2017). Like acute respiratory illness, vitamin D deficiency results in greater intestinal barrier disruption and inflammation (Assa et al., 2014). Evidence in rodents suggest that vitamin D may protect the intestinal barrier through the expression of VDR-associated intracellular junction proteins (Zhang et al., 2013). This also makes vitamin D a promising agent for relieving the burden of both exercise-induced intestinal damage and acute illness.

In Chapter 5, we have showed for the first time that a combined supplement containing 17.5g raw turmeric root (700 mg curcumin), 1000 mg of vitamin C, 3000 IU of vitamin D_3, and 200 mg black pepper (10 mg of piperine) is effective at reducing GI damage following a competitive football match in elite male footballers. To the authors knowledge this is also the first time that I-FBAP has been assessed during a competitive 90-minute football match. Players also experienced fewer days with URS and less gastrointestinal discomfort during the supplementation period. We witnessed an 85% difference between the post-match plasma I-FABP concentrations in the control and supplementation periods. This was greater than what has previously been reported by Symanski et al. (2018), who observed a 40% difference immediately post exercise in favour of the turmeric group. It is unclear why there was such a heightened response in the current study, but it may be

due to a longer intervention period (112 days vs 3 days) or a greater dose of curcumin (700mg vs 500mg). Exercise-induced gastrointestinal damage occurs due to splanchnic hypoperfusion, ischemic stress and elevated core temperature. This can result in LPS-induced inflammation within the intestinal barrier causing further disruption. LPS-induced inflammation is heavily influenced by the proinflammatory mediator IL-1β (Jiang et al., 1999). Evidence in mice found that curcumin supplementation can downregulate the production of proinflammatory cytokines including IL-1β from response ischemia-reperfusion injury (Zhange et al., 2015; Zhong et al., 2016). As discussed in chapter 5, the reduction of LPS-induced IL-1β may also activate p38 MAPK. Activation of this pathway can reduce the expression of myosin light kinase which can strengthen the tight complexes that form the intestinal barrier (Wang et al., 2017).

A previous study observed alterations in gut microbiome composition with a greater average alpha diversity following curcumin supplementation than placebo ($\alpha = 6.31$ vs $\alpha = 6.15$) (Peterson et al., 2018). Specifically, Peterson et al. (2018) found an average increase in 69% of species with notable increases in Clostridium spp., Bacteroides spp., Citrobacter spp., Cronobacter spp., Enterobacter spp., Enterococcus spp., Klebsiella spp., Parabacteroides spp., and Pseudomonas spp. A smaller dose was used in chapter 5 (700mg vs 6000mg per day) but as the intervention was longer (16 vs 8 weeks) there may have been similar alterations in the gut microbiome. Gut bacteria play an important role in the maturation and formation of the intestinal barrier as their absence led to a smaller intestinal surface and shorter villi within the intestinal barrier (Gordon & Bruckner-Kardoss 1961; Abrams et al., 1963; Meslin et al., 1999). Gut bacteria also aid mucus production which is essential for preventing pathogenic bacteria from disrupting the intestinal barrier. Therefore, it is plausible to suggest that curcumin may have reduced exercise induced GI damage and symptoms through these mechanisms. However, as stool samples were not collected and assessed in this study future research is required to assess the influence of this supplement on the human microbiome and gain a greater understanding of the potential mechanisms for lower GI damage.

Like curcumin, evidence also suggests that vitamin D may have aided the attenuation of exercise-induced GI damage. Vitamin D receptors transcriptionally regulate claudin-2, 5, 12 and 15, while a lower number in vitamin D receptors results in lower claudin levels and a loss in GI barrier integrity (Chatterjee et al., 2021; Zhang et al., 2021). Studies from in vitro have shown that the expression of vitamin D receptors correlate with tight junction expression (Liu et al., 2017; Wang et al., 2019; Palmer et al., 2001). Research from in vivo is lacking but one murine based study did observe upregulated occluding and claudin within the intestine following vitamin D_3 supplementation which was coupled with attenuated permeability (Lee et al., 2021). Interestingly, one study in healthy monozygotic twins found that a smaller dose of vitamin D (2000 vs 3000 IU) elevated vitamin D concentrations and the expression of vitamin D receptors (Franco Pires Medeiros et al., 2020). Furthermore, it is possible to speculate that the vitamin D component of the supplement elevated vitamin D receptors within the intestine and upregulated tight junction proteins that enhance integrity within the intestinal barrier. But like curcumin, future research is needed to elucidate the mechanisms at which vitamin D can influence the intestinal barrier in humans.

In Chapter 5, the combination of turmeric root, vitamin D_3 and vitamin C was able to alleviate the burden of URS. This is in corroboration with previous studies that observed improvements in URS following vitamin D_3 and vitamin C supplementation in taekwondo athletes and endurance runners (Jung et al., 2018; Peterson et al., 1993). Due to the combined nature of the supplement used in chapter 5 the exact mechanism of action is unclear. Vitamin D is proposed to support the structure of the respiratory tract. Specifically, vitamin D can upregulate tight junctions within upper respiratory tract, enhancing paracellular integrity and limiting the infiltration of bacteria and viruses (Chen et al., 2018). Vitamin C has anti-inflammatory and antioxidant properties which can help protect cells from oxidative damage, alleviate neutrophil oxygen radical production, and cytokine production (Dwenger et al., 1992; Schwager & Schulza, 1998; Bijur et al., 1999). Collectively this may have reduced inflammation within the respiratory tract and reduced the severity of URS. Like vitamin C, curcumin can also provide antioxidant and anti-inflammatory effects (Zielinska et al.,

2020). Curcumin has been shown to suppress intracellular NF-kB, MAPKs and JAKs/STATs pathways which can regulate the expression and suppression of inflammatory mediators (Yaw-Syan Fu et al., 2021). Although it is not proven in the current chapter, it is possible that such anti-inflammatory effects influenced the respiratory tract. It is also possible that curcumin provided anti-viral effects via its interaction with viral coat proteins, virus -specific enzymes or RNA polymerase and abolish virus replication (Yaw-Syan Fu et al., 2021). Overall, this data suggests that this supplement is effective at alleviating URS but further research is required to identify whether it is a specific ingredient or the combination that is driving the positive responses.

Recommendations based on the findings of Chapter 5 are that the combination of raw turmeric root, vitamin C, vitamin D_3, and black pepper is effective at reducing exercise induced GI damage during a competitive football game and effective at alleviating URS and GI discomfort across the course of a season in professional footballers. These data highlight that the daily supplementation of this ready-to-drink product may be beneficial for reducing the discomfort caused by acute illness and improve player availability for training and competition.

A limitation of the study in chapter 5 was that it was impossible to blind participants to the study intervention. Future studies should attempt to blind participants to the intervention with a suitable placebo. Another limitation was differences in the duration of the control and intervention periods and the fact that the control and supplement periods were during different times of the year. This meant that we could not assess the duration of illness using the same method as Chapter 6 and 7. Instead, illness was characterised as a ratio (illness per 1000 player days). This method is used elsewhere (Dvorak et al., 2011) and did still show positive effects, but we may have not seen the full benefits of the supplement regarding illness recovery. The omission of a control group during the intervention period makes it more difficult to compare as it is unclear whether a control group would have experienced fewer or greater URS episodes. As this study involved professional athletes during a competitive season, we could not control diet, supplement history and the timing of which

they ate. However, as each player was from the same squad, it is likely that all players followed a similar eating pattern. During the study, participants were allowed to continue using their usual dietary supplements, but they were instructed to avoid additional vitamin C, D and turmeric consumption. This is a limitation as alternative supplements may interact with the GI and immune system, influencing the results but it should be noted that we still found positive results despite this. Future studies are advised to control for such factors but the study design in the current chapter allowed 'real life' responses in the field which are very valuable in a practical sense.

As discussed previously, the gut microbiome has a significant influence on host immune and gastrointestinal function. Prebiotics are a non-digestible food ingredient that encourage the growth and increased activity of commensal gut bacteria. In Chapter 6, the aim was to assess whether the daily consumption of the commercially available Bimuno-galactooligosaccaride could reduce the burden of URS and GIS in elite rugby union players during a competitive season. This was conducted using a double-blind, randomised control design to bypass some of the limitations from Chapter 5.

Chapter 6 reports that the daily supplementation of B-GOS reduced the duration of the URS episodes by 2.4 days but the not the incidence or severity. This supports previous research that used multi-strain probiotics in elite rugby union players and recreational runners (Haywood et al., 2013; Gleeson et al., 2011). To our knowledge, this was the first study to assess the impact of prebiotics on acute illness in elite athletes. Like Gleeson et al. (2011) we also saw an increase in IgA which may indicate that the modulation of immune function, specifically salivary IgA could have been one of the underpinning mechanisms for the alleviation of URS. SCFA can enhance sIgA production through binding to G-protein-coupled receptors free fatty acid receptor FFA3 and FFA2 and activate the sympathetic nervous system (Kimura et al., 2013). Indeed, intestinal SCFA concentrations were correlated with increased sIgA flow rate following fructooligosaccharide supplementation in rats (Yamamoto et al., 2016). As B-GOS has previously been shown to be extremely bifidogenic and produce SCFAs, it is plausible that the production of SCFAs in current study was heightened, which

may have influenced IgA production via a sympathetic nervous pathway. SCFA production was also the likely contributor to the lower incidence and severity of GIS. Elevated SCFA concentrations can improve gut epithelial integrity and mucosal immunity (Mariadason et al., 1997; Hernot et al., 2009), which may have reduced local inflammation and gut permeability. Together, these findings suggest that the daily supplementation of B-GOS is effective at alleviating the burden of URS and GIS in elite rugby union players during a competitive season which may lead to greater player availability and performance.

As Chapter 6 showed that intervention with B-GOS can significantly reduce the burden of URS and GIS while potentially enhancing immune function it is still unclear whether similar protective effects occur in response to a bout of exercise. As shown in Chapter 5, a competitive football match induces significant GI damage demonstrated by elevated I-FABP concentrations. It also demonstrated the occurrence of URS was 1 episode per season and GIS was present during 70% of season. It is becoming more common for team-sport players to perform in hot climates. Evidence suggests that exercise in the heat can exacerbate GI damage, symptoms, and immunosuppression (Walsh et al., 2002; Laing et al., 2005; Yeh et al., 2013). In murine models, GOS has appeared to enhance the epithelial structure within the intestine and the production of goblet cells which contribute to barrier integrity (Bhatia et al., 2014; Hu et al., 2012; Cheng et al., 2018). This provides a strong rationale for using B-GOS as a form of therapy to protect against exercise-induced GI damage in the heat. Chapter 7 presents novel evidence that manipulation of the gut microbiome, and potential direct action on the gut barrier through prebiotic supplementation could attenuate exercise-induced GI damage and discomfort during football specific exercise in the heat. Forty-two days of B-GOS intervention significantly reduced peak Δ plasma I-FABP by 20% with no change in the placebo group ($P = 0.042$). This is similar to the improvements seen in Chapter 5 and seen elsewhere following curcumin supplementation (Syzmanski et al., 2017). But to our knowledge, this is the first study that has observed improvements in GI damage following either a probiotic or prebiotic intervention. This finding is also significant and novel as it shows that B-GOS can be effective during exercise in the

heat when GI damage is likely to be worse than exercise in thermoneutral conditions. Thus, as with Chapter 5, B-GOS intervention was shown to provide protective effects against exercise-induced GI damage.

As discussed in Chapters 5 and 6, acute respiratory illness and GI discomfort are prevalent in team-based sport. Both interventions in Chapter 5 and 6 were shown to alleviate URS and GIS on a day-to-day basis during a competitive season in elite athletes. Like Chapter 6, the findings in Chapter 7 confirm that a B-GOS intervention can reduce the duration of URS and be effective in as little as 6-weeks. During the 42-day B-GOS intervention the average URS episode was 5.7 days shorter than Placebo (3.2 ± 5 vs 8.9 ± 5.4; P = 0.007). Unlike Chapter 6, URS severity was also lower in B-GOS than Placebo (12.0 ± 16.5 vs 34.5 ± 22.5; P = 0.017). In addition to the alleviation of URS, a greater maintenance of sIgA secretion rate was observed immediately after exercise in the B-GOS group but not placebo following the 6-week intervention. This heightened immunity could be a contributing factor to the alleviation of URS in this cohort. As mentioned previously, prebiotics can influence IgA production via SCFA interactions which may have protected against URS but there are other potential mechanisms of action. Butyrate has been shown to increase antimicrobial peptides in the lung epithelial line (Steinmann et al., 2009). It is also possible for prebiotics to be absorbed into systemic circulation and reach the lungs where they can have direct anti-inflammatory effects by inhibiting TLR-4 expression, the MAPK/NF-κB pathway and cytokine production (Mussatto & Mancilha, 2005; Cai et al., 2021). It is interesting to note that GI discomfort during the FSTIP was lower after the B-GOS intervention when compared to day-0. This complements the findings in Chapter 5 and 6 but also shows that it has protective effects during the intervention period and during a bout of exercise in the heat. As discussed earlier it is possible that the modulation of the gut microbiome and production of SCFAs may have attenuated local and systemic inflammation, and strengthen the epithelial barrier of the intestine, alleviating the onset of GI discomfort. These findings indicate that B-GOS is an effective treatment strategy for maintaining GI barrier integrity

and sIgA during intermittent team-sport exercise in the heat and for alleviating URS and GI discomfort.

Regarding the implications of this thesis in the field, the primary aim of this thesis was to investigate whether dietary interventions that target the gut microbiome could help enhance respiratory and gastrointestinal health in athletic cohorts. As previously highlighted, illness poses a significant burden in elite sport as lower athlete availability is associated with a lower chance of success (Hägglund et al., 2013). As there are no medications that can reduce the burden of common colds or gastrointestinal discomfort, simple, cost-effective alternatives are warranted. Based off the collective findings from chapters 5, 6 and 7, we can confidently recommend the ingestion of either prebiotic GOS or a ready-to-drink supplement contain turmeric, vitamin D_3 and vitamin C can reduce the burden of URS and GIS during competitive seasons and in preparation for events that will be performed in hotter environments.

In addition to symptoms, this thesis showed that the daily administration of prebiotic B-GOS was able to enhance salivary IgA both at rest and following intense exercise in the heat, implying an increase in immunity. Likewise, the daily dose over 6-weeks was able to reduce circulatory concentrations of I-FABP following intermittent running in the heat, indicating greater intestinal barrier resistance. These findings support the recommendation for ingesting B-GOS during periods where immune function or the intestinal barrier can become compromised.

8.3 Limitations

The specific limitations of each study are discussed in the relevant experimental chapters. One limitation from the studies in Chapters 5, 6 and 7 was that upper respiratory illness was assessed using self-reported questionnaires. Although this does not guarantee that the URS present were from an infection the subjective questionnaire used has been validated against a GP diagnosis. Future research should attempt to obtain a GP diagnosis or assess bacterial or viral load at the time

of illness as this will clarify if an URTI was present. Despite the potential issue of self-reported questionnaires, it would not have been feasible to obtain a GP diagnosis or sputum analysis as majority of the supplementation period was remote. Also, as the participants in Chapters 5 and 6 were elite individuals at professional clubs they may have been reluctant to approach medical staff to state the present of an illness to avoid deselection from competition.

Chapters 5, 6 and 7 successfully showed that dietary interventions that target the gut microbiome can reduce the burden of URS and GIS through randomised controlled trials in relatively small cohorts of elite and recreationally active individuals. Although it is positive that we still observed improvements with smaller sample sizes it may have masked some other positive effects. For example, in Chapter 6 we failed to see any improvements in systemic inflammation despite there being a potential trend in favour of B-GOS for plasma CRP. There was also a trend for URS severity which did not reach statistical significance in Chapter 6 which did reach significance in Chapter 7. Therefore, replicated studies with larger cohorts are needed to confirm other positive effects in athletes and support the findings from this thesis.

A limitation of the studies in Chapters 5 and 6 are that no measures of training or competition attendance or medication usage were taken. Furthermore, it is not evident whether the reductions in URS and GIS improved player availability. Additionally, there was no performance measures taken. Previous research using probiotic interventions have shown improvements in exercise performance yet no studies using prebiotics have elucidated this. Future research should look to include player attendance and a performance outcome to assess the direct effects of prebiotics on performance.

8.4 Significance of findings and future research direction

As discussed, acute respiratory illness and gastrointestinal discomfort are some of the leading causes of athlete absence or impaired performance (Soligard et al 2015; Soligard et al. 2013; Engebretsen et al 2010; Engebretsen et al. 2013; Soligard et al., 2023). The novel findings from Chapters 5, 6 and 7

give significant scope to implement dietary interventions to alleviate these issues in athletic populations. The occurrence of acute illness or GI discomfort should not be the defining factor in elite sport. This thesis expands the current knowledge of what impact extreme environments, particularly hypoxia can have on exercise-induced GI damage and discomfort. But it also gives promise for using a combined supplement of turmeric, vitamin D and vitamin C, or a prebiotic B-GOS to reduce acute illness, GI discomfort while potentially enhancing immune and GI function. This novel research opens the door for future research to gain a better understanding of the therapeutic, mechanistic and ergogenic benefits of turmeric, vitamin D, vitamin C and B-GOS in athlete cohorts:

- In Chapter 5, the combined daily supplementation of turmeric (17.5g raw turmeric root), vitamin C (1000mg) and Vitamin D_3 (3000 IU) significantly reduced exercise induced GI damage as shown by lower plasma I-FABP concentrations following a 90-min football match. As the intervention included a combination of three separate supplements it is unclear whether the reduction in GI damage was due to one specific supplement or the combination of the three. Therefore, future research should investigate the effect of each supplement on plasma I-FABP concentrations following exercise.
- Further to this, investigations in both in vivo and in vitro are needed to confirm the improvements and to gain a greater understanding into the mechanisms by which the combined supplementation or each isolated supplement reduces exercise-induced GI damage.
- Previous literature has observed alterations in the gut microbiome following turmeric supplementation. Furthermore, future research studies should collect faecal samples to confirm to assess the impact of turmeric on the diversity of gut microbiome.
- A study with a larger cohort, equal intervention periods for both conditions and a placebo arm should be conducted to confirm or refute the present findings.

Across Chapters 6 and 7, we have shown for the first time that a prebiotic B-GOS intervention can significantly reduce the duration and severity of URS, the severity and incidence of GIS, enhance salivary IgA secretion rate and reduce exercise-induced GI damage following exercise in the heat. Although positive impacts were observed, the exact mechanisms of action remain to be elucidated. Future research studies should attempt to:

- Collect faecal samples before and after the intervention to assess the athletes' gut microbiome diversity at baseline and post intervention. It would also confirm the bifidogenic effects of B-GOS in this type of cohort.
- Chapter 7 confirmed the findings of Chapter 6, where B-GOS reduced URS and GIS, but it also reduced plasma I-FABP following exercise. A larger scale research study is required to confirm or refute this finding. Investigations should also be conducted in field-based studies to assess its impact following competitive matches and training.
- There are several methods to try and elucidate the mechanisms of action of prebiotic B-GOS on exercise-induced damage and symptoms further. The use of dual sugar probes is considered the gold standard method for assessing gastrointestinal permeability, therefore this may provide a greater insight into the impact of prebiotic B-GOS of GI barrier integrity. Prebiotic B-GOS has previously been shown to reduce systemic inflammation in healthy controls (Williams et al., 2016). Furthermore, the assessment of pro-inflammatory cytokines, CD-14, T Cell and Foxp3 expression through flow cytometry may show any anti-inflammatory properties.
- Evidence implies that the lung microbiome plays an important role in respiratory illness and inflammation. It is possible to assess the lung microbiome through the use of the epithelial brushing technique. This can establish any changes in the lung microbiome pre and post prebiotic B-GOS intervention.
- Future research studies should assess whether beneficial effects of B-GOS can be translated into other sports (i.e. endurance and combat events) and military personnel. URS and GIS

should be assessed in the field against competition success to identify any relationships between B-GOS, athlete availability and competition success.

- Some evidence suggests that targeting the microbiome can directly impact exercise performance (Huang et al., 2020), recovery, and carbohydrate metabolism (Pugh et al., 2020). Therefore, future studies should assess the ergogenic effect of prebiotic B-GOS.
- Previous literature has shown that probiotics containing lactobacilli can reduce the burden of acute illness in athletes and increase salivary IgA. Furthermore, a combination of B-GOS with strains of lactobacilli and bifidobacteria in the form of a synbiotic may enhance immunity and strengthen the GI barrier to a greater degree than that shown in Chapters 6 and 7.
- As the mechanisms of action may be different, future research could investigate whether there is a combination effect of turmeric, vitamin C, vitamin D_3 and prebiotic B-GOS.

8.5 Conclusion

In conclusion, this thesis provides novel evidence of how exercising in extreme environments can acutely disrupt the structure of the GI tract and how two nutritional interventions can alleviate exercise-induced GI damage and acute illness in athletic populations. We have shown for the first time that exercising in normobaric hypoxia when intensity is matched exacerbates GI damage and permeability as shown by higher plasma concentrations of I-FABP, LBP and Claudin-3. This highlights the issues that athletes face when exercising in extreme environments and the need for therapies to alleviate them. In Chapter 5 it was showed for the first time that the daily consumption of a combined supplement containing turmeric, vitamin C and vitamin D is effective at alleviating GI damage following a 90-min football match and the burden of URS and GIS during a competitive season in professional footballers. In Chapter 6, we showed for that the daily supplementation of prebiotic B-GOS can help reduce the duration of URS and the incidence and severity of GIS in professional rugby union players during a competitive season. Observations in Chapter 7 showed

that the same prebiotic B-GOS intervention can also reduce the volume of GI damage following a 90-min simulated football match in the heat in recreational team-sport athletes. It also confirmed the effectiveness of the prebiotic B-GOS for alleviating URS and GIS. Furthermore, our novel findings show for the first time in athletic cohorts that the combination of turmeric, vitamin C and vitamin D or prebiotic B-GOS can positively alter immune function, enhance GI barrier integrity and alleviate the burden of acute illness in both elite and recreational team-sport athletes. This data adds to the growing body of evidence that nutritional interventions that target the gut microbiome and GI tract can have a significant impact on GI function and immunity beyond the GI tract making it a suitable target for future research in athletes and exercising individuals.

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Appendix 1a – Chapter 6 Participant Information Sheet

Participant Information Sheet

The effects of long-term prebiotic supplementation on markers of immunity and upper respiratory illness in elite rugby union players.

- Brief Introduction:

Within athletic populations there is a heightened incidence of upper respiratory tract infections (URTI) and gastrointestinal (GI) illness during heavy training periods and competitions. This increased likelihood of illness likely relates to a short-term reduction in immune function post exercise and/or long term suppression after frequent intense training and competition. Therefore, athletes may be at higher risk of developing illnesses during these periods, potentially impairing training and competition performance.

Increasing evidence suggests dietary interventions which modify the gut microbiota (the trillions of microorganisms which live in the gut) can help reduce the frequency, duration and severity of URTI and GI symptoms in athletes. The majority of research has focused upon the supplementation of probiotics (ingestion of live bacteria promoted as having a health benefit). However, currently there is no consensus on which probiotic bacteria are most effective. A suitable alternative is daily consumption of a prebiotic (a non-digestible carbohydrate food ingredient) that acts a fertilizer for the health promoting bacteria in the gut. This may improve URTI and GI symptoms by increasing the number of beneficial microbes within the gut. Regular consumption of prebiotics has previously been shown to improve markers of immunity and reduce inflammation in the general population and high-risk groups (elderly, obese & asthmatics). Therefore, we hypothesise that prebiotics may improve immunity and reduce the risk of URTI and GI symptoms within elite athletic populations.

This study aims to assess the effects of a long-term prebiotic supplementation period on markers of immunity, URTI and GI symptoms in a professional rugby squad throughout an entire domestic rugby season.

- Study Requirements:

To be eligible to take part, you must:

- Be 18-45 years of age
- Member of London Irish Rugby Football Union Club
- Be a non-smoker

Unfortunately, you will not be able to take part if any of the following apply to you:

- Routine consumption of prebiotic, and / or probiotic supplements
- Take a daily dose of aspirin or other NSAIDs
- Intake of drugs which affect gastrointestinal mobility, laxatives in the 4 weeks before the study
- Vegetarian or vegan diet
- Previously diagnosed with COPD, emphysema, chronic bronchitis or similar respiratory illness
- Asthma exacerbation within the last 12 months (course of steroids, or hospital visit)
- History of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease

- History of recurrent chest infections
- Smoker
- Acute infection within the last four weeks
- Major operation within the past four months
- History of gastrointestinal drug reaction
- Use of antibiotics in the past 3 months
- History or current evidence of gastrointestinal disease e.g. chronic constipation, diarrhoea, irritable bowel syndrome, Crohn's Disease
- Milk allergy

- Location:

You will be asked to visit 4 experimental trials at London Irish's training ground. Each visit will last approximately 30 minutes.

Address:

Hazelwood Centre

Hazelwood Drive

Sunbury-on-Thames

TW16 6QU

- Restrictions During Testing:

You will be required to report to each visit at pre-arranged set times that will be the same on each occasion. No alcohol or caffeine consumption 12 hours prior to testing will be allowed. You will be required to keep a record of all illnesses, upper respiratory and gastrointestinal symptoms experienced between visits. You will be provided with these record sheets at the training ground. You will be allowed to administer non-prescribed and prescribed medication when needed, although this must be recorded. You will administer two doses (2.9g) of either the prebiotic or placebo every day for 36 weeks. This must be logged to track supplement adherence. The consumption of any additional ergogenic supplements must also be recorded.

- Testing Protocol:

Following initial briefing and consent you will then complete a health screen and provide baseline measurements (visit 1) before the completion of three further visits separated by 12 weeks each (visit 2, 3 & 4).

What's involved?

Visit 1 – briefing and consent

At least 24 hours prior to visit 1 you will attend a briefing of the study design and read the participant information sheet; you will then provide written informed consent to take part in the study.

Visit 2 – Pre-season measurements

Following consent, you will complete a health and medical questionnaire then have height and weight measured before baseline measurements are collected. A venous blood sample will be obtained. A saliva sample and a faecal sample will also be collected. You will also be asked to complete upper respiratory and gastrointestinal symptom questionnaires. Once all measurements are obtained you will be randomly allocated to receive the first dose of either a prebiotic or placebo. You and the researchers will be blinded to which supplement you will be consuming over the course of the season.

Visit 3 – Mid-season measurements

Following 12-weeks of supplementation, you will attend the first mid-season experimental visit. During this visit a venous blood and saliva sample will be obtained. You will also complete upper respiratory and gastrointestinal symptom questionnaires as per visit 2.

Visit 4 – Mid-season measurements

Following 24-weeks of supplementation you will attend the last mid-season experimental visit. During this visit, a venous blood and saliva sample will be obtained. You will also complete upper respiratory and gastrointestinal symptom questionnaires as per visits 2 & 3.

Visit 5- End of season measurements

Following 36-weeks of supplementation and at the end of the domestic season, you will attend the final experimental visit. Venous blood samples using vacutainers will be obtained. A saliva sample and a faecal sample will also be collected. You will also be asked to complete upper respiratory and gastrointestinal symptom questionnaires, as per visit 2.

What are the possible benefits to you for taking part?

You will be provided with information pertaining your gut microbiota. You may also benefit from reduced illness rates throughout the season, increasing training and match day availability. Advice can also be provided on nutrition for enhancing the gut microflora and immunity.

What are the possible risks to taking part?

Some discomfort may be experienced during venepuncture for the blood sample. You will be instructed to apply pressure to the area afterwards. The venepuncture procedure has been risk assessed and in relation to current experience at Nottingham Trent University it is safe. Staff who perform the procedure are appropriately trained and sterile procedures are used at all times.

Although rare, prebiotics may cause dose dependent gastrointestinal side effects, including; bloating and flatulence. Most common in the first 7-14 days of taking the supplement these will then subside. Any prolonged discomfort must be reported to the medical team and recorded as an adverse event. These will be monitored by medical staff, where considered appropriate, you will be removed from the study.

If at any point you decide to withdraw from the study your data will be destroyed.

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Appendix 1b – Chapter 6 Consent Form

Participant Statement of Consent to Participate in the Investigation Entitled:

The effects of long-term prebiotic supplementation on markers of immunity and upper respiratory illness in elite rugby union players.

1) I, *[name of participant]* agree to partake as a participant in the above study.

2) I understand from the participant information sheet (Dated… Version…), which I have read in full, and from my discussion(s) with *[name of investigator]* that this will involve me completing a 36-week supplementation of either a prebiotic or placebo over the course of a professional rugby union season. I also understand that I will have measures of height, weight, respiratory and GI symptoms measured during the study. I also understand that I will be asked to provide blood and saliva samples during each experimental visit and faecal samples during the baseline and final visit.

3) It has also been explained to me by *[name of investigator]* that the risks and side effects that may result from my participation are as follows: some discomfort may be experienced during venepuncture for the blood sample. You will be instructed to apply pressure to the area afterwards. The venepuncture procedure has been risk assessed and in relation to current experience at NTU it is safe. Staff who perform the procedure are appropriately trained and sterile procedures are used at all times. Although rare, prebiotics may cause gastrointestinal side effects, including bloating and flatulence these normally occur in the first 7-14 days of taking the supplement and then subside. If issues do arise you must inform the medical team. Prolonged symptoms will be monitored by the medical team and where appropriate, will be recorded as an adverse event and were appropriate you will be removed from the study.

4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction.

5) I undertake to abide by University regulations and the advice of researchers regarding safety.

6) I am aware that I can withdraw my consent to participate in the procedure at any time and for any reason, without having to explain my withdrawal and that my personal data will be destroyed and that my medical care or legal rights will not be affected.

7) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by individuals relevant to the performance of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous.

8) I confirm that I have had the University's policy relating to the storage and subsequent destruction of sensitive information explained to me. I understand that sensitive information I have provided through my participation in this study, in the form of anthropometric data, questionnaires, blood, saliva and faecal samples will be handled in accordance with this policy.

9) I understand that as part of this study I will be consuming a supplement. I am aware that elite sports people (i.e. international or national standard) may undergo either out-of or in-competition (or both) doping tests and appreciate that the supplement being studied could be contaminated with a substance that appears on the banned lists.

10) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research.

11) If appropriate) I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers.

Participant signature: Date:

Independent witness signature: Date:

Primary Researcher signature: Date:

Appendix 1c – Self Reported Health Screen

Name or Number .....

Please complete this brief questionnaire to confirm fitness to participate:

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

(a) on medication, prescribed or otherwise Yes ☐ No ☐

(b) attending your general practitioner Yes ☐ No ☐

(c) on a hospital waiting list Yes ☐ No ☐

(d) Take a daily dose of aspirin or other NSAIDs Yes ☐ No ☐

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

(a) consult your GP Yes ☐ No ☐

(b) attend a hospital outpatient department Yes ☐ No ☐

(c) be admitted to hospital Yes ☐ No ☐

(d) use antibiotics Yes ☐ No ☐

(e) intake drugs which affect gastrointestinal mobility (e.g. laxatives) Yes ☐ No ☐

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

(a) Convulsions/epilepsy Yes ☐ No ☐

(b) Asthma Yes ☐ No ☐

(c) Eczema Yes ☐ No ☐

(d) Diabetes Yes ☐ No ☐

(e) A blood disorder Yes ☐ No ☐

(f) Head injury Yes ☐ No ☐

(g) Digestive problems Yes ☐ No ☐

(h) Heart problems Yes ☐ No ☐

(i) Problems with bones or joints Yes ☐ No ☐

(j) Disturbance of balance / coordination Yes ☐ No ☐

(k) Numbness in hands or feet Yes ☐ No ☐

(l) Disturbance of vision Yes ☐ No ☐

(m) Ear / hearing problems Yes ☐ No ☐

(n) Thyroid problems Yes ☐ No ☐

(o) Kidney or liver problems Yes ☐ No ☐

(p) Allergy to nuts, alcohol etc. Yes ☐ No ☐

(q) Any problems affecting your nose e.g. recurrent nose bleeds Yes ☐ No ☐

(r) Any nasal fracture or deviated nasal septum Yes ☐ No ☐

(s) History or current evidence of gastrointestinal disease e.g. chronic constipation, diarrhoea, irritable bowel syndrome, Crohn's Disease Yes ☐ No ☐

(t) History of gastrointestinal drug reaction Yes ☐ No ☐

4. **Has any,** otherwise healthy, member of your family under the age of 50 died suddenly during or soon after exercise? Yes ☐ No ☐

5. Are there any reasons why blood sampling may be difficult? Yes ☐ No ☐

6. Have you had a blood sample taken previously? Yes ☐ No ☐

7. Have you had a cold, flu or any flu like symptoms in the last Month? Yes ☐ No ☐

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

Appendix 1d – Supplement disclaimer form

As part of this study you will be consuming a supplement. If you are an elite sportsperson i.e. international or national standard who may undergo either out-of and in-competition (or both) doping tests it is important that you consider the following:

1) The supplement being studied could be contaminated with a substance that appears on the banned list. There is evidence from research that around 15% of supplements can be contaminated accidently with prohormones of testosterone and nandrolone [1]. Even well-known brands from UK and USA have been found to be contaminated.
2) You are responsible for what goes into your body and unless it can be guaranteed that what you take is "clean" then you should not take it.

Reference:

1) *Greyer et al.* Int J Sports Med. **2004 Feb; 25(2):124-9** Analysis of non-hormonal nutritional supplements for anabolic-androgenic steroids - results of an international study.

Participant Name (Please print):

Signature: Date:

Appendix 2a – Chapter 7 Participant Information Sheet

Study title

The effects of a prebiotic on gastrointestinal damage, permeability, and symptoms during a simulated football match in the heat

- Brief Introduction:

Gastrointestinal (GI) disturbances during exercise activity are well documented across a variety of athletic events and populations. Symptoms likely occur due to the redistribution of blood flow away from the gut which can result in gut damage and increased permeability. Damage to the gut and symptoms of nausea, bloating, stich and flatulence can increase when exercise at high intensity and/or long duration in the heat.

Football is a team-based sport characterised by repeated bouts of high intensity activity separated by low intensity recovery periods. It is becoming more popular for football and similar team sports to be performed in hot ambient temperatures, putting them at a greater risk of GI complaints. Identifying interventions that can alleviate GI damage and symptoms during exercise in the heat are warranted.

Increasing evidence suggests dietary interventions which modify the gut microbiota (the trillions of microorganisms which reside in the gut) can help reduce GI symptoms in athletes. Majority of research has focused upon the supplementation of probiotics (ingestion of live bacteria promoted as having a health benefit). However, currently there is no consensus on what probiotics are most effective. A suitable alternative may be dietary intervention of a prebiotic (a non-digestible carbohydrate) that acts as a fertilizer the good bacteria in the gut. Regular consumption of prebiotics has previously been shown to improve markers of immunity and reduce inflammation in the general population and high-risk groups (elderly, obese & asthmatics). Therefore, it could be hypothesised that prebiotics may reduce GI damage and symptoms in response to exercising in the heat.

This study aims to assess if a short-term (42 days) prebiotic supplementation period reduces GI damage and symptoms following a football specific treadmill protocol in the heat.

- Study Requirements:

To be eligible to take part, you must:

- Be 18-45 years of age
- Physically active and team sports player (training/performing > 4hrs a week)
- Have a body mass index 20-25 $kg \cdot m^{-2}$ (we can work this out for you using your weight and height)
- Be a non-smoker

Unfortunately, you will not be able to take part if any of the following apply to you:

- Routine consumption of ω-3 PUFA supplements, supplements with antioxidants
- Routine consumption of prebiotic, and / or probiotic supplements
- Take a daily dose of aspirin or other NSAIDs
- Intake of prebiotics and probiotics,
- Intake of drugs which affect gastrointestinal mobility, laxatives in the 4 weeks before the study
- Vegetarian or vegan diet
- Previous history of acute mountain sickness, or adverse effect from altitude exposure
- Previously diagnosed with COPD, emphysema, chronic bronchitis or similar respiratory illness
- Asthma exacerbation within the last 12 months (course of steroids, or hospital visit)
- History of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease
- History of recurrent chest infections
- Smoker
- Acute infection within the last four weeks
- Major operation within the past four months
- History of gastrointestinal drug reaction
- Use of antibiotics in the past 3 months
- History or current evidence of gastrointestinal disease e.g. chronic constipation, diarrhoea, irritable bowel syndrome, Crohn's Disease
- You were previously hospitalised for COVID-19 in the last 4 months.
- You are continuing to suffer from symptoms associated with "Long-COVID".

- Location:

  You will be asked to visit Nottingham Trent University Clifton Campus on 6 occasions. Visits 1 will last 1 hour, visit 2 will last up to 2 hours, and visits 3 & 5 will last up to 8 hours. Visit 4 & 6 will last up to 30 mins.

  Address:

  Sport Science Labs and Environmental Chamber

  Erasmus Darwin Building

  Clifton Campus

  Nottingham Trent University

  Clifton Lane

  Nottingham

  NG11 8NS

- Restrictions During Testing:

You will be required to report to the laboratory at pre-arranged set times that will be the same on each occasion. No strenuous exercise in the 48 hours prior to visiting the laboratory, or alcohol and caffeine consumption 12 hours prior to testing will be allowed. You will be required to follow a standardised diet 24 hours before each trial.

- Testing Protocol:

Following initial briefing and consent (visit 1) you will then complete a health screen and maximal oxygen uptake test (visit 2) before complete the two main experimental trials (visit 3-10).

Visit 1 – briefing, consent, health screening and maximal oxygen uptake testing

At least 24 hours prior to visit 2 you will attend the laboratory for a briefing of the study design and to read the participant information sheet; you will then provide written informed consent to take part in the study. Following consent, you will complete a health and medical questionnaire then have height and weight measured before completing a standardised incremental treadmill test for the assessment of maximal oxygen uptake.

Speed Lactate Test

The test involves participants completing an incremental treadmill run starting at 9 $km \cdot h^{-1}$, 1 % gradient with the speed increasing by 1 $km \cdot h^{-1}$ every 3 minutes. At the end of each stage the participant will stop and a capillary blood sample will be collected from the fingertip for the assessment of lactate. The test will end once there has been an increase > 1 mmoL/l when compared to the previous stage. Heart rate and rating of perceived exertion will also be measured at the end of each 3 minute stage.

Standard incremental treadmill test

You will complete a standard incremental treadmill test for the determination of VO_{2PEAK} and gas exchange threshold. The test involves you completing an incremental treadmill run to fatigue starting at 9-10 $km \cdot h^{-1}$, 0 % gradient with the speed increasing by 1 $km \cdot h^{-1}$ each minute until a speed of 18 $km \cdot h^{-1}$. After 1 min at 18 $km \cdot h^{-1}$, the treadmill gradient will increase by 1 % each minute until volitional fatigue. Heart rate will be measured every minute, and respiratory variables will be measured breath-by-breath and recorded every 30 s through the use of a face mask and breath-by-breath analyser. Exhaled air is analysed to determine oxygen consumption (O_2), carbon dioxide production (CO_2) and minute ventilation (VE). Arterial oxygen saturation using pulse oximetry will be measured continuously throughout the test.

Visit 2 – Familiarisation in the heat

You will perform one half of the football specific intermittent treadmill protocol (FSITP) in the heat (as described below).

Visit 3 & 6 – Experimental trials

Following the incremental treadmill test and determination of your GET and $\dot{V}O_{2max}$ you will then undergo two visits separated by a 28-day supplementation period to complete a 90minute FSITP in the heat (33°C). On each visit you will arrive 4 hours prior to the start of exercise having fasted overnight, a venous blood sample, saliva and urine sample will be collected. You will then be provided a standardised breakfast 2 hours prior to the beginning of the trial and another blood sample will be collected. Immediately before the beginning of the trial another blood and urine sample will be collected, and you will then sit in the environmental chamber for 10 mins. You will then start the FITP which will involve two 45-minute halves separated by a 15 min half-time. Throughout each 45-minute half you will run at a range of speeds between 0-25 km/h, simulating a football match. A venous blood and urine sample will be collected at half-time and immediately post 90 mins. Finger prick blood samples will be collected every 22.5 mins for lactate and glucose, while heart rate, oxygen saturation and core body temperature will be recorded throughout the exercise task. Subjective assessment of gastrointestinal discomfort, RPE and thermal discomfort will be assessed throughout the FSITP will also be assessed every 22.5 mins. You will be asked to perform a battery of cognitive tests before, during half-time and post FSITP, this will include the stroop, visual search, memory block and rapid visual information processing test. Once you have completed the trial you will rest for another hour outside the environmental chamber and provide another blood sample at 60 mins post exercise. You will also be given a yellow wristband after pill ingestion. It is a requirement that a warning wrist band is worn continuously for 7 days after as you cannot have medical scans using magnetic fields (such as an MRI/NMR) with the pill still inside your body.

Visits 4 & 7 – 24 hour post-trial visit

You will be asked to return to the lab 24 hours post exercise to provide another venous blood and saliva sample. You will then be given your supplement for the next 42 days, this will be either the prebiotic or a placebo. You and the researcher will be blinded to what supplement you are taking. You will be asked to consume a daily dose with your breakfast, mixing the supplement with a hot drink is the most effective. If you miss a day, please do not take a double dose the following day. You will be asked to return all empty and missed capsules so supplement adherence can be calculated. Once you have reached 28 days you will return to the lab to repeat the experimental trial protocol and then you will begin the 2-week washout period. During this period, you will not need to take a supplement. Once 2-weeks are finished, you will repeat the experimental trial and begin supplementation with the alternative supplement.

Visit 5

Halfway through the supplementation period (3-weeks) you will be asked to come to the laboratory to collect the 3-weeks worth of supplements and to provide a saliva sample. This visit will be no longer than 30 minutes in duration.

Data Protection

All personal information and data will be stored pseudonymised under a unique identification code on a password protected file for confidentiality. Pseudonymising your data will allow us to remove your personal information and data set if you were to withdraw from the study.

COVID Special measures (Please include information on COVID).

In the morning of your visit you must have taken and received a negative test result for COVID-19 using a lateral flow test. These can be collected from the research team, or you can book into the NTU campus testing here https://myntuac.sharepoint.com/sites/LateralFlowTesting

- Potential Risks to You:

Some discomfort may be experienced during venepuncture for the blood sample. You will be instructed to apply pressure to the area afterwards. The venepuncture procedure has been risk assessed and in relation to current experience at NTU it is safe. Staff who perform the procedure are appropriately trained and sterile procedures are used at all times.

Although it is extremely unlikely, high intensity exercise has been known to reveal unsuspected heart or circulation problems and very rarely these have had serious or fatal consequences.

You may feel slight discomfort when swallowing the telemetric pill. This may vary from individual to individual but is not a significant discomfort. Unfortunately, if you have any issues swallowing the pill (impairment of the gag reflex, gastrointestinal problems) then you will be excluded from participating in the study. The pill will be passed naturally over time.

If at any point you decide to withdraw from the study your data will be destroyed.

- Contacts:

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Appendix 2b – Chapter 7 Informed Consent Form

Participant Statement of Consent to Participate in the Investigation Entitled:

The effects of a prebiotic supplementation on markers of gastrointestinal damage and symptoms following a football specific treadmill protocol in the heat.

1) I, *[_____]* agree to partake as a participant in the above study.

2) I understand from the participant information sheet (Dated… Version…), which I have read in full, and from my discussion(s) with *[_____]* that this will involve me completing an incremental test to exhaustion to measure VO_{2peak} in the heat followed by 4 subsequent visits to the environmental chamber to perform a football specific treadmill protocol in 33^{O}C heat, separated by a 42 day supplementation period of either a prebiotic or placebo. I also understand *that I will have measures of height, weight, heart rate, blood oxygen saturation and cognitive function measured during the study. I also understand that I will be asked to provide blood and saliva samples upon each visit to the lab.* I understand I must wear a warning wrist band for 7 days post-trial so medical personnel know to not perform any scans that use magnetic fields whilst the pill is in my body.

3) It has also been explained to me by *[name of investigator]* that the risks and side effects that may result from my participation are as follows: *Some discomfort may be experienced during venepuncture for the blood sample. You will be instructed to apply pressure to the area afterwards. The venepuncture procedure has been risk assessed and in relation to current experience at NTU it is safe. Staff who perform the procedure are appropriately trained and sterile procedures are used at all times.*

   Although it is extremely unlikely, high intensity exercise has been known to reveal unsuspected heart or circulation problems and very rarely these have had serious or fatal consequences.

   When exercising in the heat there is the potential for you to feel light headed, dizzy, nauseous and uncomfortably hot. You will always be monitored and will be immediately withdrawn if core body temperature increases above 40^{O}C.

   Before attending each visit you will be asked to provide a negative lateral flow test for covid-19 and to wear a face mask throughout.

4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction.

5) I undertake to abide by University regulations and the advice of researchers regarding safety.

6) I am aware that I can withdraw my consent to participate in the procedure at any time and for any reason, without having to explain my withdrawal and that my personal data will be destroyed and that my medical care or legal rights will not be affected.

7) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by individuals relevant to the performance

of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous.

8) I confirm that I have had the University's policy relating to the storage and subsequent destruction of sensitive information explained to me. I understand that sensitive information I have provided through my participation in this study, in the form of *[Health screen questionnaire, blood and saliva samples.]* will be handled in accordance with this policy.

10) I understand that as part of this study I will be consuming a supplement. I am aware that elite sports people (i.e. international or national standard) may undergo either out-of or in-competition (or both) doping tests and appreciate that the supplement being studied could be contaminated with a substance that appears on the banned lists

11) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research.

12) If appropriate) I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers.

13). (If appropriate) I agree to my General Practitioner being informed of my participation in the study. / I agree to my General Practitioner being involved in the study, including any necessary exchange of information about me between my GP and the research team.

14) It has been explained to me that there may be additional risks arising from the current COVID pandemic. I have read the NTU recommendations for undertaking 'Research with human participants' and undertake to abide by the special measures which have been explained to me for this study together with such Government Guidelines that are at the time prevailing.

Participant signature: Date:

Independent witness signature: Date:

Primary Researcher signature: Date:

*When completed: 1 for participant; 1 for researcher site file; 1 to be kept in medical notes (if appropriate).